



# **PLANT GROWTH-PROMOTING MICROORGANISMS FOR SUSTAINABLE AGRICULTURAL PRODUCTION**

EDITED BY: Everlon Cid Rigobelo, Saveetha Kandasamy and  
Duraishamy Saravanakumar

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# PLANT GROWTH-PROMOTING MICROORGANISMS FOR SUSTAINABLE AGRICULTURAL PRODUCTION

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# Editorial: Plant Growth-Promoting Microorganisms for Sustainable Agricultural Production

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**Keywords:** plant growth, soil microbiology, endophytes, rhizobacteria, rhizosphere

## Editorial on the Research Topic

### Plant Growth-Promoting Microorganisms for Sustainable Agricultural Production

Global food insecurity is a chronic issue that is likely to deteriorate following climate change, rapid population growth, and the increasing scarcity of arable land. To keep up with food demand, it was seemingly necessary to increase the application of agrochemicals such as fertilizers and pesticides for improved crop production. The intensive use of agrochemicals has led to severe consequences, including soil and environmental deterioration.

Soil is heterogeneous and contains different microenvironments in each centimeter depth. Therefore, soil contains totally different kinds of microorganisms. Soil is able to shelter aerobic and anaerobic microbes, for example, in the same place and at the same moment. Additionally, soil contains microbes responsible for biochemical processes, such as nitrogen, phosphorus, sulfur, and potassium cycling.

Soil microbiomes are important because they produce essential compounds used for medicine, industry, and biotechnology.

Studies related to the soil microbiome have been deepened, and our understanding has increased. Methods using RNA and DNA, metagenomics, and metatranscriptomics have revolutionized our understanding of soil microorganisms and their potentialities.

Soils contain rhizospheric microorganisms with several abilities to promote plant growth. These abilities can affect plants directly or indirectly. The abilities that affect plants directly are related to phytohormone production, improvement of nutrient availability, nitrogen fixation, and phosphorus and potassium solubilization. The indirect effects on plant growth are biocontrol, interference of quorum sensing, and induced systemic resistance.

New methodologies have generated large soil datasets that are analyzed using algorithms and deep learning, promoting an unprecedented advance in soil microbiology.

Methodologies such as synthetic communities, SynComs, and microbiome engineering have been used to better understand the diversity and complexity of microbe-microbe and plant-microbe interactions, as well as microbial diversity and its relevance to the mechanisms of suppressing plant diseases and health and the wealth of several microbial niches in soil and its importance to diversification processes and soil bacterial genome adaptations.

This Research Topic constitutes a thorough update of the relevant topics in plant growth promotion.

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It covers all relevant topics in plant growth-promoting microorganisms, intending to provide a broad understanding of this fascinating interdisciplinary area that encompasses aspects of soil science, biophysics, ecology, physiology, genetics, biotechnology, biochemistry, and microbiology.

Msimbira et al. reviewed relevant information on the roles of plant growth-promoting microbes in enhancing plant tolerance to acidity and alkalinity stress.

Santos et al. reviewed the use of plant growth-promoting rhizobacteria in maize and sugarcane.

Mokhtar et al. showed the reduction of detrimental effects caused by salt stress on date palm by applying arbuscular mycorrhizal fungi and compost.

Hernández-Esquivel evaluated the application of *Azospirillum brasilense* lipopolysaccharides to promote early wheat plant growth.

Chifetete et al. comprehensively reviewed mycorrhizal interventions for sustainable potato production in Africa.

Santos and Rigobelo showed some rhizobacteria isolated from sugarcane to promote its growth.

Diagne et al. evaluated the effect of plant growth-promoting rhizobacteria along with arbuscular mycorrhizal fungi on the salt stress tolerance of *Casuarina obesa*.

Díaz-Garza et al. found temporal dynamics of rhizobacteria in Pequin pepper, soybean and orange trees growing in a semiarid ecosystem.

Novinscak and Filion showed that strains of *Pseudomonas fluorescens* and *Pseudomonas synxantha* promote plant growth in talc and peat formulations.

Ochieno et al. presented a very good review of the rhizobium-linked nutritional and phytochemical changes in a multitrophic functional context in sustainable food systems.

Shahid et al. evaluated different profiles of *Bacillus* metabolites and their application in sustainable plant growth promotion and biocontrol effects.

Aguégué et al. verified the positive effects of organic fertilizer based on rhizophage intraradices for maize production.

Bal and Adhya showed the reduction of stress in rice seedlings using the rhizobacteria ACC deaminase.

Koskey et al. showed an interesting review of the potential use of beneficial microbes for soil amelioration and phytopathogen control.

Volkogon et al. evaluated the potential nitrogen fixation and denitrification in rhizosphere soil under potato cultivation and verified that this crop responded to *Azospirillum* inoculation.

Lopes et al. comprehensively reviewed successful plant growth microbes to achieve sustainable development and environmental conservation.

Gitonga et al. compared the genetic and morphological diversity of indigenous *Bradyrhizobium*-nodulating soybean in both organic and conventional production systems.

Mitter et al. approached an interesting review suggestion of a rethinking of crop nutrition in times of modern microbiology.

Araujo et al. presented interesting results of bacteria improving and modifying the essential oil rose crop.

Rivas-Franco et al. verified the biocontrol effect against *Fusarium graminearum*-producing microsclerotia from entomopathogenic fungi.

Boleta et al. verified the effect of *Azospirillum brasilense* on productivity on the nutritional accumulation of wheat cultivars.

Alberton et al. reviewed what we learned from plant growth-promoting rhizobacteria grass associations through proteomic and metabolomic approaches.

Maldonado et al. approached the importance of phosphate-solubilizing bacteria as an option for enhancing sustainable agriculture.

Ceretto et al. showed the contrasting expression of rhizobial phytase in nodules of two soybean cultivars grown under low phosphorus availability.

Duong et al. reviewed the coffee microbiota and its potential use in sustainable management.

Díaz-Rodrigues et al. provided important knowledge about the current and future role of microbial culture collection in food security worldwide.

Molina-Romero et al. showed a bacterial consortium that interacts with different varieties of maize, promoting plant growth and reducing the application of chemical fertilizer under field conditions.

Modesto et al. showed the yield and production components of corn under the straw of Marandu paleade grass inoculated with *Azospirillum brasilense*.

Hakim et al. reviewed and gathered important information about rhizosphere engineering with plant growth-promoting microorganisms for agricultural ecological sustainability.

Yarzabal and Chica addressed microbial-based technologies for improving smallholder agriculture in the Ecuadorian Andes.

Ilangumaran et al. reported that a strain of rhizobacteria from root nodules enhanced salinity tolerance in soybean.

Ullah et al. provided important information regarding climate change and salinity effects on crops and chemical communication between plants and microorganisms.

Grover et al. reviewed PGPR-mediated alterations in root traits.

Madhaiyan et al. reported exciting results on *Burkholderia* and its interactions with some important tree species.

Romero-Perdono et al. addressed phosphorus nutrition and plant growth in cotton crops.

Jaiswal et al. reviewed rhizobia as a source of plant growth-promoting molecules.

Diedhiou-Sall et al. verified the spatial and temporal distribution of soil microbial properties in two intercropping systems.

Scagliola et al. showed bioinoculants as a promising complement of chemical fertilizers.

Delitte et al. addressed plant microbiota beyond farming practices.

Ouversen et al. showed the temporal soil bacterial community response.

Sinong et al. reported distinct root microbial communities in nature farming.



Subramanian et al. showed thurvicin production and proteome differences in *Bacillus thuringiensis*.

Armin et al. reported an evaluation of the Apple Root-associated endophytic *Streptomyces pulveraceus*.

Ambardar et al. evaluated the diversity of the rhizo-bacteriome of *Crocus sativus* grown in various geographic locations.

Nemr et al. verified the culture media based on leaf strip root segments.

Agbodjato et al. showed the efficacy of biostimulants formulated with *Pseudomonas putida*.

Jhuma et al. isolated endophyte bacteria and showed resistance of salinity stress. Finally, Kalu et al. reviewed the response and bioremediation potential for agriculture production.

This special issue brings together many reviews and research articles focused on plant growth-promoting microorganisms and their abilities and impact on plant growth and health. Here, we summarize some of the highlights derived from the 49 articles published in this special issue.

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# The Roles of Plant Growth Promoting Microbes in Enhancing Plant Tolerance to Acidity and Alkalinity Stresses

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Plant growth often occurs under a range of stressful conditions, including soil acidity and alkalinity. Hydrogen ion concentration, which determines pH of the soil, regulates the entire chemistry of plant nutrient colloidal solutions. Beyond certain levels of pH, multiple stresses such as hydrogen ion toxicity, and nutrient imbalance, toxicities and deficiencies are induced in plants. Breeding for stress coupled with suitable agronomic practices has been a way to deal with this situation in agriculture. However, plant growth promoting microbes (PGPM) have shown potential as sustainable plant growth enhancers and have potential to help with a range of stresses in their environment. Considering the long-term evolutionary relationships between plants and microbes, it is probably that much remains unknown about potential benefits of microbes that could be harnessed from PGPM. This article reviews the current understanding of acidity and alkalinity stress effects on plants and various approaches have or could address these stresses. This review provides a detailed account of the current understanding regarding the role of PGPM in acidity and alkalinity stress management, including when agronomic practices and plant breeding are combined. Approaches already evaluated have shown limitations because acidity and alkalinity in soils are gradual and progressive conditions. Greater exploitation of PGPM in this regard, would be interesting to explore as they have the potential to address multiple stresses in a more sustainable fashion. Future crop production will require further breeding for pH stress resistance, but also implementation of microbial technologies that provide enhanced tolerance to pH stress.

**Keywords:** acidity, alkalinity, PGPM, soil pH, plant stress

## INTRODUCTION

Abundant microscopic life resides in the soil including bacteria, algae, protozoa and fungi (Glick, 1995; Müller et al., 2016), together with below-ground plant parts. The vigor of the microbes in the soil depends on nutrient availability, temperature, water and pH, among others. Plants, partition significant amounts of photosynthetically synthesized carbon to their root systems (Zhalnina et al., 2018) for the important roles of root growth and maintenance. Additionally, this C partitioning, to a large extent, is released from the roots into the rhizosphere in the form of exudates and sloughed off cells, together called rhizodeposition (Paterson et al., 1997; Badri and Vivanco, 2009).

Rhizodeposition serves as the main reduced C source for the microscopic life inhabiting in the soil and sustains diverse groups of microbes and microbial feeding life forms (Nguyen, 2009). The root exudates are organic compounds which include: organic acids, sugars, fatty acids and amino acids (Huang et al., 2014); all these have nutritional, preferential microbe selection and soil colloidal effects. Exudates therefore, control interactions between plants and microbes in part because they contain signal molecules that facilitate the interactions (Bulgarelli et al., 2013). Many studies have shown that plants expend this C to attract beneficial microbes such as rhizobia, involved in biological nitrogen fixation in legumes (Msimbira et al., 2016; Chagas et al., 2018) and mycorrhizal associations (Van Der Heijden et al., 2015). The legume-rhizobia symbiosis is a plant growth promoting mechanism acting through nitrogen fixation that is well described as compared to other plant-microbe associations (Oldroyd, 2013); it is reported to contribute to about 50% of the biologically fixed nitrogen on earth (Lindström et al., 2010). The second-most largely studied mechanism is that of a set of plant-fungal interactions, which involves about 90% of all plant species on planet Earth, by way of mycorrhizal symbioses (Gough and Cullimore, 2011; Zifcakova, 2020). Such interactions benefit plants by improving nutrient acquisition, water uptake and ability to survive various stresses. The usefulness of plant-microbe interactions has been the focus of intensive research, focused on unlocking key unanswered question, since their first description (Kloepper et al., 1989). Since then, beneficial interactions and better understanding of the mechanism(s) involved in microbial enhancement of plant growth have been demonstrated (Lira, 2015; Smith et al., 2015).

Beneficial symbiotic associations between early plants and mycorrhizal fungi are thought to have evolved to overcome limitations of terrestrial ecosystems, such as restricted water and nutrient availability (Kenrick and Crane, 1997; Kenrick and Strullu-Derrien, 2014). In soils, nutrient availability is related to hydrogen ion concentration ( $H^+$ ), which is the measure of soil pH. The pH variation in the environment has a direct impact on the availability of nutrients and plant growth; the critical and important effects of these conditions on microbial communities are not well understood. In soils, pH is an important driver for soil microbial community structures. Microbial survival and colonization in such conditions requires the capacity to sense, and adapt to, environmental changes (Biswas et al., 2007).

Recent years have witnessed considerable interest in unraveling the role and potential of microbes in the success of plants and animals. Of all, the human microbiome is the most studied, as reviewed by Gilbert et al. (2018); more recently, much attention has been focused on plants and their associated phytomicrobiome (Compant et al., 2019); terrestrial plants being the entry point of most energy into the terrestrial biosphere. Much has been done, mostly on symbiotic microbes and particularly under optimum conditions for plant growth. This review investigates the current understanding of one of the common, but complex and less explored abiotic factors, pH, as a determinant factor of the distribution and survival of microbes and plants. This review also wishes to understand how much is known regarding aspects of acidity and alkalinity

stress alleviation related to the evolutionary understanding that microbes have co-evolved with plants, each benefiting the other. Microbes, having a large surface area to volume, are very exposed to environmental stressors, so that their mode(s) of adaptation is of great importance for survival and, potentially of high impact, if these could be translated to multicellular organisms. While optimum pH is a crucial factor for survival in an evolutionary context, challenging conditions improve fitness over the course of evolution. Other factors of great importance include temperature and nutrients, which will also be touched upon as related to pH.

## PLANT MICROBE INTERACTION

Plants do not exist alone; always have complex interactions with microbes. Plants co-live with microorganisms (fungi, bacteria and archaea), allowing them to inhabit almost all of their tissues; and the resulting assemblage of microbes is collectively known as the plant-microbiome or phytomicrobiome (Knack et al., 2015; Smith et al., 2017). This perspective has helped, in recent years, to start answering some common evolutionary questions regarding how microbes have evolved, together with their host organisms, from their original ancestors. It is of critical importance to understand how plant adaptation has been influenced by their interactions with microbes, though much remains unknown. Plant-microbe interactions are a lifelong process for plants, as some microbes may be leaving the plant-associated community, while others will be entering the community (Baltrus, 2017). The ability of plants and microbes to communicate prior to physical contact being established is a very important (Chagas et al., 2018) as it helps the partners maximize the chance of benefiting from one another, without harm. There are a number of phytomicrobiome groupings, for instance depending on the plant part colonized by microbes: rhizomicrobiome – roots, caulomicrobiome – stem, phylломicrobiome – leaves, anthromicrobiome – flowers, carpo microbiome – fruit, or degree of intimacy with the plant tissue which are termed as endophyte (interaction inside plant parts), epiphyte (on the surface of plant structures such as shoots, stems, leaves, flowers and fruits) (Laksmanan et al., 2014; Chagas et al., 2018). The rhizosphere phytomicrobiome richness, activities and diversity is far greater than the phyllosphere (Laksmanan et al., 2014). This is primarily because much of the root exudation and sloughed off cells contain nutrient rich compounds for microbes associated with roots (Meharg and Killham, 1990; Beneduzi et al., 2012; Daguerre et al., 2017). Even with many questions unanswered about their full potential role in each of their tissues of colonization, plant-associated microbes provide promising insights around some best ways to augment plant growth and productivity, and understanding continues to expand.

For agriculture, microbes hold great promise in promoting productivity through synergistic interactions with host plants. All agricultural production taking place under field conditions face a range of challenges. This means increasing production with a constant, or even decreasing, land resource, and the need for a breakthrough to find possible sustainable means

for food production under field conditions. Plant growth promoting elements in the rhizomicrobiome provide promising potential for sustainable crop production and there has been increased interest in optimizing their use under a range of stresses, such as salinity and drought (Booth et al., 2002; Subramanian et al., 2016). As the world faces changing, and generally harsher, crop-growth conditions related to ongoing climate change, preparedness requires multiple options for sustainability. Apart from drought and salinity two other key growth stressors of the phytomicrobiome are increasing acidity and alkalinity of the soils.

Many recent studies have identified beneficial microbes that help plants, including crop plants, to survive the stresses they encounter, including nutrient imbalance (Mylona et al., 1995; Yazdani et al., 2009), salinity (Subramanian et al., 2016), and drought (Lim and Kim, 2013), with much less being reported regarding soil acidity and alkalinity. Thus, there is potential to understand better and move forward with a more sustainable agriculture based on knowledge at hand on each physical stress, and the role microbes can play in agricultural management of these stresses. A key factor in microbial proliferation in the wild is pH. The acidity and alkalinity of soils has been linked directly to soil and plant associated microbial population dynamics (Biswas et al., 2007; Zhahnina et al., 2015). Despite its obvious importance much seems unclear as to why microbes behave the way they do at varying pH levels.

## SOIL: A CENTER FOR PLANT MICROBE INTERACTION

Soil is a reservoir of basic natural resources, such as nutrients, for animals, plants and microbes. It is a life support system that provides a wide range of necessary ecosystem goods and services ranging from storage of carbon, to water purification, soil fertility and agricultural production (Rojas and Caon, 2016). Variation in soil characteristics throughout the world is affected by weather/climate and how it is geopositioned on the globe. Apart from nutrients, soil also contains plant-available water which plays a key role in creating an aqueous nutrient solution, the form taken up by plants (Sassenrath et al., 2018). The fact that all living biological cells are water-based systems makes a cell's survival very dependent on aqueous equilibria. For any aqueous solution reaction to occur, presence of anions and cations is needed. The necessity of appropriate pH in a biological system is crucial as it helps maintaining biochemical equilibria, correct levels of proton dissociable groups and maintain the cell pH at near neutral all the time. Like any other living cells, microbes need an appropriate pH balance to maintain physiological functions.

### Soil pH

The measure of soil reaction (alkalinity or acidity) is expressed as pH. It is mostly measured in water solutions and to lesser extent, for research purposes, 0.01 M calcium chloride is used (Blake et al., 1999). Soil pH is a key condition with substantial influence on soil biology, chemistry and physical processes which

have direct impacts on plant growth and development. It is clear soil and crop productivity are linked to pH. The United States Department of Agricultural National Resources Conservation Service has categorized soil pH as follows: ultra-acidic (<3.5), extremely acidic (3.5–4.4), very strongly acid (4.5–5.0), strongly acidic (5.1–5.5), moderately acidic (5.6–6.0), slightly acidic (6.1–6.5), neutral (6.6–7.3), slightly alkaline (7.4–7.8), moderately alkaline (7.9–8.4), strongly alkaline (8.5–9.0) and very strongly alkaline (>9.0) (Burt, 2014).

Agricultural crop production is generally conducted within the range of slightly acidic to slightly alkaline, a window that is associated with optimal availability of soil nutrients. In all soils, solubility, mobility and bioavailability of trace elements is strongly affected by pH. However, soils which fall outside of the range of optimum nutrient availability are grouped as either acidic or alkaline and pose a range of challenges to plants. Though plants differ in their tolerance to extreme pH, most agricultural plants perform optimally at a pH near neutrality (Läuchli and Grattan, 2012). In the context of crop production, pH variation is associated with all the ways the soil is managed before, during and after crop production, which includes; soil tillage, planting of cover crops, fertilizer application and lime addition, as well as precipitation and other climate variables.

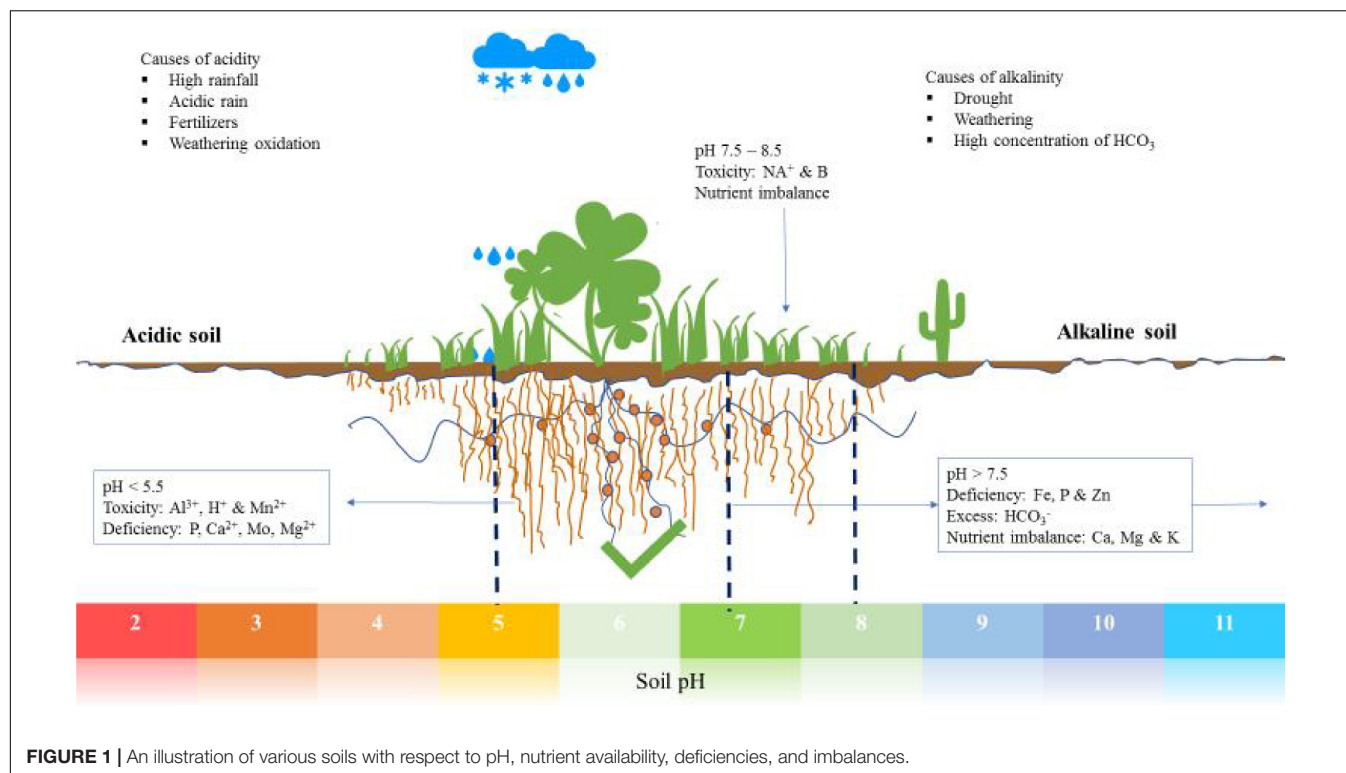
A full understanding of pH is necessary for optimizing nutrient cycling, soil remediation and plant nutrition, as it affects the entire interacting system. In order to establish ways to deal with various aspects that are affected by soil pH, one should initially understand what causes variation in the soil pH. One of the major causes of pH variation is the inherent mineral composition of the parent soil material. In this review, acidification and alkalization of soil are discussed to enlighten our understanding of causes of pH changes in the soil below and above neutrality.

### Soil Acidification

Soil acidification is the result of various direct and indirect factors interacting with the soil; these include nutrient cycling and organic matter decomposition, high and acidic rainfall, fertilizer application, crop growth and weathering (Figure 1). Acidification is a gradual and progressive process which is influenced by agricultural practices and now by climate change (Bolan et al., 1991; Filipek, 1994; Hao et al., 2019). It is the result of increased  $H^+$  concentration with the  $H^+$  released from Carbon (C), Nitrogen (N) and Sulfur (S) during transformation and cycling. For example S and N oxides released from burning of fossil fuels react with rain water to form tetraoxosulfate (vi) acid and trioxonitrate (v) acid (Oshunsanya, 2018). Mineralization and oxidation of organic N and S release  $H^+$ , thus lowering the soil pH. Organic matter decomposition causes release of  $CO_2$  into the soil air which, when dissolved in soil water, forms  $H_2CO_3$  which causes a decline in pH (Bolan et al., 1991).

High rainfall is also a cause of soil acidification because rainwater is slightly acidic (around pH 5.7), a result of reaction with atmospheric  $CO_2$  forming carbonic acid, hence reducing the pH of soil. In addition, water in the soil causes leaching of basic cations, such as bicarbonate, leaving more  $Al^{3+}$  and  $H^+$  relative to other cations in the soil (Oshunsanya, 2018).





**FIGURE 1** | An illustration of various soils with respect to pH, nutrient availability, deficiencies, and imbalances.

In agricultural soils a major contributor to acidity is the application of ammonium-based fertilizers, urea, sulfur and legume cultivation. The salts from applied fertilizers have strong effects on acidification of the soil through nitrification. This happens only when  $\text{NH}_4^+$  undergoes nitrification and/or  $\text{NO}_3^-$  is leached but not when the nitrate is taken up by plants (Marschner, 2011). The scope of the problem is becoming worrisome as the occurrence of acid rain and continued intensive use of synthetic fertilizers. In addition,  $\text{N}_2$  fixation also has impact on soil acidification. Comparatively, legumes are known to cause more soil acidification than non-legumes, due to excessive uptake of cations relative to anions during  $\text{N}_2$  fixation, and also leaching of nitrates eventually resulting from fixed N (Tang, 1998; Tang et al., 1999). However, variation in  $\text{N}_2$  fixation among legumes exists, which results in variation of the acid generated with a range of 0.2 to 1 mol  $\text{H}^+$  for each mol of fixed N (Bolan et al., 1991). Other factors which influence acidification by legumes are soil nutrients and nitrogen (Yan et al., 1996; Marschner, 2011).

Crop growth is another factor which causes localized soil acidification as a result of nutrient uptake. Plants take up nutrients from the soil solution in ionic form with a preference for cations over anions, which leads to cation reduction in the soil (Tang and Rengel, 2003). To counteract the effect of charge imbalance, plants release  $\text{H}^+$  from roots to the rhizosphere, hence lowering soil pH. In addition, roots naturally exude organic acids which cause acidification of the soil.

## Soil Alkalization

Soil alkalinity can be a result of natural weathering processes or man-made conditions. Weathering of silicates, aluminosilicates

and carbonate containing compounds such as  $\text{Na}^+$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  is linked to silicates being hydrolyzed and subsequent  $\text{OH}^-$  release, which increases soil pH. Irrigation is also associated with alkalinity of the soil, especially when the used water contain large quantities of bicarbonates (Oshunsanya, 2018). Drought is another natural cause of soil alkalinity due to insufficient water to leach soluble salts, allowing their accumulation in the upper soil profile. Alkaline soils are characterized by high concentrations of carbonates ( $\text{CO}_3^{2-}$ ) and bicarbonates ( $\text{HCO}_3^-$ ) which have the ability to neutralize acids (Bailey, 1996). As a result, alkaline soils are associated with desertification in most parts of the world, and this is also closely associated with soil salinity. Recently, the demand for aluminum in the world has contributed to increased alkalinity in surrounding ecosystems because mining and disposing of the alkaline bauxite residue (Kong et al., 2017). Lastly, over liming also leads to alkalization of soil. Therefore, liming should carefully consider the knowledge of soil acidity so that required liming material can be calculated before it can result in soil alkalization.

## MICROBIAL COMMUNITIES IN RELATION TO SOIL Ph

Environmental factors are the main drivers of the phytomicrobiome composition (Chu et al., 2016; Baltrus, 2017) with soil pH exerting a large effect in microbial community structure (Zhahnina et al., 2015). According to Graham et al. (1994), prokaryotic lifeforms are profoundly influenced by the

pH of their environment. For all living cells there are optimum pH requirements for normal physiological functions. The pH range 5.5–6.5 is optimal for plant growth as the availability of nutrients is optimal. This is also so for most soil microbes, in part because in this range plants grow well and produce more root exudates as a carbon source available for survival and multiplication of microbes. Though, some microbes have the ability to alter soil pH by acidifying their surroundings, as a way to outcompete other microbes, most bacteria do best around neutral pH. Fungal activities on the other hand are favored by at least somewhat acidic pH conditions, which explains why they are dominant in forest acidic soils compared to range land soils and sub-humid and arid prairies which are mildly acidic and are dominated by bacteria (Zifcakova, 2020).

Bacteria are among the single celled organisms most able to adapt to and thrive under harsh environmental pH conditions. Acidic soils are dominated by *Acidobacteria* and *Alphaproteobacteria* (Shen et al., 2019) while *Actinobacteria* abundance increases toward alkalinity (Jeanbille et al., 2016). However, the most sensitive component of the cell to pH changes is its workhorse, the protein (Hyyryläinen et al., 2001). Slight changes in pH have been reported to interfere with amino acid functional group ionization and impair hydrogen bonding, as a result protein folding is changed, leading to denaturation and cessation of enzymatic or other activities (Booth et al., 2002).

Variation of pH in the environment has a direct impact on the availability of Al, Fe, Mn, Cu, and plant growth; the critical and important effects of these conditions on microbial communities are not well understood. Graham et al. (1994), reported that there were two pH related mechanisms influencing microbial communities, the direct and indirect, the latter being the spillover effects of pH.

## Acidity Tolerance in PGPM

In general, organisms have developed mechanisms to survive environmental variation. Among other abiotic factors, most organisms need to sense and adapt to hydrogen ion concentration (pH) (Booth et al., 2002). In soils, pH is an important driver for soil microbial community structures. Microbial survival under such conditions requires the capacity to sense, and adapt to, environmental changes (Biswas et al., 2007).

However, little is known about optimal pH ranges and nutrient availabilities for many species of microorganisms (Ratzke and Gore, 2018). Plant–microbe interactions such as that of legumes and rhizobia are affected by Ca, P, Fe, and Mo; they influence rhizobia and their optimal growth, which is near pH 6. Biochemical properties and activities of microbes are partly affected by pH, leading to diversity effects in microbial community structure (Roe et al., 1998).

Microbes have developed various means to tolerate extreme pH changes. Production of extracellular polysaccharides by rhizobia is one of the reported behaviors (Gopalakrishnan et al., 2015). *Rhizobium tropici* demonstrated an ability to tolerate acidic pH by producing glutathione, a tripeptide (Muglia et al., 2007; Wang et al., 2018). Some rhizobia are known for their ability to accumulate high levels of potassium and phosphorus as a means to tolerate low pH, as compared to acid sensitive strains

(Watkin et al., 2003). However, the relationship between these microbial survival mechanisms and plant growth promotion is not well understood.

Cells' major functions, such as nutrient acquisition, cytoplasmic pH homeostasis and protection of DNA and proteins are largely affected by low pH (Booth et al., 2002). Mechanisms involved in the induction of protective systems pose a considerable challenge. The advent of proteomics (Blankenhorn et al., 1999) has complemented the genome information in this area, for example, *Lactobacillus* spp., like many microorganisms, produces a thin biofilm composed of polysaccharides and proteins, which protects the cell against changes in the pH of the environment (Wang et al., 2018).

## Alkalinity Tolerance in PGPM

High pH disrupts the bonds holding together the DNA helix strands, and lipid hydrolysis occurs more readily as the environment becomes more basic (Rousk et al., 2010; Shen et al., 2019). Most microbes adjust their surrounding medium to near neutrality as a way to survive high pH. Sodium is very important in intracellular pH maintenance for microbes because it allows exchange of  $H^+/Na^+$  antiporters into the culture media (Satyanarayana et al., 2005). Furthermore, the  $H^+$  concentration gradients across the membranes plays an important role in producing ATP during cellular respiration, through proton motive force (Celiker and Gore, 2013). It is not very clear how PGPM are able to use sodium-dependent ATP synthases as an alkali tolerance mechanism.

## ACIDITY AND ALKALINITY STRESS IN PLANTS

### Acidity Stress

Acidification of the soil is currently a major limit for sustainable agricultural production in the world. Acid soil covers about 30–40% of the arable land worldwide, and about 70% of the world's potential agricultural land (Von Uexküll and Mutert, 1995; Kochian et al., 2004). In the soil plant roots are in constantly adjusting to varying pH as a result of water status variability (Misra and Tyler, 1999). Soil pH has significant influence on plants because it affects almost every aspect of nutrient uptake by them. In acid soil plants face three major toxicities,  $Al^{3+}$ ,  $Mn^{2+}$  and  $H^+$ , which inhibit plant growth. In any acidic soil Al toxicity is the major and often first limitation to plant growth. Effects of Al toxicity include; inhibition of root growth, inhibition of root cell division, modification of the cytoskeleton and inhibition of nutrient uptake (Bojórquez-Quintal et al., 2017; Kaur et al., 2019). In many cases direct Al toxicity effects are not obvious, instead they are manifest as P deficiency symptoms with overall stunting, dark green leaves, late maturity and purpling of stems, leaves and leaf veins. All these P deficiency symptoms occur because of delocalized P metabolism by Al. Also P ends up being fixed by Fe in most acidic soils, degrading conditions for crop production (Kaur et al., 2019).

The second prevalent toxic metal in acidic soil is Mn. In contrast with Al toxicity, Mn as an essential plant nutrient,

toxic when plants absorb it in excess. Mn toxicity is prevalent at pHs as high as 5.6; this makes it more important as a constraint in crop production, in some acid mineral soils, than Al (Sumner et al., 1991).

Low pH stress has been associated with inhibition of root growth (Yang et al., 2005) by facilitating  $H^+$  influx into roots, which causes poor plant growth. High  $H^+$  influx causes depolarization of the plasma membrane, impacting the acidity of the cytoplasm (Babourina et al., 2001). Thus generally, low pH stress caused by  $H^+$  adversely affects root tissues, which leads to reduced growth and development of crops. There is a meaningful lack of knowledge regarding how various plants respond to low pH conditions; low pH tolerance would be a good trait to select for in plant breeding programs.

## Alkalinity Stress

Most land desertification in the world is linked with soil alkalization and lower water availability and retention ability, soil erodibility and also reduced biodiversity. Alkaline soils are characterized by high concentrations of carbonates ( $CO_3^{2-}$ ) and bicarbonates ( $HCO_3^-$ ) which have the ability to neutralize acids (Bailey, 1996; Rashid et al., 2019), high pH and poor amounts of organic carbon, leading ultimately to poor availability of nutrients. Other minor contributors to soil alkalinity are those which result from hydroxides ( $OH^-$ ), borates, organic bases, silicates, phosphates and ammonia. The problem of alkalinity in soils is prevalent, as a secondary effect of drought in many places. Most arid and semi-arid regions of the globe experience soil alkalinity since concentrations of salts decrease and levels of carbonates and bicarbonates increase, leading to alkalinity of the soils. Alkalinity stress effects on crop plants are remarkably similar to those of salt stress (Xu et al., 2013), though it has remained a less researched area. Most of the studies to date have dwelt on the relationship between salinity stress and alkalinity stress by showing a strong link between them (Bui et al., 2014). The stress caused by carbonate salts is sometimes higher than that of salinization by NaCl and  $NaSO_4$  (Shi and Sheng, 2005).

The higher pH of sodic soils results in nutrient imbalance stress in crop production by affecting bioavailability of phosphorus, iron, copper, boron and zinc (Chen et al., 2011). However, it is important to note that under high pH (more  $OH^-$  than  $H^+$ ) the activity of the  $OH^-$  ions comes into play, increasing alkalinity at pHs greater than 11, whereas below pH 11 forms of carbonates are responsible for alkalinity (Whipker et al., 1996). In alkaline soils with pH < 11 there are major effects of pH on plants that are largely due to carbonate ions, rather than hydroxide ions.

Nutrient availability for plant uptake is related to soil chemistry, which is predominantly influenced by pH. When addressing pH related stresses, many other associated stresses come into play; under alkalinity stress, apart from essential element stresses, there are also osmotic, ion-induced injury or high pH effects that are automatically problematic (Lynch and Clair, 2004).

Most plants under alkalinity stress manifest stunted growth due to poor nutrient uptake and leaf chlorosis due to high and low

uptake of  $Na^+$  and  $Fe^{+}$ , respectively (Zhang et al., 2012; Singh et al., 2018). High levels of  $Na^+$  interfere with stomatal closure, which worsens the problem of water loss for plants (Bernstein, 1975), a common phenomenon under saline conditions, which can be similar under alkalinity conditions. Bicarbonates reduce Fe absorption and sometimes increase internal precipitation of Fe (Norvell and Adams, 2006), all of which affects synthesis of chlorophyll, and hence leads to chlorosis. Chlorosis, which is linked to diminished photosynthesis, has an ultimate impact on plant growth. Similarly, in calcareous soils reports have shown Fe and lime-induced chlorosis are dominant factors leading to iron deficiency (Coulombe et al., 1984). Conversely, the concentration of bicarbonate ions in the soil is known to induce minor to severe stunting in plants. For instance, cucumber plants were reported to experience negative effects of  $HCO_3^-$  on their growth (Rouphael et al., 2010).

Alkalinity stress causes inhibition of root growth due to high concentrations of  $HCO_3^-$  in the soil solution, though this varies among crop species. The suppression of root growth by  $HCO_3^-$  is associated with inhibition of respiration by the roots (Alhendawi et al., 1997). The inhibition of root growth may also result from excessive accumulation of organic acids (OAs) in root cells. Of the OAs commonly reported to stop root elongation, malate is a problem when concentrated in the elongation zone, as a result of bicarbonate in calcifuge grass species (Lee and Woolhouse, 1969). Furthermore, plant hormones are known for variation in their activity under a range of stresses, leading to alleviation of negative impacts on plants, though their final mechanisms of action are often unclear. Under alkaline stress abscisic acid (ABA) is secreted by roots into the rhizosphere, which negatively impacts root growth as water becomes limiting (Slovik et al., 1995).

## MANAGEMENT OF ACIDITY AND ALKALINITY STRESSES IN PLANTS

Acid soil management is the application of indirect and direct means to ensure that production potential of a particular soil is regained or attained. Some of the direct acidic soil amendments include correction of acidity by liming and manipulation of agricultural practices for optimum crop yield (Yirga et al., 2019). Liming is one of the major known ways to manage acid soils. However, breeding for acidity tolerance and use of PGPM are also becoming established mechanisms to address soil acidity stress. The combination of two or more of these methods could be more helpful than single method strategies. The problem is recurrent for soils that are prone to acidification.

To ensure optimum plant growth, soil pH should be monitored and the soil amended to optimal pH levels, near neutrality. Generally, it has been established that soils with high organic matter, >5%, have pHs between 5.0 and 5.5. In contrast with soil acidity, which can be tolerated by some crops, very few crops survive in even moderately alkaline soils, due to restricted nutrient mobility and availability. Alkaline soil amendment for crop production involves the use of cultural practices (conservation tillage, crop covers and rotation, organic



matter amendments, avoiding bare fallow), use of PGPM and production of alkali stress tolerant crop varieties.

## PGPM Enhance Plant Resilience to Acidity and Alkalinity Stresses

### Acidity

Sustainable agricultural innovations are not immune to the effects of acidic soils. Considerable effort has been made regarding the use of PGPM as a strategy for dealing with various environmental stresses of plants. Current understanding indicates that about 2–5% of culturable rhizobacteria are plant growth promoters, either directly and or indirectly (Dutta and Bora, 2019); the need to exploit this resource in agriculture is increasing. Legume symbioses with rhizobia, a well-studied beneficial plant microbe interaction, are constrained with regard to nodule formation and poor and/or failed bacterial survival (Correa and Barneix, 1997) by various stresses, including soil acidity. Many reports share a similar perspective, indicating that selecting for acid-soil tolerant symbiotic partners can improve the survival and productivity of crop plants (Zhang et al., 2020). With *Sinorhizobium*, for instance, the genetic control of acid tolerance is becoming increasingly understood (Draghi et al., 2016). In legume symbioses, as with other interactions, there is a requirement for specific recognition of signal molecules produced by both bacterial and plant partners. One of the factors affecting the signal molecule exchange and recognition process is pH, with effects on both plant and bacterial partners (Zhang et al., 2020). Though there have been few advances in understanding the direct effect of PGPM on acid stress, there is a substantial body of research literature indicating the potential for using microbes to address secondary effects of acidity in the soil, such as Al toxicity (Zerrouk et al., 2016) and P deficiency (de la Luz Mora et al., 2017; Delfim et al., 2018).

It has been established that, within the legume-rhizobia nitrogen fixing symbiosis, rhizobia isolated from acidic soils have more ability to colonize and improve plant growth under acidic conditions. Several genes contributing to rhizobial survival under acidic conditions have been identified. Some of them are those which code for stress tolerance proteins, including ActA (apolipoprotein N-acyl transferase) and ActR (response regulator) (Tiwari et al., 1996a,b). Despite advances regarding tolerance and ability of legumes to nodulate under low pH conditions, much remains unknown regarding signal molecules in this capacity.

The other highly studied plant microbe interaction is mycorrhiza associations, which are associated with about 90% of all terrestrial plants. With this degree of interaction, it is clear that for a large proportion of acid dominated soils where these plants occur there are mycorrhizal associations with the plants growing there. There are two main groups of these fungi: ectomycorrhiza and endomycorrhiza (Bonfante and Genre, 2010). Endomycorrhiza reside inside plant cells and form arbuscules within cortical root cells, which are directly involved in the symbiosis beneficial effects. This type of mycorrhizal association is as old as the evidence of first terrestrial plants on the earth (Chagas et al., 2018) and is the most widespread type. Endomycorrhiza are further divided into arbuscular mycorrhiza

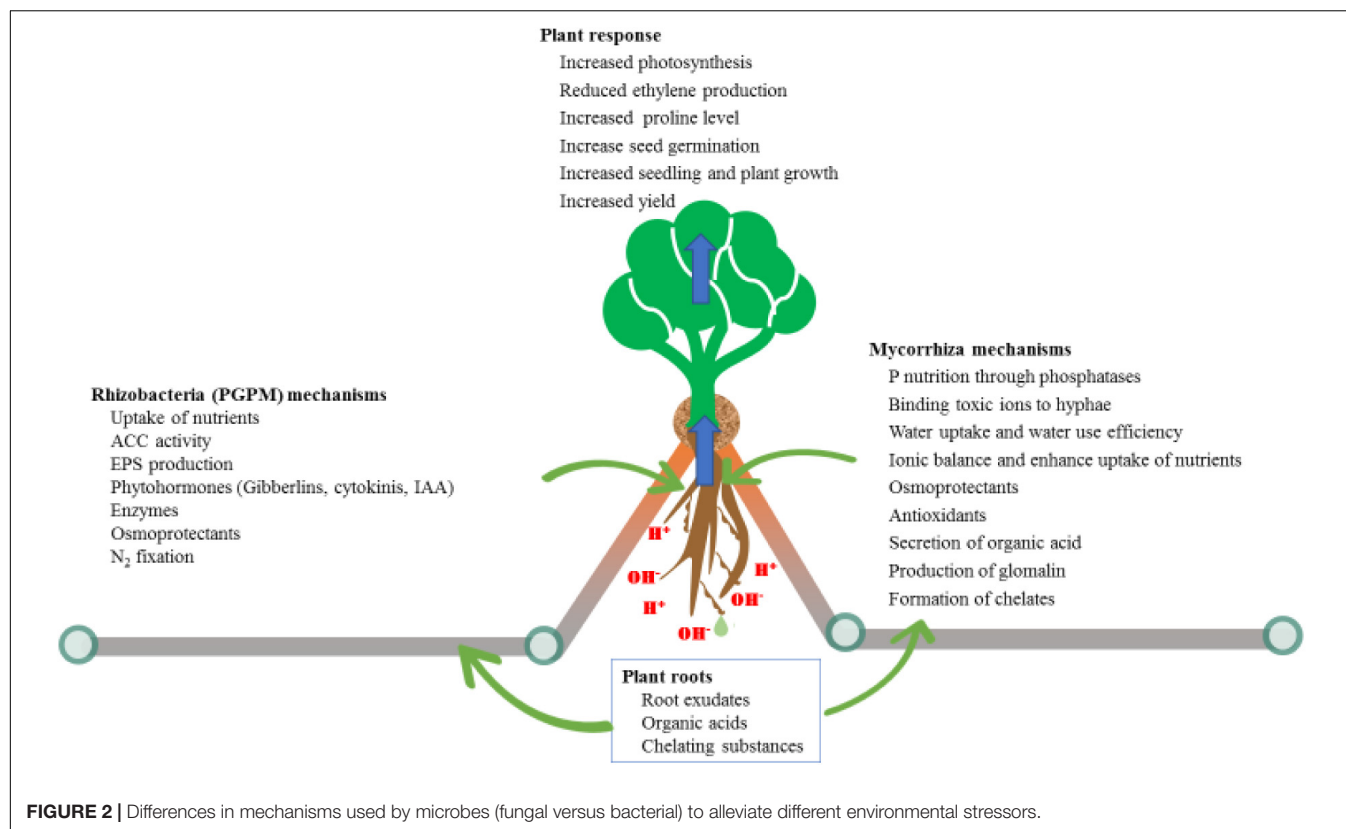
(AM), ericoid and orchidoid associations (Parniske, 2005). The promiscuity of AM make them associate with a wide range of hosts and assist most plant species with a range of stresses, and assist plants under nutrient imbalance situations, most notably P deficiencies (Zhu et al., 2007). Plants depend on AM particularly for P uptake from the nutrient stressed soils and the fungi involved depend on plants for their C requirements (Mishra et al., 2017). Acidic soils are also associated with metal toxicities, ultimately resulting in decreased root growth, which hinders overall plant growth and development. The major constraint to plant growth in acidic soil is the toxic effects of Al, Mn, and Fe, together with P deficiency. AM associations with plants is one of the most important plant-microbe associations, due to its ability to help plants with multiple stresses, compared to other association which may address only one stress. Many reports have shown that a wide variety of AM fungal species exist in acidic soils and help plants survive in such conditions; dealing with soil pH is always complex as it has numerous effects on both roots and mycorrhizal associations (Clark, 1997; Bloom et al., 2006). Even though more investigation is required to determine the best ways to exploit the potential benefits of both partners, some development has already been achieved. Studies of plants associations with AM fungi as a strategy to thrive in acid soils has revealed that these fungi provided benefit to plant growth through the ability to bind to toxic ions, secrete organic acids and glomalin (Thangavelu et al., 2014; **Figure 2**). Like plants AM fungal species also vary in their tolerance to acidic soils. AM fungal colonization of plant roots in soils is decreased at pH < 4 (Higo et al., 2011), which can explain differences in the ability of various AM fungal species to enhance plant growth under acidity stress and their variation in mechanisms to be employed (**Figure 2**).

Despite the advantages of AM to plants their beneficial impact on plants in acidic soils is less well documented than in non-acidic soils. The few studies that have examined the potential of AM fungi to assist plants in dealing with acidity stress (**Table 1**) have shown clearly that there are numerous benefits that can be acquired by plants. Clark (2002), revealed that use of various species of AM resulted in variable effects on switchgrass growth, but that all AM fungi caused greater yields than the control.

### Alkalinity

Developing proper and economically beneficial techniques for managing major challenges in crop plants, such as alkalinity stress, requires intensive research. PGPM with dual (salinity and alkalinity) stress tolerance, termed as haloalkaliphilic, have the ability to alleviate both salinity and alkalinity stresses of plants and improve growth (Siddique et al., 2011; **Table 2**). Bacteria with the ability to maintain their intrinsic pH below 9 when external pH is 9–11 are called alkaliphilic. This tolerance to alkalinity is achieved through a cytoplasmic membrane proton transfer system (Torbaghan et al., 2017). The Bacillaceae family of rhizobacteria is among the best described in this capacity, and has a wide range of host plants; the group of PGPM consists of 15 genera which include *Alkalibacillus*, *Bacillus* and *Haloalkalibacillus*, among others (Radhakrishnan et al., 2017; Torbaghan et al., 2017). The use of alkalinity tolerant





**TABLE 1 |** Effectiveness of PGPM on plant growth under acidity stress.

Plant species	Microbe	Stress type	Response of plant to microbe	References
Alfalfa	<i>Sinorhizobium meliloti</i> LPU63	Acidity	Ability to nodulate under low pH and increase plant growth	Segundo et al., 1999
Rice	<i>Burkholderia thailandensis</i> , <i>Burkholderia seminalis</i> <i>Sphingomonas pituitosa</i>	Acidity	Promote plant growth by increasing root volume and seedling dry weight	Panhwar et al., 2014
Mung bean	AM	Acidity	Increase and decrease of plant dry weight dependent on the AM species	Lin et al., 2001
Cowpea	AM	Acidity	Increase in biomass of plants with decreasing pH when inoculated with AM	Rohyadi, 2008
Chickpea	AM ( <i>Rhizophagus clarus</i> )	Acidity	Increased shoot dry matter	Alloush et al., 2000
Wheat	AM	Acidity	Significant increase in straw and grain yield	Suri et al., 2011
Switchgrass	AM	Acidity	Increased shoot dry weight	Clark, 2002
Sweet potato	AM	Acidity	Increased biomass of the plants	Yano and Takaki, 2005

rhizobacteria, with ability to produce indole acetic acid (IAA) and 1-aminocyclopropane-1-carboxylate (ACC), has the advantage of reducing ethylene production, and increasing K levels (Figure 2). This increases the relative humidity in plants and helps maintain ion homeostasis (Soleimani et al., 2018) which increased wheat growth. Several other mechanisms are also reported (Figure 2) by which PGPM alleviate abiotic stress in plants, such as by modulating hormones, enzymes, photosynthesis, secretion of organic acids and secondary metabolites (Bisht et al., 2019; Dixit et al., 2020). Moreover, rhizobacteria are involved in cycling of key nutrients such as N and C, which ensures long term reserves of nutrients in the soil.

The use of legumes is also regarded as a means of alkalinity management and has been applied in various parts of the world, as they have an inherent ability to acidify the soil. The combination of legumes and their symbionts to alleviate alkalinity problems is a sustainable way to redeem unproductive soils for agricultural use again. The use of AM fungi and rhizobia are among the oldest and best documented plant symbioses, and our understanding of them has shed light on helping plants withstand a range of stress conditions (Kumar et al., 2009). Abd-Alla et al. (2014) demonstrated that rhizobia and mycorrhizae can work together on faba bean to help the crop grow well under alkaline stress conditions; they found clear and

**TABLE 2 |** Effectiveness of PGPM on plants growth under alkalinity stress.

Plant species	Microbe	Stress type	Response of plant to microbe	References
Wheat	<i>Bacillus clausii</i>	Alkalinity	Increased root and shoot growth, increased grain yield	Torbaghan et al., 2017
	<i>Virgibacillus marismortui</i>	Saline-alkaline	Increased root and shoot growth, increased grain yield	Torbaghan et al., 2017
	<i>Lysinibacillus</i> sp.	Alkalinity	Improved seed germination and vegetative growth	Damodaran et al., 2019
	<i>Enterobacter</i> sp.	alkalinity	Increased photosynthetic rate	
Corn	<i>Bacillus</i> sp. NBRI YN4.4	Alkalinity	Photosynthetic pigment and sugar content improvement and decreased level of proline in corn	Dixit et al., 2020
Soybeans	<i>Burkholderia</i> spp. PER2F	Alkalinity	Increased plant growth and N/P ratio	Fernández et al., 2007
Chrysanthemum	<i>Bacillus licheniformis</i>	Alkalinity	Increased plant survival rate, photosynthesis and yield	Zhou et al., 2017
Faba beans	<i>Bacillus subtilis</i>	Alkalinity	Increased germination percentage, seedling growth and yield due to increased production of IAA by microbe at high pH	Yousef, 2018
Tall fescue	<i>Klebsiella</i> sp. D5A	Saline-alkaline	Increased plant growth through the activity of ACC	Liu et al., 2016
Wheat	<i>Bacillus simplex</i>	Alkalinity	Significant decrease in pH of the rhizosphere and increased plant growth and root P concentration	Hansen et al., 2020
Chickpea	<i>Mesorhizobium ciceri</i>	Alkalinity	Efficient in nodulation under high pH and increase plant growth	Müller et al., 2016

synergistic contributions of inoculated symbionts to alkalinity stress resilience of faba bean, and that this resulted in increased nodulation, nitrogenase activity and yield.

## Plant Breeding and PGPM to Improve Crop Yield Under Conditions of Acidity and Alkalinity Stresses

### Use of Alkalizing Agents in Acidic Soils

Acidic soil causes negative effects on plant growth and development (Rengel, 2011) leading to poor or no crop yield. Liming is a practice that corrects soil pH, moving it toward neutrality by addition of alkalizing materials rich in Ca and Mg. This material can be in solid (limestone, chalk) or liquid (hydrated lime) forms. Liming is the most common amendment technique and uses  $\text{CaCO}_3$  or  $\text{MgCO}_3$  as they have substantial ability to neutralize acids, hence increasing soil pH. By increasing soil pH, Al toxicity, which inhibits root growth is alleviated; P, which is fixed by Fe, becomes available leading to improved crop productivity and yield. This is the reason for the popularity of liming (Fageria and Baligar, 2008) as an amendment for acidic soils. How much lime to be applied for soil acidity amendment depends mainly on the type of lime, fertility status, crop grown, management practices and cost benefit analysis (economic) considerations.

### Use of Acid-Tolerant Crop Varieties

In acidic soils some plants are more tolerant than others, with variation even among genotypes of the same species; choosing the right crops based on the pH status of the soil is important (Hede et al., 2001). Though the soil may continue acidifying if no liming is done, it is advisable to include management practices, such as reducing the use of nitrogen fertilizers to reduce nitrate leaching, which increases soil acidity. Plowing crops or pasture into the soil will help reduce acidification of soil. In addition, rotation of legumes, which are rhizosphere acidifiers, with less acidifying crops would be of great importance in reducing soil acidity effects.

### Use of Acidifying Agents

Lowering soil pH to reduce alkalinity can be achieved by adding organic carbon to the soil; minimum tillage also helps to improve water retention and soil structure improvement, as does the use of cover crops and legumes, all of which acidify the soil. Furthermore, the use of elemental sulfur acidifies the soil by neutralizing alkalinity (Goulding, 2016).

The use of manure and compost is a common practice for management alkaline soils, as it improves organic carbon pools and soil structure. Manure and compost decrease soil pH greatly, as a result of releasing  $\text{NH}_4^+$ ,  $\text{CO}_2$  and organic acids during microbial decomposition (Walker et al., 2003). All these sum up to improvement of soil structure, water holding capacity and nutrient availability and, hence, soil health and productivity is regained.

Planting of cover crops helps alkaline soils in many ways, including reduction of exposure of the soil to agents of erosion. The cover crops also help in dissolution of carbonates, through root exudates, improvement of the soil structure and addition of organic matter to soil. Best cover crops for alkaline soils are sorghum and legumes with high carbon sequestration by stabilizing soil organic matter and structure, hence carbon and nitrogen concentration in the soil is increased (Williams et al., 2016).

Ammonium sulfate and other sulfur containing fertilizers cause quick declines in soil pH, which makes them good fertilizer options for alkaline soils. When oxidized elemental S and  $\text{SO}_2$  from the atmosphere produce acids which decrease the pH of alkaline soils. Similarly, nitrogen fixation by legumes forms  $\text{NH}_4^+$  inside the nodule, and excessive uptake of  $\text{K}^+$  causes charge imbalance, both of which leads to proton release by the roots to balance the charge (Marschner, 2011), resulting in rhizosphere pH decline.

### Use of Alkalinity Tolerant Crop Varieties

Plants have developed various strategies in response to environmental stimuli, such as activation of various metabolic

defense molecules. Among the metabolic molecules produced by plants to enhance defense capacity are salicylic acid, ethylene, calcium and jasmonic acid (Klessig and Malamy, 1994). Of the mentioned defense molecules salicylic acid has been confirmed to confer alkalinity tolerance to tomato plants, when applied exogenously, by reducing reactive oxygen species (ROS) generation and improving antioxidant defense against alkaline stress (Khan et al., 2019). Similarly, it was demonstrated that SA applied in combination with Si had positive effects on alkalinity tolerance in tomatoes (Khan et al., 2019). From such reports, it is clear that much is still to be understood regarding how different tolerance molecules and beneficial elements work together in helping plants grow under such alkaline stress conditions.

Plant breeding is one important approach to ensuring crop productivity in stress prone areas, including alkalinity of soil and water. A range of plants have shown various mechanisms of tolerance to alkaline stress, most of them showing early seed germination and seedling establishment. Cultivars of lentil tolerant to alkalinity stress are known to have shoots with a thicker epidermis than sensitive cultivars (Singh et al., 2018). Similarly, tolerant lentils (Singh et al., 2018), finger millet (Krishnamurthy et al., 2014) and *Lotus tenuis* (Paz et al., 2012) minimize  $\text{Na}^+$  uptake by having intact pericycle and stele regions. Despite the presence of tolerance mechanisms for alkalinity stress by plants much remains unknown in relation to other related stresses, such as salinity and drought. According to Bui et al. (2014), the success of breeding for salinity tolerance required that increased attention also be placed on alkalinity tolerance.

As previously indicated a major limiting nutrient under alkaline stress is Fe; plants have developed two strategies to deal with this problem. Firstly, most plants optimize Fe uptake via transporter by first reducing  $\text{Fe}^{+++}$  to  $\text{Fe}^{++}$  in the root plasma membrane. Secondly, development of specific iron uptake by release of phytosiderophores for chelating iron (Curie et al., 2001). The iron chelators have high affinity to  $\text{Fe}^{+++}$  when released into the rhizosphere; the chelated iron is then taken up by the plant through yellow stripe-like (YSL) transporters a family of protein used by maize (Yordem et al., 2011).

Plants have also been shown to have mechanisms for alkaline stress management through acidification of the rhizosphere by  $\text{H}^+$  ATPase activity related to plasma membrane proton extrusion (Xu et al., 2013).

## FUTURE PERSPECTIVES

From various studies of tolerance to extreme pH there is a wider range of adaptation of microbes than plants. Our limited knowledge on the full information available in the genome of microbes that help them in adapting to such extremes will pave the way to understanding and broadening their application in biotechnology and crop production. The constant need to explore the unknown potential of microbes in helping enhance plant productivity under various unfavorable conditions of growth and is currently developing quickly and contributing to improvement of plant growth under stressful environmental conditions.

Currently we use synthetic fertilizers as part of our approach to feeding the growing global population. The uptake efficiency of these fertilizers by plants is generally 30–50%, leading to economic losses and large environmental impacts, due to large quantities of the fertilizer being lost to water bodies and the atmosphere (Adesemoye and Kloepper, 2009). PGPM have shown their ability to increase the efficiency of nutrient release from fertilizer and subsequent uptake by plants. Bearing in mind that soil acidification and alkalization are both gradual and progressive processes, preparing ahead of time is not optional. The future of crop production sustainability meaningfully depends on better understanding of PGPM in conferring stress alleviation and ways to effectively introducing them under field conditions, to provide the same results that are observed under controlled environment conditions.

## CONCLUSION

The importance of pH in agriculture is well understood, and similar to a patient's temperature in humans. Most of the literature has acknowledged pH is a “master” variable in productivity of agricultural soils as it controls soil chemistry. This review started with an evolutionary perspective regarding plants and microbes, an interaction that has always been present, to the benefit of both members of the partnership. It is becoming clearer that the interaction has always provided plants with a mechanism of survival even in harsh environmental stress conditions. With the ongoing development of climate change conditions and associated multiple stresses, potentially occurring simultaneously and impacting plant productivity. This is quite similar to pH stress which is accompanied by other stresses in its effects. The reviewed literature has shown that most of the acid and alkaline soil remediation measures are focused on cultural practices and breeding for tolerance. Little has been established regarding utility of PGPM as a mechanism of dealing with acidity and alkalinity stresses. Our future will require further breeding for pH stress and the help of microbes that provide enhanced tolerance to pH stress. With this consideration in mind the potential for alleviation of extreme pH stress by PGPM has become clearer and there is a need to now focus more research effort specifically on acid and alkaline stresses in this regard.

## AUTHOR CONTRIBUTIONS

LM structured and prepared manuscript initially. DS provided the conceptual framework for the manuscript as well as feedback and guidance during manuscript development. Both authors contributed to the article and approved the submitted version.

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# Alleviation of Detrimental Effects of Salt Stress on Date Palm (*Phoenix dactylifera* L.) by the Application of Arbuscular Mycorrhizal Fungi and/or Compost

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The date palm is a commercially important woody crop and is a good target plant for improving agricultural yields in extreme environments. However, salinity has been the primary abiotic stress complicating its cultivation and damaging its production worldwide. This study investigated the effect of alleviating salt stress on date palm growth and development by using arbuscular mycorrhizal fungi (AMF) and/or compost. The experiment was arranged in a completely randomized design with eight treatments. The treatments comprised control without inoculation or amendment and application of compost (made from green waste) and AMF (an autochthonous consortium) individually or in combination under non-saline (0 mM NaCl) or saline (240 mM NaCl) conditions. Growth, physiological characteristics, nutrient uptake, chlorophyll content, oxidative stress markers, and antioxidant enzyme activities were assessed. Salt stress increased sodium (Na<sup>+</sup>) and chlorine (Cl<sup>-</sup>) content, lipid peroxidation and proline, soluble sugar, and H<sub>2</sub>O<sub>2</sub> content. However, it reduced growth parameters, AMF colonization, leaf water potential, nitrogen (N), phosphorus (P), potassium (K<sup>+</sup>), calcium (Ca<sup>2+</sup>), and chlorophyll content. The application of AMF and compost separately or in combination mitigated the deleterious effects induced by salinity. AMF inoculation contributed to plant salt tolerance through strategies such as increased nutrient uptake (particularly P and Ca<sup>2+</sup>), chlorophyll content, relative water content, stomatal conductance, antioxidant enzymatic activities (superoxide dismutase, ascorbate peroxidase, catalase) and by decreasing lipid peroxidation and H<sub>2</sub>O<sub>2</sub> content. Plants grown in soil amended with compost under salt stress showed an improvement particularly in K<sup>+</sup> and proline content and a decrease in H<sub>2</sub>O<sub>2</sub> concentration compared to controls under the saline condition. In the presence of NaCl stress, the dual application of the compost and AMF consortium maximized plant growth, stomatal conductance, leaf water potential, all antioxidant enzyme activities and P, K<sup>+</sup>, N, and Ca<sup>2+</sup> uptake as well as proline and soluble sugar content. However,



it reduced  $\text{Na}^+$  and  $\text{Cl}^-$  uptake and oxidative stress marker content. In conclusion, our study suggests that the application of AMF with compost has the potential to improve the tolerance of date palm seedlings to salt stress more than AMF or compost applied separately.

**Keywords:** biofertilizers, mycorrhiza, nutrient uptake, oxidative stress, salinity, sustainable agriculture

## INTRODUCTION

Climate change causes perturbations to the surrounding environment and significantly induces soil salinization. In arid and semi-arid regions, sporadic rainfall and high temperatures have resulted in increased evapotranspiration rates, which, when combined with the use of saline water for irrigation and excessive use of chemical products, has led to increasing soil salinization (Munns, 2005; Trenberth et al., 2014). It has been estimated that saline soils account for around 8% of the earth's surface land and are increasing worldwide (Hajiboland, 2013). Soil salinity is a global problem, damaging agricultural lands and causing a >20% reduction in agricultural yields (Porcel et al., 2012; Setia et al., 2013). The increase in soil salinity results in osmotic as well as specific ion effects, which induce secondary stress in plants known as oxidative stress (Evelin et al., 2019; Abdel Latef et al., 2020). Consequently, salt stress causes adverse effects on physiological and biochemical activities such as mineral homeostasis, seed germination, osmotic balance, photosynthesis, and respiration processes (Porcel et al., 2012; Ben Laouane et al., 2019). Oxidative salt-induced stress leads to the production of reactive oxygen species (ROS) that induce deleterious effects on normal metabolism and growth (Gill and Tuteja, 2010). However, plants can tolerate both osmotic and oxidative stress caused by soil salinity through several mechanisms (Evelin et al., 2019). Osmotic adjustment is one of the defense mechanisms that help plants to handle the osmotic and ion toxicity effects by the exclusion of the salts and compartmentation of  $\text{Na}^+$  ions in the vacuole or apoplast (Apse and Blumwald, 2007). Salinity tolerance can also occur *via* the production of osmolytes such as glycine-betaine, proline, and soluble sugars (Zhu, 2003). Furthermore, plants possess an efficient antioxidant defense system to scavenge ROS (Sharma et al., 2012). Enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), and glutathione reductase (GR) have been previously reported as the main antioxidant enzymes under salinity stress (Abdel Latef and Chaoxing, 2014; Foyer, 2018; Evelin et al., 2019).

Arbuscular mycorrhizal fungi (AMF) have generated growing interest as an efficient alternative to promote plant growth and stress tolerance. They may also contribute to increased yield and nutrition of host plants for sustainable agriculture (Baslam et al., 2011a, 2012; Baslam and Goicoechea, 2012; Bouamri et al., 2014; Bowles et al., 2017; Rillig et al., 2019; Zhang et al., 2019). This symbiosis is known to mitigate the harmful effects of salt stress on plants although the fungi are adversely affected by salinity themselves (Abdel Latef and Chaoxing, 2011; Ait-El-Mokhtar et al., 2019). In the saline environment, AMF improve

the rhizospheric condition of the soil and enhance the mineral nutrition and the water uptake of the host plant (Augé et al., 2014; Hodge and Storer, 2014; El Kinany et al., 2019). AMF colonization has been widely reported to reduce the influx of toxic ions (sodium and chlorine) into the root system (Daei et al., 2009), and stimulate the photosynthetic apparatus and enhance the effectiveness of antioxidant defense system (Hidri et al., 2016; Boutasknit et al., 2020). These features led to in recent years the commercial production of AMF as a “biofertilizer” for field use, to reduce the intensive use of agrochemicals with no loss in yield. However, the merits of such biofertilizers are currently under debate owing to the variable effects of AMF on plant biomass (Berruti et al., 2016; Ryan and Graham, 2018; Rillig et al., 2019) which are driven by intrinsic (host plant and AMF identities) and extrinsic/environmental (nutrient and water) factors.

Previous studies have reported the utilization of organic compost fertilizers for the restoration of salt-affected soils (Meena et al., 2016; Mbarki et al., 2018). When incorporated into the soil, compost is mineralized, thereby providing a sustained release of available nutrients to plants (D'Hose et al., 2014; Ngo and Cavagnaro, 2018; Ou Zin et al., 2020). Compost can alleviate salinity stress in plants by improving soil fertility (Meena et al., 2016; Raklami et al., 2019), promoting nutrient availability and plant growth (Duong et al., 2012; Trivedi et al., 2017) and stimulating respiration, photosynthesis, and chlorophyll content (Lakhdar et al., 2008).

The date palm (*Phoenix dactylifera* L.) is one of the most important fruit crops in the arid and semiarid areas of North Africa and the Middle East where it is cultivated for its nutritional, socio-economical, and environment value (Chao and Krueger, 2007). The fruits of the date palm are rich in essential nutrients such as sugars, proteins, fibers, minerals, antioxidants, and vitamins (Vayalil, 2012; Kamal-Eldin and Ghnimi, 2018). To date, several studies have analyzed date palm responses to salinity stress (Sperling et al., 2014; Yaish and Kumar, 2015; Meddich et al., 2018; Ait-El-Mokhtar et al., 2019; Al Kharusi et al., 2019) but few have addressed the effects of AMF inoculation in alleviating salt stress in date palms (Meddich et al., 2018; Ait-El-Mokhtar et al., 2019). Furthermore, no study has yet tested the role of the combined application of AMF and compost in the salt tolerance of date palms and the underlying mechanisms. Therefore, the present study was carried out to investigate the effects of both fertilizers alone and in combination on growth, physiological, and biochemical traits of date palm seedlings under saline conditions. This was accomplished by using native AMF and locally produced compost to improve plant tolerance, as well as a clear understanding of the mechanisms implicated in AM-compost-induced salt stress tolerance. The study paves the

way for the use of biological fertilizers to strengthen plants' adaptability and to further studies on the defense mechanism and functioning of AMF and/or compost in the palm family of plants in response to changing environments.

## MATERIALS AND METHODS

### Biological and Organic Materials and Treatments

Seeds of *Phoenix dactylifera* cv. Boufeggous obtained from Aoufous palm grove (Errachidia, Morocco) were surface-sterilized by immersion for 10 min in 10% (v/v) sodium hypochlorite solution followed by several washings with sterile distilled water. Seeds were germinated in plastic bowls containing a sterile sandy substrate collected from "Oued Lahjar" river limiting the Northeastern palm grove of Marrakesh, Morocco. The river sand has the following characteristics: pH: 9.31, EC: 0.29 dS cm<sup>-1</sup>, 0.001% P (Phosphorus), 0.001% K (Potassium), 0.006% Mg (Magnesium), 0.012% Fe (Iron), 0.01% Ca (Calcium), 0.002% Na (Sodium), and 0.01% Al (Aluminum) (Anli et al., 2020). The date palm seeds were incubated for 3 weeks at 38°C in the dark. Two months after germination, seedlings were transplanted into plastic pots containing 2.3 kg of river sand (same substrate as above) previously sterilized for 3 h at 180°C during three consecutive days.

The native mycorrhizal *Aoufous* consortium (MAC) had been isolated earlier from the rhizospheric soil of *P. dactylifera* from the Tafilalet palm grove (500 km southeast of Marrakesh) and it contained a mixture of native species: (i) *Glomus* sp. (15 spores/g soil), (ii) *Sclerocystis* sp. (9 spores/g soil), and (iii) *Acaulospora* sp. (one spore/g soil) (Meddich et al., 2015).

The trap culture protocol was used to propagate MAC under greenhouse-controlled conditions for 3 months using *Zea mays* L. as a host plant. The mycorrhizal fungi inoculum was a mixture of spores, soil, hyphae, and infected root fragments from the trap culture. The most probable number (MPN) test (Sieverding, 1991) was used to assess inoculum potential infectivity and to determine the doses to be applied. According to the MPN test, the trap culture inoculum contained 1056 infective propagules/100 g. The application of mycorrhizal inoculum was carried out when transplanting seedlings. The inoculated dosage, added to the corresponding pots, was 10 g of inoculum per pot containing approximately 110 spores with Ma = 78.8%. Non-inoculated pots received the same amount of autoclaved mycorrhizal fungi inoculum.

Compost was prepared from green waste as described previously by Meddich et al. (2016). The compost had the following characteristics based on dry matter: pH (6.87), organic matter (527.2 g kg<sup>-1</sup>), organic C (306.5 g kg<sup>-1</sup>), total N (21.9 g kg<sup>-1</sup>), C/N ratio (14.0), ashes (490 g kg<sup>-1</sup>), available P (2.5 mg g<sup>-1</sup>), NH<sub>4</sub><sup>+</sup> (0.03 10<sup>-3</sup> mg g<sup>-1</sup>), NO<sub>3</sub><sup>-</sup> (0.07 10<sup>-3</sup> mg g<sup>-1</sup>), NH<sub>4</sub><sup>+</sup>/NO<sub>3</sub><sup>-</sup> ratio (0.44), bacterial population (2.12 10<sup>5</sup> CFU g<sup>-1</sup>), and fungal population (9.75 10<sup>4</sup> CFU g<sup>-1</sup>). The compost was added to the corresponding pots, when transplanting seedlings, in a proportion of 5% (w/w).

After transplantation, date palm seedlings were irrigated regularly with distilled water for 5 months after germination, and then transplants were subjected to two salt concentrations (0 and 240 mM NaCl). The NaCl concentration was gradually added (60, 120, 180, and 240 mM NaCl) to the pots on alternative days for 2 weeks to reach 240 mM NaCl without causing osmotic shock (Estrada et al., 2013). Excess water due to drainage was collected and added to the soil of the corresponding pots. Plants were irrigated with either distilled water (control) or saline solution (NaCl), with EC fixed of 24 dS/m until harvest. EC of the saline solution was monitored regularly and adjusted (and pH level) when necessary using NaCl and distilled water. The pH and EC were measured using a pH meter HI 9025 and a conductivity meter HI-9033 (Hanna Instruments, Padova, Italy), respectively. All plants were grown for 14 months without adding any fertilizer.

### Experimental Design and Plant Growth Conditions

The experimental design consisted of eight treatments (untreated plants under normal condition, Control 0 mM; seedlings treated with compost alone under 0 mM, +compost 0 mM, seedlings inoculated with AMF alone under 0 mM, +AMF 0 mM, seedlings treated with compost and inoculated with AMF under 0 mM, +compost+AMF 0 mM, untreated plants under 240 mM NaCl, Control 240 mM; seedlings treated with compost alone under 240 mM, +compost 240 mM, seedlings inoculated with AMF alone under 240 mM, +AMF 240 mM, seedlings treated with compost and inoculated with AMF under 240 mM, +compost+AMF 240 mM) with a factorial combination of 2 × 2 × 2 CxMxS (CompostxAMFxSalinity). Pots were arranged in a completely randomized block design. Ten replications (one plant per pot) were sampled in each treatment.

Plants were grown for 14 months under semi-controlled environmental conditions in a greenhouse at the Faculty of Science Semlalia (Cadi Ayyad University, Marrakesh, Morocco) under natural light (photon flux density ranged from 500 to 750 μmol m<sup>-2</sup> s<sup>-1</sup>). The average temperature was 25.5°C and a relative humidity average of 68%.

### Determination of Plant Biomass Production and Root Colonization

At harvest, plants were first rinsed with tap water and then with distilled water. The shoot and root system were separated and the growth parameters (plant height, number of leaves, leaf area, and shoot and root dry weights) were measured. The leaf area was determined using an LC 4800 scanner and Winfolia v. 2004 software.

To assess AMF colonization, roots fragments, from the lateral root system, were carefully washed, cleared with 10% of KOH and stained with Trypan blue (Phillips and Hayman, 1970). Thirty 1 cm long fine root segments, for each replicate sample, were observed under a Zeiss Axioskop 40 microscope at 40–100× magnification. The number of root segments colonized by different AMF structures was used to calculate AMF colonization (McGonigle et al., 1990). AMF infection frequency and intensity

were determined using the equations below:

$$\begin{aligned} \text{AMF infection frequency (Fa)(\%)} &= \left( \frac{\text{Infected root segments}}{\text{Total root segments}} \right) \times 100 \\ \text{AMF infection intensity (Ma)(\%)} &= \frac{(95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1)}{\text{Total root segments}} \end{aligned}$$

Where “n5” corresponds to the number of roots with infection level of 5 (infection rate 90–100%). “n4” corresponds to the number of roots at level 4 (infection rate 50–90%). “n3” corresponds to the number of roots at level 3 (infection rate at 10–50%). “n2” corresponds to the number of roots at infection level 2 (infection rate 1–10%). “n1” corresponds to the number of roots at level 1 (infection rate 0–1%).

### Determination of Nutrient Concentration

Oven-dried shoots and roots were powdered to determine the mineral concentration. P content was estimated using the Olsen method (Olsen and Dean, 1965). Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> content in the plant was estimated by flame photometry (JENWAY, PFP7) as described by Wolf (1982). The Nitrogen (N) content was determined by colorimetry after the Kjeldahl digestion and chlorine (Cl<sup>−</sup>) content was measured using the silver nitrate (AgNO<sub>3</sub>) titration method.

### Determination of Photosynthetic Efficiency and Stomatal Conductance

The third youngest, fully expanded and attached leaves from five plants for each treatment were used for measurements. An average of four records from different parts of each leaf was considered for each replicate. The efficiency of photosystem II was evaluated by measuring chlorophyll fluorescence using a portable fluorometer (Opti-sciences OSI 30p) for dark-adapted leaves. Leaves were acclimated to dark for 30 min using leaf clips before measurements were taken. Chlorophyll fluorescence variables were recorded: Initial (F<sub>0</sub>), maximum (F<sub>m</sub>), variable (F<sub>v</sub> = F<sub>m</sub> − F<sub>0</sub>) fluorescence as well as F<sub>v</sub>/F<sub>m</sub> ratio (Baker, 2008).

Stomatal conductance (g<sub>s</sub>) was measured on five replications per treatment on a sunny day before harvest and by using a porometer system (Leaf Porometer LP1989, Decagon Device, Inc., Washington, USA) following the user manual instructions. The second youngest leaf from five different plants from each treatment was used for measurements.

### Determination of Leaf Relative Water Content and Leaf Water Potential

Leaf portions were taken from two plants per replicate (the third leaf from the top) to determine the fresh weight (FW), dry weight (DW), and turgid weight (TW). Values of FW, TW, and DW were used to calculate leaf relative water content (LRWC) using the following equation (Barrs and Weatherly, 1962):

$$\text{LRWC (\%)} = \frac{\text{FW} - \text{DW}}{\text{TW} - \text{DW}} \times 100$$

The leaf water potential (LWP) was measured on newly mature leaves from each plant (five repetitions per treatment) using a pressure chamber according to Scholander et al. (1965).

### Determination of Photosynthetic Pigments Content

Photosynthetic pigments were extracted from leaf samples in 80% acetone according to Arnon (1949). The extracted material was centrifuged at 10,000 × g for 10 min. The optical density of the supernatants was recorded at 480, 645, and 663 nm using a UV-vis spectrophotometer (UV-3100PC spectrophotometer). Values of absorbance were used to calculate the content of photosynthetic pigments. A blank with 80% acetone served as the control.

### Determination of Lipid Peroxidation and Hydrogen Peroxide Content

Lipid peroxidation was measured in terms of malondialdehyde (MDA) content according to the method of Dhindsa et al. (1981). Fresh leaf and root material (0.25 g) was homogenized in 10 mL of 0.1% trichloroacetic acid (TCA) and centrifuged at 18,000 × g for 10 min. Two-milliliter aliquots of the supernatant were mixed with 2 mL of 20% TCA containing 0.5% thiobarbituric acid (TBA). The mixture was heated at 100°C for 30 min, quickly cooled and then centrifuged at 10,000 × g for 10 min for clarification. The absorbance of the supernatant was measured at 532 nm (A532). The unspecific turbidity was corrected by subtracting A600 from A532.

Hydrogen peroxide content in leaves and roots was determined as described by Velikova et al. (2000). 0.25 g of fresh material was homogenized in a cold mortar with 5 mL 10% (w/v) TCA and then centrifuged at 15,000 × g for 15 min at 4°C. The supernatant was then recovered to determine the content of H<sub>2</sub>O<sub>2</sub>. 0.5 mL of potassium phosphate buffer (10 mM, pH 7) and 1 mL of iodic potassium (1 M) was added to 0.5 mL of the supernatant. The absorbance at 390 nm was recorded after 1 h of incubation in the dark. The blanks were made by replacing the sample extract with 10% TCA.

### Determination of Proline and Soluble Sugar Content

Proline from the date palm leaves and roots was extracted following the method of Bates et al. (1973). Fresh plant material (0.1 g) was ground in a mortar with 5 mL of 3% (w/v) aqueous sulphosalicylic acid and then centrifuged at 12,000 × g for 15 min. The supernatant (2 mL) was mixed with 2 mL of glacial acetic acid and 2 mL of acid ninhydrin reagent and incubated at 100°C water bath for 1 h. The reaction was terminated by placing the mixture in an ice bath followed by extraction with toluene. The absorbance was read at 520 nm and the proline concentration was calculated from a calibration curve using proline as standard.

A sample of 0.1 g of frozen material from leaves and roots was homogenized with 4 mL of ethanol (80%) then the homogenate was centrifuged at 5,000 rpm for 10 min. The resulting supernatant was collected, while the rest was resuspended again



by adding 2 mL of ethanol and recentrifuged. Both supernatants were used to quantify total soluble sugar according to the method described by Dubois et al. (1956). The supernatant (1 mL) was mixed with 1 mL of phenol solution (5%) and 5 mL of concentrated sulfuric acid. After 5 min, the absorbance was measured at 485 nm in a UV-3100PC spectrophotometer. Soluble sugar content was determined using glucose as a standard.

## Determination of Antioxidant Enzyme Activities

Enzyme extraction was performed according to optimized protocols described by Benhiba et al. (2015). Fresh leaf and root material (0.5 g) were frozen in liquid nitrogen and the powder was extracted at 4°C in a 5 mL solution containing 0.1 M potassium phosphate buffer (pH 7.0), 0.1 g polyvinylpyrrolidone (PVPP), and 0.1 mmol ethylenediaminetetraacetic acid (EDTA). The homogenate was centrifuged at  $18,000 \times g$  for 10 min at 4°C, and the supernatant was kept at -20°C for subsequent enzyme assays.

Superoxide dismutase (SOD, EC 1.15.1.1) activity estimation was based on the ability of SOD to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) and was assayed by the method of Beyer and Fridovich (1987). One unit of SOD was defined as the amount of enzyme required to induce a 50% inhibition of NBT reduction at 25 °C. The activity of SOD was expressed at unit  $\text{min}^{-1} \text{mg}^{-1}$  protein. Catalase (CAT, EC 1.11.1.6) activity was determined by monitoring the decrease in absorbance at 240 nm for 3 min following the consumption of  $\text{H}_2\text{O}_2$  (Aebi, 1984). The reaction mixture consisted of 0.1 M potassium phosphate buffer (pH 7.0), 0.1 mM EDTA, 20 mM  $\text{H}_2\text{O}_2$  and 100  $\mu\text{L}$  of enzyme extract in a 2 mL volume. The activity of ascorbate peroxidase (APX, EC 1.11.1.11) was measured according to the method of Amako et al. (1994). APX was assayed as a decrease in absorbance at 290 nm for 1 min. The assay mixture contained 50 mM potassium phosphate buffer (pH 7.0), 0.5 mM  $\text{H}_2\text{O}_2$ , 0.1 mM ascorbate, and 100  $\mu\text{L}$  of enzyme extract. The reaction was started by adding the enzyme extract and the decrease in absorbance was recorded. Peroxidase (POD, EC 1.11.1.7) activity was measured using the guaiacol test by following the change of absorbance at 470 nm. The activity was assayed for 3 min in a reaction solution containing 100 mM potassium phosphate buffer (pH 7.0), 20 mM guaiacol, 10 mM  $\text{H}_2\text{O}_2$ , and 0.1 mL enzyme extract in a 3 mL volume (Polle et al., 1994). The protein concentration in the different extracts was determined following the protocol of Bradford (1976) by using bovine serum albumin (BSA) as a standard.

## Statistical Analyses

The experiment data presented are mean values based on five replicates  $\pm$  standard error (SE) per treatment. Statistical analysis was carried out with the software package SPSS 10.0 for Windows. All results were subjected to a three-way multivariate analysis of variance (MANOVA) for main effects (Salinity, S; Compost, C; AMF inoculation, AMF) and their interactions. A principal component analysis (PCA) was performed using XLSTAT-software v. 2016, to explore the different interactions among variables and different applied

treatments. The comparisons among means were assessed using Duncan's test calculated at  $P < 0.05$ .

## RESULTS

### Growth and Mycorrhizal Colonization

Salinity had a significant adverse ( $P < 0.001$ ) (Table 1 and Figure S1) effect on the growth of date palms by reducing plant height, the number of leaves, leaf area and shoot and root dry matter (Table 1). The impact of salinity was more pronounced in roots than shoots. Root dry matter was reduced by 42% while shoot dry weight was reduced by 34% compared with non-stressed plants. Application of AMF and compost separately or in combination improved all the plant growth characters affected by salt. The dual application of AMF and compost significantly mitigated the effects of salt stress on all growth parameters followed by the AMF treatment, which in turn was better than the compost treatment. Under the saline condition, the maximum improvement was recorded for the shoot dry matter of plants grown in the presence of AMF and compost with an increase of 226% and the minimum improvement was recorded for the plant height of seedlings grown in the presence of compost with an enhancement of 12% in comparison to untreated stressed plants. Interactions among  $C \times M \times S$  treatments were significant ( $P < 0.05$ ) for the number of leaves and leaf area (Table 1 and Figure S1).

As shown in Table 1, no root colonization was observed in non-inoculated plants. Mycorrhizal colonization was particularly high in the absence of salinity stress and compost (Fa = 100%, Ma = 79%). Under NaCl stress, the frequency and intensity of mycorrhization decreased by 33 and 57%, respectively ( $P < 0.001$ ). In the same conditions, root colonization was further reduced by the application of compost (Fa by 53% and Ma by 63%). Significant interactions between  $C \times M \times S$  treatment were observed for these two parameters ( $P < 0.01$ ) (Table 1 and Figure S2).

### Nutrient Content

Results from Figure 1 indicate that the application of the different treatments had considerable effects on the different mineral uptakes of date palm plants under saline and non-saline conditions. P, N,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  content was significantly ( $P < 0.001$ ) decreased with the application of 240 mM NaCl in comparison with the control plants.  $\text{K}^+$  content was the most affected with ca. 40% decrease and the least affected was P content with ca. 25% decrease. However, the uptakes of  $\text{Na}^+$  and  $\text{Cl}^-$  were significantly ( $P < 0.001$ ) higher in salt-affected plants. As a result, the  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  ratios were significantly ( $P < 0.001$ ) reduced by NaCl stress (Table 2). Under saline stress, the dual application of AMF and compost showed a greater increase in P, N,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  nutrients and a maximum decrease in  $\text{Na}^+$  and  $\text{Cl}^-$  content. This was followed by the application of AMF alone, which recorded a greater increase in P and  $\text{Ca}^{2+}$  uptakes and maximum decrease in  $\text{Na}^+$  content. In contrast, the application of compost alone showed only a high increase in  $\text{K}^+$  content (Figure 1). Accordingly, and under the same conditions, plants grown in the presence of AMF+compost had greater values for

**TABLE 1** | Growth traits and AMF infection of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	PH (cm)	NL	LA (cm <sup>2</sup> )	SDW (g plant <sup>-1</sup> )	RDW (g plant <sup>-1</sup> )	Fa (%)	Ma (%)
0 mM	– AMF	– compost	34.5 ± 0.9 <sup>d</sup>	4.2 ± 0.4 <sup>d</sup>	24.2 ± 0.8 <sup>f</sup>	2.8 ± 0.1 <sup>e</sup>	1.9 ± 0.2 <sup>d</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>e</sup>
		+ compost	38.8 ± 1.3 <sup>c</sup>	6.2 ± 0.4 <sup>c</sup>	35.7 ± 0.1 <sup>c</sup>	6.7 ± 0.3 <sup>b</sup>	3.3 ± 0.4 <sup>b</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>e</sup>
	+ AMF	– compost	42.5 ± 1.2 <sup>a</sup>	7.2 ± 0.4 <sup>b</sup>	38.0 ± 0.3 <sup>b</sup>	6.3 ± 0.3 <sup>bc</sup>	4.1 ± 0.3 <sup>a</sup>	100.0 ± 0.0 <sup>a</sup>	79.0 ± 4.1 <sup>a</sup>
		+ compost	40.7 ± 1.1 <sup>b</sup>	8.2 ± 0.4 <sup>a</sup>	40.3 ± 0.6 <sup>a</sup>	8.8 ± 0.4 <sup>a</sup>	4.1 ± 0.4 <sup>a</sup>	100.0 ± 0.0 <sup>a</sup>	48.7 ± 3.6 <sup>b</sup>
240 mM	– AMF	– compost	29.6 ± 1.2 <sup>e</sup>	3.8 ± 0.4 <sup>d</sup>	14.5 ± 0.4 <sup>h</sup>	1.8 ± 0.1 <sup>f</sup>	1.1 ± 0.1 <sup>e</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>e</sup>
		+ compost	33.2 ± 1.3 <sup>d</sup>	5.8 ± 0.4 <sup>c</sup>	21.2 ± 0.5 <sup>g</sup>	4.5 ± 0.6 <sup>d</sup>	2.0 ± 0.3 <sup>d</sup>	0.0 ± 0.0 <sup>d</sup>	0.0 ± 0.0 <sup>e</sup>
	+ AMF	– compost	34.8 ± 1.7 <sup>d</sup>	5.8 ± 0.4 <sup>c</sup>	27.0 ± 0.5 <sup>e</sup>	4.6 ± 0.6 <sup>d</sup>	2.7 ± 0.3 <sup>c</sup>	66.5 ± 3.7 <sup>b</sup>	34.1 ± 2.8 <sup>c</sup>
		+ compost	34.0 ± 2.0 <sup>d</sup>	6.2 ± 0.4 <sup>c</sup>	28.0 ± 0.3 <sup>d</sup>	6.0 ± 0.7 <sup>c</sup>	2.1 ± 0.1 <sup>d</sup>	47.3 ± 4.5 <sup>c</sup>	18.1 ± 0.7 <sup>d</sup>
Significance level									
S			***	***	***	***	***	***	***
C			**	***	***	***	***	***	***
M			***	***	***	***	***	***	***
SxC			ns	ns	***	***	*	***	**
SxM			*	**	ns	*	**	***	***
CxM			***	**	***	***	***	***	***
SxCxM			ns	*	***	ns	ns	***	**

The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing a three-way MANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi; PH, plant height; NL, number of leaves; LA, leaf area; SDW, shoot dry weight; RDW, root dry weight; Fa, AMF infection frequency; Ma, AMF infection intensity. <sup>ns</sup> non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .

$K^+/Na^+$  and  $Ca^{2+}/Na^+$  in both shoots and roots compared to the untreated plants, whereas the mycorrhizal plants only showed greater values for  $Ca^{2+}/Na^+$  (Table 2). The interaction between  $C \times M \times S$  application had a significant effect on  $Na^+$  and  $K^+$  uptakes and  $K^+/Na^+$  ratio ( $P < 0.05$ ) (Figure 1 and Table 2).

## Physiological Parameters and Water Status

The effect of salt stress and non-saline treatment on leaf relative water content (LRWC), leaf water potential (LWP), stomatal conductance ( $g_s$ ), and photosynthetic efficiency variables is shown in Table 3. In general, the untreated date palm plants were more sensitive to salinity than treated plants. The parameter most affected by salinity was  $g_s$ , which decreased 42% in non-treated stressed plants. Under salt stress, LRWC and photosynthetic efficiency recorded the maximum improvement (20 and 92%, respectively) with mycorrhizal plants and best leaf water potential and stomatal conductance enhancement (53 and 107%, respectively) were obtained with the AMF+compost application. Interactions among  $C \times M \times S$  treatment had a significant effect ( $P < 0.001$ ) on stomatal conductance and photosynthetic efficiency (Table 3). Salt stress caused a severe decrease in the quantum efficiency of photosystem II ( $F_v/F_m$ ) in non-treated plants. The presence of AMF and/or compost had significantly higher  $F_v/F_m$  as compared to untreated plants independently of the salinity conditions.

## Photosynthetic Pigments Content

As shown in Table 4, salinity caused a significant ( $P < 0.001$ ) decline in photosynthetic pigment concentrations. Indeed, the application of NaCl stress in untreated plants decreased chlorophyll a (Chl a) by 58%, chlorophyll b (Chl b) by 51%, carotenoids by 47%, and total chlorophyll by 56% in comparison with non-stressed plants. The application of compost and AMF

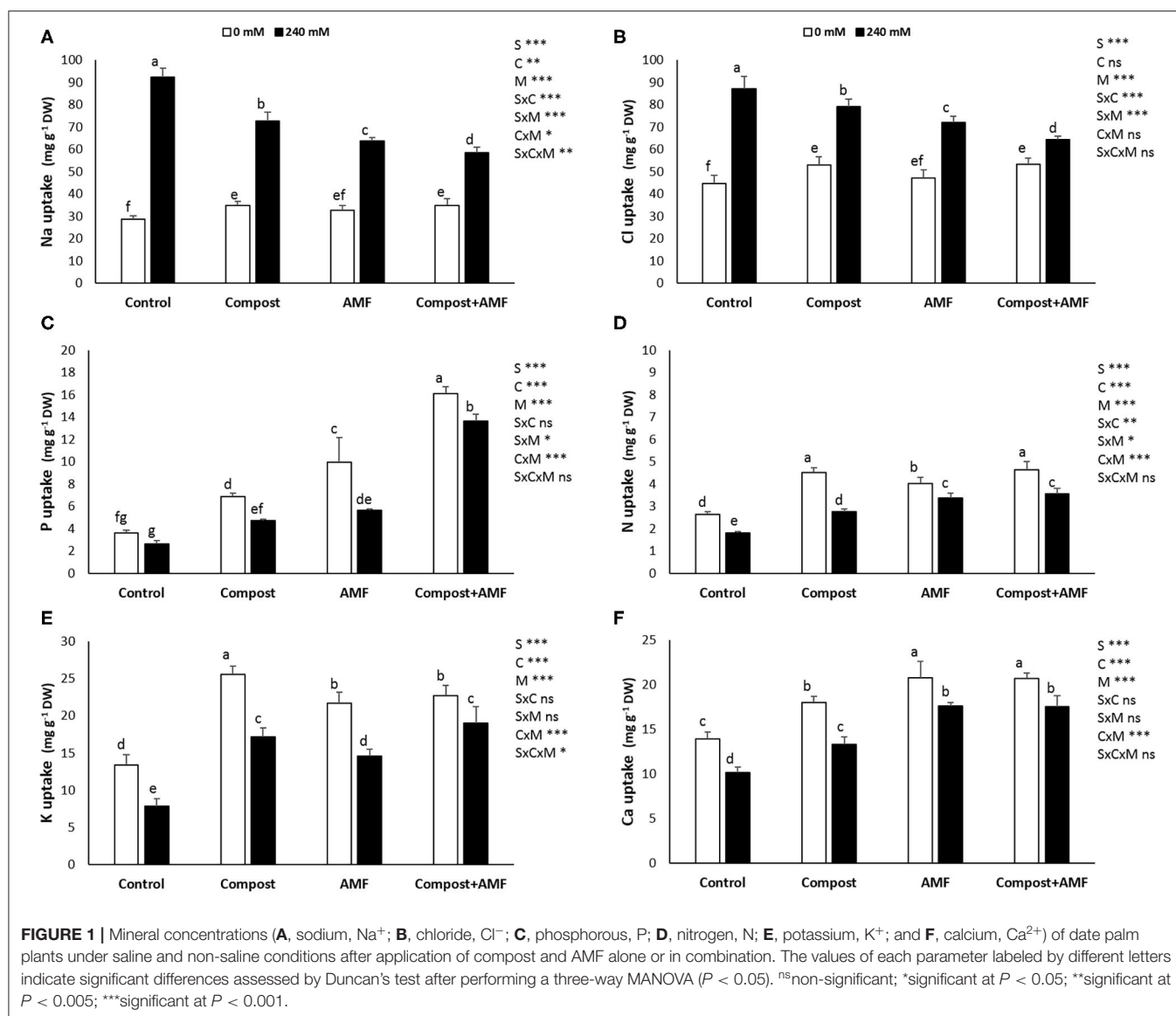
separately or in combination significantly improved the content of different photosynthetic pigments under saline conditions. Date palm seedlings grown in the presence of salt stress and the application of AMF alone or in combination with compost yielded marked increases in photosynthetic pigment concentrations (207 and 190%, respectively for Chl a, 222, and 212% for Chl b, 172 and 167%, respectively for carotenoids, and 213 and 198% for total chlorophyll) compared with untreated salt-affected plants. Significant interactions among  $C \times M \times S$  treatment were observed for chlorophyll a and carotenoid content ( $P < 0.05$ ) (Table 4).

## Hydrogen Peroxide and Malondialdehyde Content

There was an apparent significant ( $P < 0.001$ ) increase in the content of hydrogen peroxide ( $H_2O_2$ ) and malondialdehyde (MDA) concentrations (in shoots and roots) with the application of NaCl stress as compared with non-salinized plants (Figure 2). A maximum increase (ca. 40%) was recorded for root  $H_2O_2$  content and a minimum increase (18%) was observed for root MDA content. In contrast, AMF and/or compost applications reduced those parameters in both stressed and non-stressed date palm seedlings as compared to untreated plants. Indeed, the lowest significant values were recorded by plants treated with AMF+compost followed by AMF-inoculated plants and finally plants treated with compost.

## Proline and Soluble Sugar Content

The results related to the effect of salt stress on proline and soluble sugar content in date palm seedlings are presented in Figure 3. Plants exposed to salinity showed significantly ( $P < 0.001$ ) greater proline and soluble sugar concentrations in comparison with non-exposed plants. In the presence of NaCl



stress, application of AMF alone or in combination with compost led to the highest percentage improvement of soluble sugar in shoots (32 and 41%, respectively) and roots (ca. 90% in both), while there were no significant differences in root proline content between untreated and plants treated with the dual application.

### Antioxidant Enzyme Activity

Exposure of date palm plants to salt stress led to a significant ( $P < 0.001$ ) increase in antioxidant enzymes APX, CAT, POD, and SOD in the shoot (Table 5) and root (Table 6) activity. POD activity was the most affected by salinity and recorded a maximum increase of 62% in roots compared to non-salt affected plants. A further significant increase was observed in antioxidant enzyme activity in plants treated with AMF and compost separately or in combination under saline condition. The highest significant improvement was recorded with the dual application of AMF and compost followed by AMF inoculated

plants and then compost amended plants. This improvement was more marked in the roots than in the shoots. CAT activity was the activity most sensitive to the treatments applied and showed an enhancement of 74% in AMF treated plant shoots in comparison to untreated plants under salinity stress.  $C \times M \times S$  treatment effects on antioxidant enzymes activity were significant ( $P < 0.001$ ) for root POD activity (Tables 5, 6).

### Principal Component Analysis

Principal component analysis (PCA) took into account all the analyzed parameters of the different treatments. Two main components accounted for 91% of the variability observed in the data with 60% for PC1 and 31% for PC2 (Figure 4 and Table S1). Under the non-saline condition, PCA analysis showed a positive correlation among the different biofertilizers (applied separately or combined) for growth, physiological and water parameters, AMF infection, nutrient (N, P, K, and Ca, and K/Na

**TABLE 2 |** Shoot and root K/Na and Ca/Na ratios of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	Shoot		Root	
			K/Na	Ca/Na	K/Na	Ca/Na
0 mM	– AMF	– compost	1.2 ± 0.04 <sup>d</sup>	0.88 ± 0.10 <sup>a</sup>	0.24 ± 0.01 <sup>c</sup>	0.36 ± 0.02 <sup>d</sup>
		+ compost	1.6 ± 0.12 <sup>a</sup>	0.86 ± 0.07 <sup>a</sup>	0.44 ± 0.00 <sup>a</sup>	0.40 ± 0.01 <sup>c</sup>
	+ AMF	– compost	1.3 ± 0.05 <sup>c</sup>	0.86 ± 0.06 <sup>a</sup>	0.39 ± 0.01 <sup>b</sup>	0.54 ± 0.03 <sup>a</sup>
		+ compost	1.4 ± 0.03 <sup>b</sup>	0.86 ± 0.06 <sup>a</sup>	0.38 ± 0.02 <sup>b</sup>	0.50 ± 0.03 <sup>b</sup>
240 mM	– AMF	– compost	0.1 ± 0.01 <sup>g</sup>	0.11 ± 0.00 <sup>d</sup>	0.05 ± 0.01 <sup>f</sup>	0.11 ± 0.00 <sup>g</sup>
		+ compost	0.4 ± 0.02 <sup>f</sup>	0.23 ± 0.01 <sup>c</sup>	0.15 ± 0.01 <sup>e</sup>	0.15 ± 0.00 <sup>f</sup>
	+ AMF	– compost	0.3 ± 0.01 <sup>f</sup>	0.30 ± 0.00 <sup>bc</sup>	0.16 ± 0.01 <sup>e</sup>	0.26 ± 0.00 <sup>e</sup>
		+ compost	0.5 ± 0.04 <sup>e</sup>	0.35 ± 0.01 <sup>b</sup>	0.21 ± 0.02 <sup>d</sup>	0.27 ± 0.01 <sup>e</sup>
Significance level						
S			***	***	***	***
C			**	ns	***	ns
M			**	**	***	***
SxC			ns	*	**	ns
SxM			***	**	***	ns
CxM			**	ns	***	**
SxCxM			*	ns	***	ns

The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing a three-way MANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi. <sup>ns</sup> non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .

**TABLE 3 |** Physiological traits and water status of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	LRWC (%)	LWP (MPa)	g <sub>s</sub> (mmol m <sup>-2</sup> s <sup>-1</sup> )	F <sub>v</sub> /F <sub>m</sub>
0 mM	– AMF	– compost	71.0 ± 1.3 <sup>b</sup>	–2.46 ± 0.05 <sup>f</sup>	24.6 ± 1.2 <sup>e</sup>	0.55 ± 0.02 <sup>d</sup>
		+ compost	75.2 ± 0.4 <sup>b</sup>	–1.73 ± 0.20 <sup>cd</sup>	44.5 ± 2.1 <sup>c</sup>	0.77 ± 0.01 <sup>ab</sup>
	+ AMF	– compost	83.5 ± 4.1 <sup>a</sup>	–1.49 ± 0.18 <sup>bc</sup>	61.6 ± 1.1 <sup>a</sup>	0.79 ± 0.01 <sup>a</sup>
		+ compost	73.9 ± 1.2 <sup>b</sup>	–1.05 ± 0.13 <sup>a</sup>	50.6 ± 0.7 <sup>b</sup>	0.77 ± 0.01 <sup>ab</sup>
240 mM	– AMF	– compost	52.7 ± 1.4 <sup>e</sup>	–2.98 ± 0.25 <sup>g</sup>	14.3 ± 0.8 <sup>g</sup>	0.40 ± 0.03 <sup>e</sup>
		+ compost	58.6 ± 2.3 <sup>d</sup>	–2.06 ± 0.05 <sup>e</sup>	22.2 ± 1.4 <sup>f</sup>	0.73 ± 0.01 <sup>c</sup>
	+ AMF	– compost	63.3 ± 1.0 <sup>c</sup>	–1.96 ± 0.08 <sup>de</sup>	26.5 ± 0.6 <sup>e</sup>	0.78 ± 0.01 <sup>a</sup>
		+ compost	57.8 ± 4.3 <sup>d</sup>	–1.41 ± 0.08 <sup>b</sup>	29.6 ± 1.3 <sup>d</sup>	0.76 ± 0.01 <sup>bc</sup>
Significance level						
S			***	***	***	***
C			ns	***	***	***
M			***	***	***	***
SxC			ns	ns	ns	***
SxM			ns	ns	***	***
CxM			***	*	***	***
SxCxM			ns	ns	***	***

The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing a three-way MANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi; LRWC, leaf relative water content; LWP, leaf water potential;  $g_s$ , stomatal conductance; F<sub>v</sub>/F<sub>m</sub>, photosynthetic efficiency. <sup>ns</sup> non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .

and Ca/Na ratios), photosynthetic pigments, and protein content, which were highly correlated to each other. The analysis also confirmed the negative impact of salt concentration on these parameters. The biplot revealed the positive correlation linking salt treatment and Na, Cl, MDA, and H<sub>2</sub>O<sub>2</sub> content. Under saline conditions, PCA showed a clear positive correlation between the antioxidant system (antioxidant enzyme and osmolytes) and the AMF application alone or combined with compost.

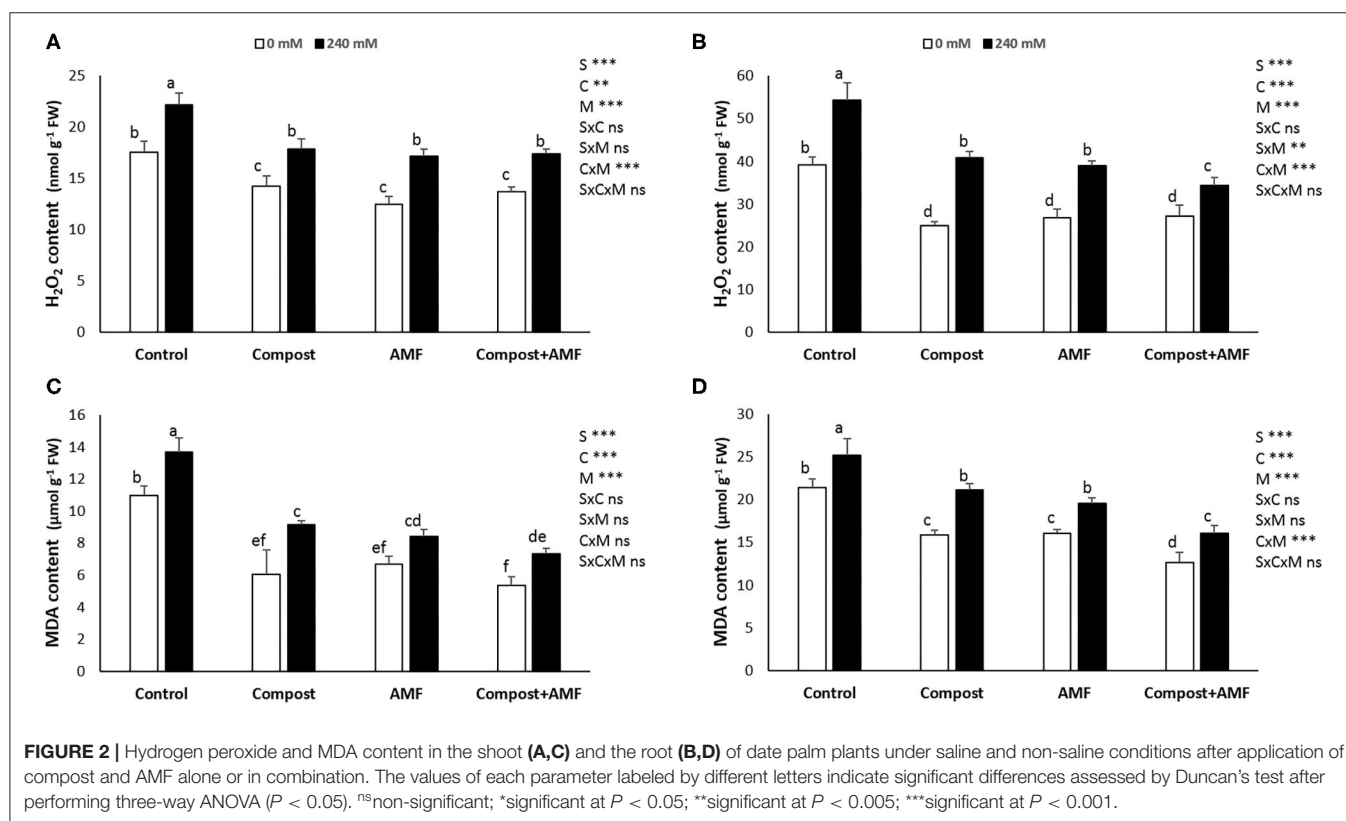
## DISCUSSION

The present study confirms, for the first time, the synergistic effect of AMF inoculation and the addition of green waste compost on improving date palm tolerance to salinity. Organic amendments (Tartoura et al., 2014; Mbarki et al., 2018) and AMF inoculation (Ait-El-Mokhtar et al., 2019; Ben Laouane et al., 2019) have been reported to confer certain complex beneficial

**TABLE 4** | Photosynthetic pigment content of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	Chl a (mg/g FW)	Chl b (mg/g FW)	Chl T (mg/g FW)	Car T (mg/g FW)
0 mM	– AMF	– compost	11.3 ± 0.3 <sup>d</sup>	6.3 ± 0.4 <sup>e</sup>	17.6 ± 0.7 <sup>e</sup>	57.5 ± 8.9 <sup>e</sup>
		+ compost	19.1 ± 0.2 <sup>b</sup>	12.4 ± 1.0 <sup>b</sup>	31.5 ± 1.1 <sup>c</sup>	111.4 ± 8.4 <sup>b</sup>
	+ AMF	– compost	21.5 ± 0.8 <sup>a</sup>	15.5 ± 1.0 <sup>a</sup>	36.9 ± 1.8 <sup>a</sup>	140.0 ± 8.7 <sup>a</sup>
		+ compost	19.6 ± 0.5 <sup>b</sup>	15.2 ± 0.3 <sup>a</sup>	34.7 ± 0.8 <sup>b</sup>	134.3 ± 5.0 <sup>a</sup>
240 mM	– AMF	– compost	4.7 ± 0.5 <sup>e</sup>	3.1 ± 0.9 <sup>f</sup>	7.8 ± 1.3 <sup>f</sup>	30.3 ± 8.3 <sup>f</sup>
		+ compost	11.48 ± 0.9 <sup>d</sup>	7.7 ± 1.0 <sup>d</sup>	19.2 ± 1.9 <sup>e</sup>	67.4 ± 9.2 <sup>d</sup>
	+ AMF	– compost	14.47 ± 0.9 <sup>c</sup>	10.0 ± 0.9 <sup>c</sup>	24.5 ± 1.8 <sup>d</sup>	82.3 ± 4.2 <sup>c</sup>
		+ compost	13.61 ± 1.3 <sup>c</sup>	9.7 ± 1.3 <sup>c</sup>	23.3 ± 2.7 <sup>d</sup>	80.8 ± 5.0 <sup>c</sup>
Significance level						
S			***	***	***	***
C			***	***	***	***
M			***	***	***	***
SxC			ns	ns	ns	ns
SxM			ns	**	ns	***
CxM			***	***	***	***
SxCxM			*	ns	ns	*

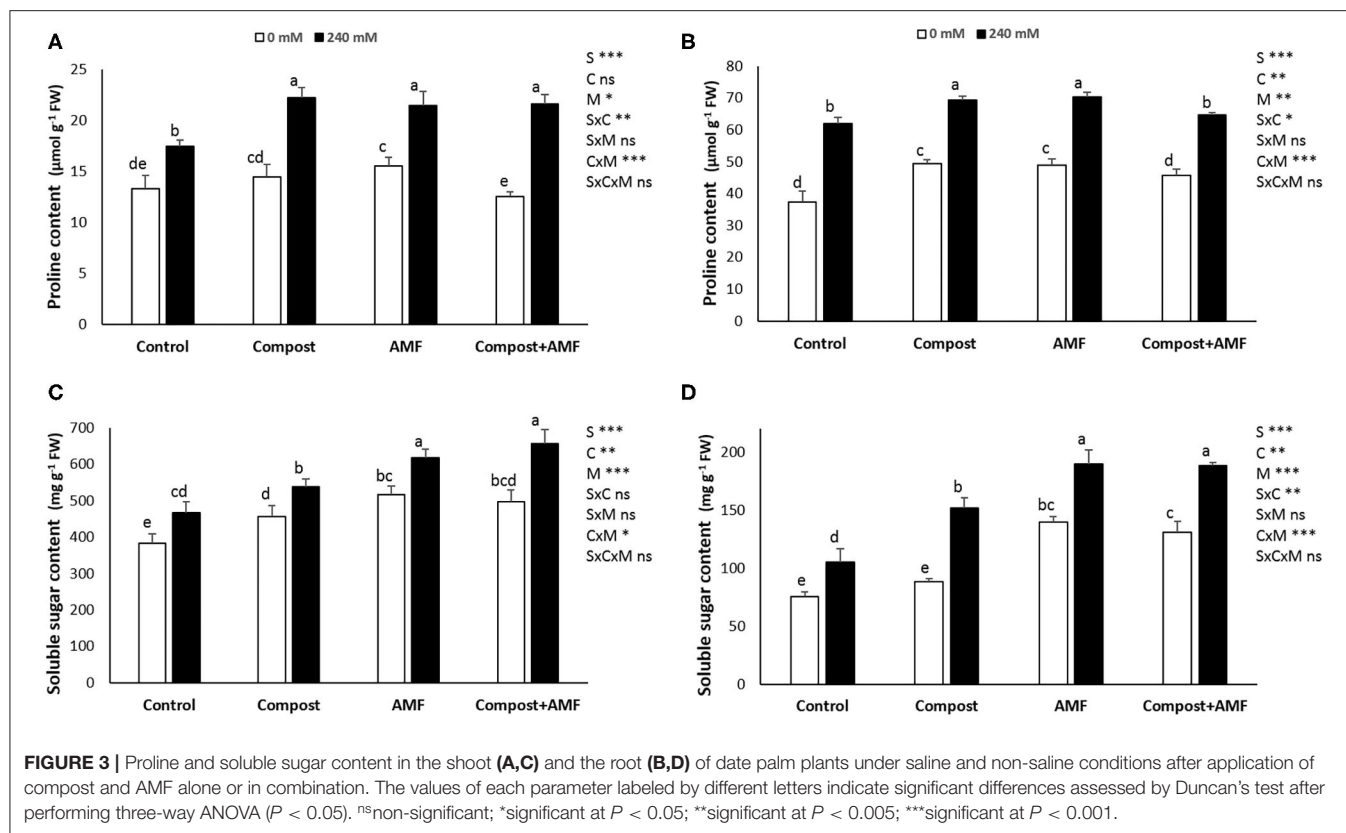
The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing three-way MANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi; Chl a, chlorophyll a; Chl b, chlorophyll b; Chl T, total chlorophyll; Car T, carotenoids. <sup>ns</sup>non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .



changes on plant growth and physiological and biochemical characters under salt stress. Adverse effects of salinity on date palm growth and development have been reported by many authors (Sperling et al., 2014; Yaish and Kumar, 2015; Ait-El-Mokhtar et al., 2019; Al Kharusi et al., 2019). In the present

investigation, salinity stress significantly reduced date palm growth parameters, with the impact of salinity being more pronounced in roots than shoots. Previous studies showed that salt stress adversely affects the establishment, growth, and development of plants due to osmotic stress and disrupts many





**TABLE 5 |** Shoot antioxidant enzyme activity (APX, CAT, POD, and SOD) of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	APX μmol Asc/mg prot./min	CAT μmol H <sub>2</sub> O <sub>2</sub> /mg prot./min	POD μmol Guaiacol/mg prot./min	SOD Unit/mg prot.	Protein mg/g FW
0 mM	– AMF	– compost	13.6 ± 1.1 <sup>e</sup>	0.9 ± 0.2 <sup>d</sup>	0.6 ± 0.1 <sup>e</sup>	1.0 ± 0.1 <sup>d</sup>	21.2 ± 1.3 <sup>d</sup>
		+ compost	16.6 ± 2.2 <sup>d</sup>	1.4 ± 0.1 <sup>c</sup>	0.8 ± 0.1 <sup>d</sup>	1.1 ± 0.1 <sup>d</sup>	22.8 ± 1.1 <sup>cd</sup>
	+ AMF	– compost	21.4 ± 2.1 <sup>bc</sup>	1.9 ± 0.0 <sup>b</sup>	0.9 ± 0.0 <sup>d</sup>	1.5 ± 0.1 <sup>c</sup>	25.2 ± 1.2 <sup>ab</sup>
		+ compost	19.9 ± 0.6 <sup>c</sup>	1.8 ± 0.1 <sup>b</sup>	1.1 ± 0.1 <sup>c</sup>	1.4 ± 0.1 <sup>c</sup>	26.7 ± 1.2 <sup>a</sup>
240 mM	– AMF	– compost	16.6 ± 0.7 <sup>d</sup>	1.4 ± 0.1 <sup>c</sup>	0.9 ± 0.1 <sup>d</sup>	1.4 ± 0.1 <sup>c</sup>	15.4 ± 1.6 <sup>f</sup>
		+ compost	22.9 ± 2.9 <sup>b</sup>	1.9 ± 0.1 <sup>b</sup>	1.3 ± 0.1 <sup>b</sup>	1.7 ± 0.1 <sup>b</sup>	18.7 ± 0.7 <sup>e</sup>
	+ AMF	– compost	28.7 ± 0.9 <sup>a</sup>	2.4 ± 0.2 <sup>a</sup>	1.4 ± 0.0 <sup>b</sup>	1.8 ± 0.1 <sup>ab</sup>	22.1 ± 1.1 <sup>cd</sup>
		+ compost	28.7 ± 1.7 <sup>a</sup>	2.3 ± 0.0 <sup>a</sup>	1.5 ± 0.1 <sup>a</sup>	2.0 ± 0.0 <sup>a</sup>	23.4 ± 1.0 <sup>bc</sup>
Significance level							
S			***	***	***	***	***
C			*	**	***	**	**
M			***	***	***	***	***
SxC			ns	ns	ns	*	ns
SxM			*	ns	ns	ns	ns
CxM			**	***	*	ns	ns
SxCxM			ns	ns	ns	ns	ns

The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing three-way MANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi; APX, ascorbate peroxidase; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase. <sup>ns</sup>non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .

metabolic processes (Porcel et al., 2012; Abdel Latef et al., 2019a,b; Evelin et al., 2019). Roots are in direct contact with the soil solution from which they absorb minerals and water. Thus,

they are the first to encounter the saline medium and play a key role in transporting these elements to the leaves. In the context of salt tolerance, roots are the first site of defense against NaCl and

**TABLE 6 |** Root antioxidant enzyme activity (APX, CAT, POD, and SOD) of date palm plants under saline and non-saline conditions after application of compost and AMF alone or in combination.

NaCl treatment	AMF treatment	Compost treatment	APX $\mu\text{mol Asc/mg prot./min}$	CAT $\mu\text{mol H}_2\text{O}_2/\text{mg prot./min}$	POD $\mu\text{mol Guaiacol/mg prot./min}$	SOD Unit/mg prot.	Protein mg/g FW
0 mM	– AMF	– compost	24.5 $\pm$ 1.5 <sup>e</sup>	2.0 $\pm$ 0.1 <sup>f</sup>	1.3 $\pm$ 0.1 <sup>d</sup>	2.5 $\pm$ 0.2 <sup>d</sup>	11.3 $\pm$ 0.7 <sup>d</sup>
		+ compost	32.9 $\pm$ 1.9 <sup>c</sup>	2.7 $\pm$ 0.1 <sup>de</sup>	2.0 $\pm$ 0.1 <sup>c</sup>	2.7 $\pm$ 0.5 <sup>cd</sup>	13.2 $\pm$ 0.2 <sup>bc</sup>
	+ AMF	– compost	34.9 $\pm$ 0.9 <sup>c</sup>	2.9 $\pm$ 0.1 <sup>cd</sup>	2.1 $\pm$ 0.2 <sup>c</sup>	3.1 $\pm$ 0.0 <sup>c</sup>	14.2 $\pm$ 0.7 <sup>a</sup>
		+ compost	35.1 $\pm$ 2.5 <sup>c</sup>	3.1 $\pm$ 0.1 <sup>c</sup>	2.0 $\pm$ 0.1 <sup>c</sup>	2.8 $\pm$ 0.1 <sup>cd</sup>	14.0 $\pm$ 0.4 <sup>ab</sup>
240 mM	– AMF	– compost	28.8 $\pm$ 1.7 <sup>d</sup>	2.6 $\pm$ 0.1 <sup>e</sup>	2.2 $\pm$ 0.1 <sup>c</sup>	3.1 $\pm$ 0.1 <sup>c</sup>	8.9 $\pm$ 0.9 <sup>e</sup>
		+ compost	38.7 $\pm$ 2.2 <sup>b</sup>	3.6 $\pm$ 0.1 <sup>b</sup>	2.4 $\pm$ 0.1 <sup>b</sup>	3.6 $\pm$ 0.2 <sup>b</sup>	11.6 $\pm$ 0.4 <sup>d</sup>
	+ AMF	– compost	40.0 $\pm$ 2.1 <sup>ab</sup>	3.7 $\pm$ 0.3 <sup>b</sup>	2.5 $\pm$ 0.1 <sup>b</sup>	4.6 $\pm$ 0.1 <sup>a</sup>	12.7 $\pm$ 0.4 <sup>c</sup>
		+ compost	42.3 $\pm$ 2.8 <sup>a</sup>	4.1 $\pm$ 0.1 <sup>a</sup>	2.9 $\pm$ 0.1 <sup>a</sup>	4.4 $\pm$ 0.3 <sup>a</sup>	13.1 $\pm$ 0.1 <sup>bc</sup>
Significance level							
S			***	***	***	***	***
C			***	***	***	ns	***
M			***	***	***	***	***
SxC			ns	ns	ns	ns	ns
SxM			ns	ns	ns	**	ns
CxM			***	***	*	**	***
SxCxM			ns	ns	**	ns	ns

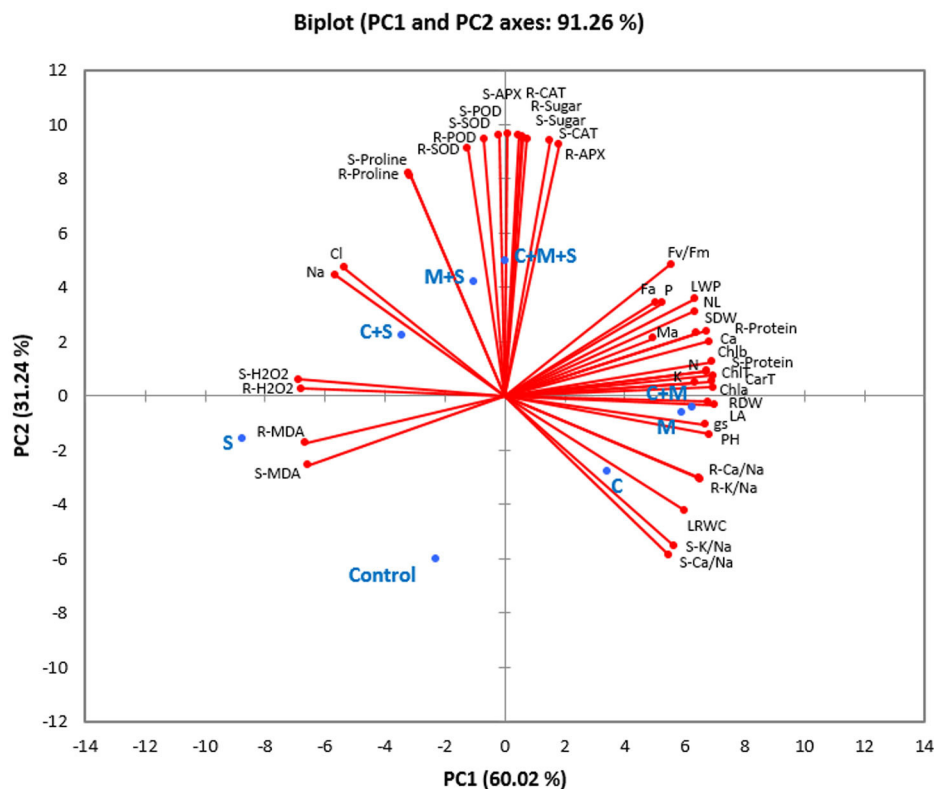
The values of each parameter labeled by different letters indicate significant differences assessed by Duncan's test after performing three-way ANOVA ( $P < 0.05$ ). AMF, arbuscular mycorrhizal fungi; APX, ascorbate peroxidase; CAT, catalase; POD, peroxidase; SOD, superoxide dismutase. <sup>ns</sup>non-significant; \*significant at  $P < 0.05$ ; \*\*significant at  $P < 0.005$ ; \*\*\*significant at  $P < 0.001$ .

may directly limit or exclude sodium uptake (Munns, 2005; Liu et al., 2015; Rana et al., 2019). Another important aspect of root systems compared to shoots is their anatomy. The stele tissues in roots are internally surrounded by cortex and epidermis, while they are distributed throughout parenchyma cells in the shoots. Evidence has emerged that the root cortex must do the heavy lifting of excluding  $\text{Na}^+$  from shoots, which requires an energy cost (ATP) used by antiporters as part of a tissue tolerance mechanism (Munns et al., 2020). Further, salt typically affects root elongation and architecture by reducing cell size and cell division and altering differentiation patterns (Potters et al., 2007; Bernstein, 2013). AMF inoculation and compost amendment significantly mitigated the inhibitory effects of salinity on growth which may be because AMF can acquire nutrients released from the composted waste (Hodge et al., 2001; Cavagnaro, 2014). The application of AMF and/or compost might improve the physicochemical and biological characteristics of the soil (Rillig and Mummey, 2006; Weber et al., 2014), and enhance the release of nutrients in the soil, which makes them more available to the plant (Astiko et al., 2013; Baslam et al., 2014). Furthermore, the spreading of AMF hyphae, beyond the root zone, provides P and other immobile nutrients to plants and thereby promotes biomass production (Porcel et al., 2012).

Under saline stress, date palm growth parameters were mostly enhanced by the dual application of AMF and compost although salinity and compost amendment hampered AMF root colonization. Zhang et al. (2017) reported that mycorrhizal colonization of trifoliate orange (*Citrus trifoliate* L.) root was negatively affected by salinity stress which was attributed to the direct effect of NaCl on the fungi. Several authors have also shown that salinity reduces mycorrhizal colonization through

inhibition of spore germination and hyphal growth or reduction of the spread of mycorrhizal colonization and the number of arbuscules (Hajiboland et al., 2010; Evelin et al., 2019). Likewise, a reduced percentage of colonization of roots by AMF in the substrate amended with compost has been reported in other research (Graceson et al., 2014; Watts-Williams and Cavagnaro, 2014). This could be explained by the release of larger amounts of mineralized P into the soil, which can result in a reduced colonization (Baslam et al., 2011b; Lehmann et al., 2011). Besides, composted organic material comprises products of decomposition, which can induce inhibition of mycorrhizal fungi (Gryndler et al., 2008).

The adverse effects of salinity stress on plant growth could be related to nutrient imbalances (Evelin et al., 2019). In this study, the uptake of P, N,  $\text{K}^+$ , and  $\text{Ca}^{2+}$  and  $\text{K}^+/\text{Na}^+$  and  $\text{Ca}^{2+}/\text{Na}^+$  ratios in date palm seedlings was lower under salinity stress. On the other hand,  $\text{Na}^+$  and  $\text{Cl}^-$  content was higher in plants irrigated with saline water—factors which induce the ionic imbalance and disrupt photosynthesis and other metabolic functions of the cell (Ramoliya et al., 2004). AMF and compost applications have been shown to affect plant physiological processes, especially nutrient absorption, and therefore plant tolerance to salt stress (Abdel Latef et al., 2016; Chaichi et al., 2017; Enebe and Babalola, 2018; Mbarki et al., 2018). In this investigation, the improvements in nutrient uptake ( $\text{K}^+$ , P, N, and  $\text{Ca}^{2+}$ ) under saline conditions were greatest with the dual application of AMF and compost. Improvements were less marked with AMF inoculation and even fewer with just compost treatments. Chaichi et al. (2017) demonstrated that tomato plants grown in the presence of compost tea enriched with AMF showed enhanced cation acquisition, including  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{Mg}^{2+}$ ,



**FIGURE 4 |** Principal component analysis of the studied traits of different biofertilizer treatments in date palm plants growing under saline and non-saline conditions. S, Salinity; M, arbuscular mycorrhizal fungi; C, compost; PH, plant height; NL, number of leaves; LA, leaf area; SDW, shoot dry weight; RDW, root dry weight; Fa, AMF infection frequency; Ma, AMF infection intensity; Na, sodium uptake; Cl, chloride uptake; P, phosphorous uptake; N, nitrogen uptake; K, potassium uptake; Ca, calcium uptake; LRWC, leaf relative water content; LWP, leaf water potential;  $g_s$ , stomatal conductance;  $F_v/F_m$ , photosynthetic efficiency. Chl a, chlorophyll a; Chl b, chlorophyll b; Chl T, total chlorophyll; Car T, carotenoids; S-K/Na, shoot K/Na ratio; R-K/Na, root K/Na ratio; S-Ca/Na, shoot Ca/Na ratio; R-Ca/Na, root Ca/Na ratio; S-H<sub>2</sub>O<sub>2</sub>, shoot H<sub>2</sub>O<sub>2</sub> content; R-H<sub>2</sub>O<sub>2</sub>, root H<sub>2</sub>O<sub>2</sub> content; S-MDA, shoot MDA content; R-MDA, root MDA content; S-Proline, shoot proline content; R-Proline, root proline content; S-Sugar, shoot sugar content; R-Sugar, root sugar content; S-APX, shoot ascorbate peroxidase activity; R-APX, root ascorbate peroxidase activity; S-CAT, shoot catalase activity; R-CAT, root catalase activity; S-POD, shoot peroxidase activity; R-POD, root peroxidase activity; S-SOD, shoot superoxide dismutase activity; R-SOD, root superoxide dismutase activity; S-Protein, shoot protein content; R-Protein, root protein content.

under salt stress. In the same vein, Sallaku et al. (2019) reported an obvious increase in the uptake of P, K<sup>+</sup>, and Ca<sup>2+</sup> in AMF cucumber seedlings, while AMF inoculation reduced the uptake of Na<sup>+</sup>. Furthermore, it has been reported that organic matter amendments improve N and P concentrations in plants grown in saline soils (Mbarki et al., 2018). The improvement of date palm tolerance to salinity by increasing P might be attributable to the role of P uptake in preserving the integrity of cell membranes and in facilitating the compartmentalization of toxic ions in vacuoles (Bothe, 2012). Moreover, the inhibition of Na<sup>+</sup> absorption through roots in mycorrhizal plants and/or the decrease of Na<sup>+</sup> mobility in compost-amended soil might be considered other strategies that improve salt adaptation in plants (Enebe and Babalola, 2018). AMF and compost application can alleviate Na<sup>+</sup> and Cl<sup>-</sup> toxicity. For example, vacuoles of AMF vesicles can store different ions such as sodium and chloride under salinity constraints (Miransari, 2010). Also, the organic amendment is directly related to mechanisms for Na<sup>+</sup> removal from soil such as Na<sup>+</sup> leaching and the Na<sup>+</sup> adsorption ratio ratio (Chaganti

and Crohn, 2015; Drake et al., 2016). Our study demonstrated that the K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratios in mycorrhizal date palm seedlings, especially in the presence of the compost application, were increased under saline conditions. The higher K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratios were found to be a key parameter to alleviate salt stress in plants, since Na<sup>+</sup> ions may compete with K<sup>+</sup> and Ca<sup>2+</sup> ions for membrane transport sites (Evelin et al., 2019). Maintenance of a higher K<sup>+</sup>/Na<sup>+</sup> and Ca<sup>2+</sup>/Na<sup>+</sup> ratios in the cytosol is a crucial aspect for the protecting physiological cellular functioning from detrimental salinity changes (Ahanger et al., 2015). Greater values of these ratios protect photosynthetic tissues by blocking Na<sup>+</sup> entry (Cuin et al., 2011) and improve hydraulic conductivity and Ca<sup>2+</sup> signaling pathways (Lovelock and Ball, 2006).

Salt stress affects the photosynthesis apparatus in many ways. The osmotic stress induced by salinity reduces stomatal conductance, which decreases the availability and assimilation of CO<sub>2</sub> (Chaves et al., 2009). Salinity disrupts the photochemical reactions of photosynthesis, especially PSII (Hidri et al., 2016).

Our study showed a decline in photosynthetic pigment content (Chl *a*, Chl *b*, carotenoids, and total chlorophyll) coupled with a decrease in photosynthetic efficiency ( $F_v/F_m$ ) under saline conditions. This adverse effect is linked to the destruction of chloroplast as a direct effect of salt stress (Zörb et al., 2009) and the decrease in the activity of photosynthetic pigment synthesizing enzymes (Murkute et al., 2006). The reduced chlorophyll content may be attributed to the low uptake of minerals, especially magnesium, in the presence of salinity (Giri and Mukerji, 2004). Application of AMF and compost resulted in a significant increase in photosynthetic attributes such as chlorophyll pigment content and stomatal conductance, reflecting a significant improvement in photosynthetic efficiency. The enhancement of N uptake owing to the AMF and compost treatment might improve photosynthetic attributes because N is considered a key component of the Rubisco enzyme (Ahanger et al., 2014). Rao and Chaitanya (2016) reported that the presence of  $Mg^{2+}$  due to organic amendment and subsequent increase in its uptake due to AMF application could be possible reasons for greater photosynthetic capacity. This might also be attributed to the accumulation of proline and glycine betaine in mycorrhizal plants which protects PSII pigment-protein complexes as well as  $CO_2$ -fixing enzymes such as RuBisCO and rubisco activase (Talaat and Shawky, 2014).

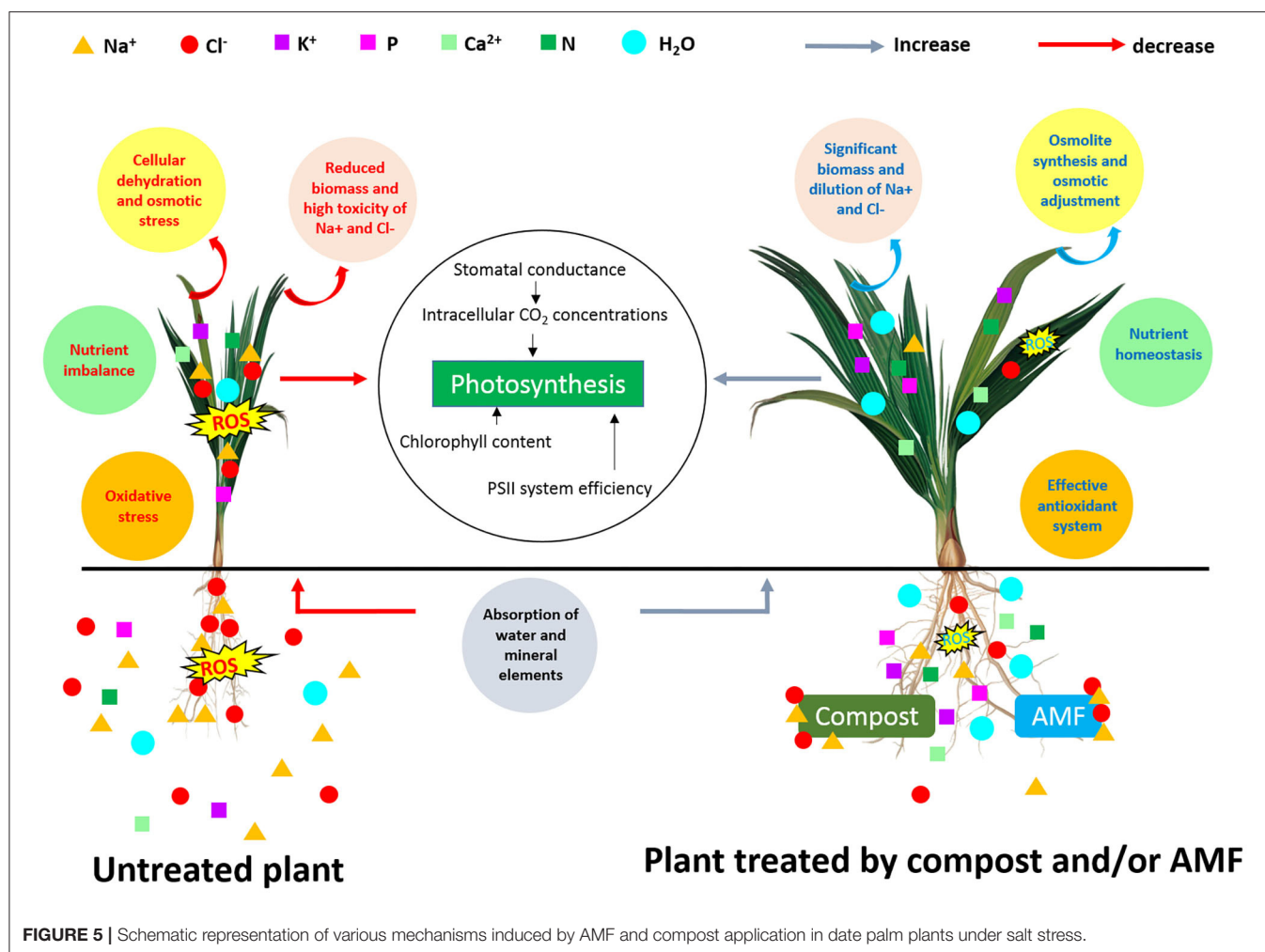
Salinization not only affected photosynthetic capacity, but also the water status of the seedlings. Plants grown under saline conditions showed a significant decrease in LRWC and leaf water potential. This decline in water status is related to the water stress generated by salinity since salt immobilizes water and makes it unavailable for the plant roots (Füzy et al., 2008). However, AMF and compost application significantly improved the water status of the plant under salt stress, as indicated by the greater LRWC and less negative leaf water potential values. This may be explained by improved hydraulic conductivity and water absorption capacity mediated by the mycorrhizae even under saline conditions (Kapoor et al., 2008). Medina and Azcón (2010) suggested that organic amendment and AMF symbiosis were able to change soil structure by inducing aggregate formation which improves water availability in soil.

The decline in date palm biomass under saline condition was accompanied by increased levels of MDA and  $H_2O_2$ . Our results are supported by earlier studies on two cultivars of *P. dactylifera* L. cv. Umsila and cv. Zabad (Al Kharusi et al., 2019), *Cicer arietinum* L. (Rasool et al., 2013), *Capsicum annuum* L. (Abdel Latef and Chaoping, 2014), and *Ephedra aphylla* (Alqarawi et al., 2014). Root tissues exhibited higher ROS accumulation and oxidative damage than shoot tissues. Plants treated with NaCl in combination with AMF and compost application showed a smaller increase in MDA and  $H_2O_2$  concentrations. The same result was reported by Rasool et al. (2013) and Tartoura et al. (2014) in the presence of salt stress. This effect can be linked to improvements in the antioxidant enzyme system which scavenges the ROS before they react with membrane lipids thus reducing lipid peroxidation. Proline and sugars figure among the most commonly used metabolites to assess the degree of response to salt stress (Evelin et al., 2019). In the present study, under stress conditions, date palm plants showed enhanced proline

and soluble sugar content, which was further enhanced by the application of AMF and compost. This may be a defensive reaction which improves the resistance of date palm seedlings to salt stress by maintaining osmotic balance and mitigating the free radical damage caused by osmotic stress (Ahanger et al., 2014). In the presence of NaCl, AMF colonization could set in motion mechanisms that launch improved proline synthesis and therefore a better tolerance of the plant to salt stress (Hashem et al., 2016). Our findings of an increase in proline and soluble sugar concentrations following the AMF and compost application under saline stress confirm the findings of Chinsamy et al. (2013) and Talaat and Shawky (2014). Proline has been shown to act as a chemical protein chaperone and to prevent protein aggregation under salt stress (Ignatova and Gierasch, 2006). The enhancement of proline in plants treated with AMF and compost helped to protect against oxidative stress given its involvement in the stabilization of redox enzymes. Hoque et al. (2008) reported that the exogenous application of proline to cell cultures was positively correlated with the increased activity of different antioxidant enzymes (i.e. SOD, CAT, and (ASC)-GSH cycle-related enzymes) under saline conditions.

Salinity tolerance in plants has been associated with the induction of the antioxidant enzyme system and the reduction of oxidative damage. Here, we show that NaCl stress significantly increased the antioxidant enzyme activity (SOD, POD, APX, and CAT) of the date palm, and this activity was further increased in treated plants, particularly those grown with the dual application of AMF and compost. Similar results were reported previously in other species (Hashem et al., 2015; Hidri et al., 2016; Ramzani et al., 2017; Ben Laouane et al., 2019). SOD is considered one of the first enzymes of defense and enables the scavenging of superoxide radicals into hydrogen peroxide which is further detoxified to water (Mittler, 2002). The conversion of  $H_2O_2$  to  $H_2O$  can be catalyzed by the action of enzymes, such as CAT, APX, and POD (Mittler, 2002). In our study, the increase in SOD activity was consistent with the improvement in the activity of hydrogen peroxide-scavenging enzymes (CAT, POD, and APX). Furthermore, the induction of the activity of antioxidant enzymes in plants treated with AMF and compost compared with untreated plants was associated with lower  $H_2O_2$  content and lipid peroxidation, thereby indicating an efficient ROS scavenging system in the date palm through a reduction in oxidative stress and less membrane damage in the treated plants.

Taken together, the results indicate that the application of compost and AMF alleviated salt stress in date palm crops in a synergistic manner and through physiological and biochemical processes (Figure 4). Even if the organic amendment decreased AMF root colonization, the combined effect of compost and AMF seems to be the most efficient way to boost the tolerance of date palm seedlings to salinity. Indeed, AMF and compost alone or in combination (right side of PC1) appear to confer multiple forms of protection to the date palm, as these application are positively correlated with growth-related parameters, plant photosynthetic traits, and nutrients homeostasis. AMF and/or compost were negatively associated with MDA and  $H_2O_2$ . Under stress, the combination AMF+compost was positively associated (PC2 axis) with antioxidant enzyme (SOD, POD,



and CAT) activity and proline biosynthesis. These associations suggest that the application of microbial inocula and compost co-occurred with enhancement in leaf gas exchange, induction in the photosynthetic apparatus and increased accumulation of pigments associated with light harvest, and alleviation of stress damages by regulating the antioxidant enzymes under high-salinity. This finding is supported by the fact that at high-salinity (240 mM NaCl) concomitant protein degradation occurred in untreated plants (as a single group, negatively correlated with agro-physiological and growth parameters) that lost antioxidant enzyme activity, while the high salt-treated plants still had a strong scavenging system consisting of antioxidative molecules. As a result, AMF and compost improved water relations without penalizing plant growth under salt stress. **Figure 5** gives an overview of the various mechanisms commonly elicited by compost and AMF which systemically counter the deleterious effects of salt stress and rectifies them, thereby ultimately improving plant growth and productivity. Date palm plants growing under saline conditions showed an ionic imbalance, physiological drought, and oxidative stress, which leads to reduced biomass. Although there are similarities in the

mechanisms induced by the application of compost and AMF to alleviate all the negative effects of salinity, there are, however, slight variations underlying the basic mechanisms involved when both biofertilizers are used. AMF can improve the water and nutrient status of host plants *via* the boosting of nutrient and water uptake by the hyphal structure effect and the activity of ion and water transporters. Similarly, compost also raises nutrient concentrations in plants, since it is a source of mineral elements (N, P, and K). Furthermore, compost may improve water uptake by increasing soil water holding capacity. The improvement of nutrient and hydraulic conductance alleviates the salinity-induced growth effects in plants. AMF and compost can also mitigate the toxicity of Na<sup>+</sup> and Cl<sup>-</sup> by preventing these ions from interfering with the plant root system. Also, AMF symbiosis and compost amendment can alleviate osmotic and oxidative stress by stimulating osmolyte synthesis and antioxidant enzymes and by reducing oxidative stress marker levels (H<sub>2</sub>O<sub>2</sub> and MDA). Furthermore, increased stomatal conductance along with chlorophyll content enhancement and PSII efficiency contribute to boost the photosynthesis machinery necessary for plant performance under saline conditions. The



symbiotic association of AMF together with compost (5% W/W with respect to culture soil) might have influenced positive plant growth by an increase in nutrient supply. The addition of compost alters the soil pH and in turn, affects nutrient availability to plants. The positive effects of the compost on AMF derived from altering the physicochemical properties of soil, changes in nutrient dynamics—mainly phosphorous availability—, the release of organic compounds, increased water content and plant available water, root hydraulic conductivity, and changes in the composition of root exudates might all have contributed to a significant increase in the plants' performance under normal and saline conditions as compared to untreated plants or those containing AMF or compost alone (Akhter et al., 2015; Kohler et al., 2015; Kranz et al., 2020). Mimmo et al. (2011) have shown the substrate-dependent variations in plants root exudation patterns. The altered response could be due to the accumulation of new compounds in root exudates which may have a stimulatory effect on mycelial growth and development (Scheffknecht et al., 2006). The growth advantage conferred by the mycorrhizal consortium in the amended soil might be related to the capacity of the fungi to acquire nutrients slow-released from the composted residue (Cavagnaro, 2014). El Kinany et al. (2019) found that the mixture of compost and AMF contributed to uptake of mobile micronutrient boron, which has a crucial role in cell wall formation and stability, maintenance of structural and functional integrity of biological membranes, movement of sugar or energy into growing parts of plants (Takano et al., 2002; Shireen et al., 2018).

Our results provide an experimental basis for the application of compost and AMF fertilizers as a sustainable method for the remediation of salt-affected soils in arid and semi-arid areas, especially in fragile ecosystems such as palm grove oases.

## CONCLUSIONS

Salinity stress reduced growth, physiological, and biochemical traits of date palm seedlings considerably. AMF-inoculated plants grown in compost-amended soil exhibited large improvements in these parameters under NaCl stress. The application of AMF and compost separately or in combination increased water status parameters, photosynthetic efficiency, and the concentration of photosynthetic pigments under saline conditions by enhancing N, P, K<sup>+</sup>, and Ca<sup>2+</sup> uptake and reducing Na<sup>+</sup> and Cl<sup>−</sup> content. The synergy between AMF and compost in salinity tolerance was manifested in the improvement in growth, soil nutrient status, and physio-biochemical traits together with the decrease

in MDA and H<sub>2</sub>O<sub>2</sub> as compared to no-mycorrhizal and compost-free plants. This dual application alleviated the oxidative stress induced by salinity by stimulating antioxidant enzyme activity and therefore the reduction of hydrogen peroxide and lipid peroxidation. All these beneficial effects protected membrane integrity and the different cell components from the adverse effects of salinity. The combined treatment of AMF and compost yielded the greatest improvement in the parameters measured in date palm seedlings under salt stress, with the application of AMF alone in second place and then finally that application of compost alone. Our findings highlight the importance of considering the dual application of AMF and compost to alleviate the detrimental effects of salinity and promote plant growth in salinized soils in arid and semiarid areas.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

AM, SW, and MB: conceptualization, validation, supervision. MA-E-M, AM, SW, and MB: methodology. MA-E-M, RB-L, MA, and AB: formal analysis. MA-E-M and MB: writing original draft preparation. AM, SW, MB, and TM: project administration. MB and TM: funding acquisition. All authors have read and agreed to the published version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.00131/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Application of *Azospirillum brasilense* Lipopolysaccharides to Promote Early Wheat Plant Growth and Analysis of Related Biochemical Responses

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While the effects of lipopolysaccharides (LPS) of plant pathogenic bacteria in induction of plant defense responses have been characterized, the role of LPS of beneficial rhizobacteria on plant growth is less clear. In this study, we assessed the *in vitro* effects of LPS from the rhizobacterium *Azospirillum brasilense* Sp245 on early growth of wheat seedlings (*Triticum aestivum*) and on some biochemical responses related to growth, like peroxidase (POD) enzyme activity and  $\text{Ca}^{2+}$  availability. Four days after treating the seedlings with various concentrations of *A. brasilense* LPS (10 to 1,000  $\mu\text{g/mL}$ ), the growth of the seedlings was enhanced as evidenced by significant increase in leaf and root lengths as well as fresh weight. These increases were similar or even higher to those resulting from inoculation with the rhizobacteria. POD enzyme activity increased significantly in roots treated with LPS and was concentration dependent. Salicylhydroxamic acid, an inhibitor of peroxidase activity, decreased POD activity and plant growth promoted by LPS. Lanthanum, an inhibitor of calcium channels, and EGTA, inhibited plant growth and POD activity promoted by LPS, while the calcium ionophore A23187 alone was able to increase plant growth and POD activity. In summary, the results suggest that isolated LPS of *A. brasilense* have the capacity to promote early wheat seedling growth and that POD enzyme activity and  $\text{Ca}^{2+}$  levels are involved in the LPS-mediated biological activity.

**Keywords:** *Azospirillum*, lipopolysaccharides, peroxidase, growth, wheat

## INTRODUCTION

Conventional agriculture is facing either reduced production or increased costs, or both, as a result in the weakening of general soil vitality, groundwater purity, and beneficial microbe (Singh et al., 2011). This have derived in the addition of increasing amount of fertilizers and pesticides to the soil. Although conventional methods enabled large increases in crop yields, their effects on soil and ecology health fail to be considered as an option for future agriculture (Singh et al., 2011). Sustainable agriculture is a broad based concept that involve agricultural management practices

and technology. Numerous soil microorganisms contribute to the sustainability of the crop yield by improving the efficiency of nutrient acquisition and enhancing plant health.

*Azospirillum* belong to soil microorganisms that can be useful in the sustainable agriculture. They are Gram-negative free-living nitrogen-fixing bacteria grouped in plant growth-promoting rhizobacteria (PGPR) (Okon, 1994; Okon and Vanderleyden, 1997; Zawoznika and Groppa, 2019). Inoculation with *Azospirillum* stimulate important changes in plant root morphology, most likely due to the bacterial production of plant growth regulating substances such as auxin and gibberelins (Fibach-Paldi et al., 2012). Plant treatment with *Azospirillum* increases the number of lateral roots and root hairs length, which maximizes the surface area available for nutrient absorption, resulting in a greater capacity for nutrient uptake and improved water status. These factors significantly contribute to the plant growth promoting effect (Lin et al., 1983; Okon, 1994; Castro-Mercado and García-Pineda, 2019). In *Arabidopsis thaliana*, inoculation of *A. brasilense* similarly causes an increase in the number of lateral roots, root hairs, elevation in internal auxin concentrations, and significant changes in the root transcriptome (Spaepen et al., 2014).

It is known that the outer cell surface characteristics of *Azospirillum* are essential for the plant–bacterium interaction contributing to the ability of the cells to colonize the root surfaces and/or to promote plant growth (Fedonenko et al., 2005). Lipopolysaccharides (LPS) are cell surface components of Gram-negative bacteria that are associated with the outer membrane of the cell envelope. In bacterial pathogens, LPS play a number of important roles in the interactions with their eukaryotic hosts (Erbs and Newman, 2003). These molecules have been associated in the virulence of many Gram-negative pathogens as key molecules in mediating host-microbe associations (Munford, 2008). In plants, LPS from various plant pathogenic bacteria trigger plant innate immune responses by acting as microbe-associated molecular pattern (MAMP) (Erbs and Newman, 2012; Desaki et al., 2018). Recently, in rice a putative protein receptor recognizing LPS from different bacterial sources such as *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Escherichia coli* has been reported. This protein, named OsCERK1, is a multifunctional receptor-like kinase that contains lysin motifs (LysMs) and is essential for the perception of chitin and peptidoglycan, fungal and bacterial MAMPs, respectively. Moreover, it seems that the different structures from the LPS are sensed by this receptor because the lipooligosaccharide and the lipid A structures stimulated the production of reactive oxygen species (ROS) at similar level than whole LPS (Desaki et al., 2018).

In relation with non-pathogenic PGPR, LPS isolated from these bacteria prime plants to respond more rapidly and/or strongly to subsequent pathogen challenges (Van Loon et al., 1998; Coventry and Dubery, 2001), and increase the  $\text{Ca}^{2+}$  influx into the cytoplasm (Dow et al., 2000; Nürnberger and Brunner, 2002; Gerber et al., 2004).

While plant defense responses to the LPS of pathogenic bacteria have been well-characterized (Erbs and Newman, 2003), there is little information regarding the effects of LPS from beneficial bacteria on plant cells. In this way, wheat plants

sprayed with *A. brasilense* LPS increased leaf length, and spike formation. Besides, the aging was accelerated and the dry weight of plants increased, suggesting that LPS affected some aspects of wheat development (Chavez-Herrera et al., 2018), however the molecular mechanism by which LPS affect plant growth remain unknown.

Responses stimulated by PGPR includes the increase in peroxidase (POD) enzyme activity, although the study of this biochemical response has been focused mostly to analyze the potential of rhizobacteria to inhibit pathogens growth (Sharma et al., 2007). POD are oxidoreductases that use  $\text{H}_2\text{O}_2$  as an electron acceptor. They take electrons from various donors to reduce  $\text{H}_2\text{O}_2$  to water (Penel and Dunand, 2009). Plant POD are grouped in class III (Welinder, 1992), and are encoded by multigene families; for example, *Arabidopsis thaliana* has 73 genes encoding for peroxidase (Tognolli et al., 2002) and *Oryza sativa* has 138 (Passardi et al., 2004). In plants, POD are mainly localized in the cell wall, the apoplast and in vacuoles. Some POD are involved in lignification, suberization, and oxidative cross-linking of proteins in the plant cell walls (Quiroga et al., 2000; Deepak et al., 2007; Mnich et al., 2020). Enzymatic activity of POD can be detected in all organs and almost all tissues, with a particularly high level in roots (Francoz et al., 2015).

The calcium ion ( $\text{Ca}^{2+}$ ) is a ubiquitous intracellular second messenger used extensively in all organisms to connect intracellular responses to extracellular stimuli and to coordinate a wide range of endogenous processes (Edel et al., 2017). It is well-known that POD activity is strongly dependent on the presence of calcium ions (Pintus et al., 2011) and they may switch a POD between different modes of action (Mura et al., 2006). Removal of calcium ions results in a considerable decrease in POD activity (Morishima et al., 1996), suggesting calcium ions function to maintain enzyme stability associated with high catalytic activity (Medda et al., 2003).

The balance of plant growth and cell expansion can be controlled by POD. They are able to either build a rigid wall by forming bonds by oxidizing aromatic cell wall compounds in the presence of  $\text{H}_2\text{O}_2$ , or loosen cell wall by regulating the concentration of  $\text{H}_2\text{O}_2$  or generating hydroxyl radical ( $\text{OH}^\cdot$ ) which break covalent bonds in cell wall polymers. They can also control cell elongation through their auxin oxidase activity (Francoz et al., 2015).

The aim of this study was to analyze the effect of LPS derived from *A. brasilense* on *in vitro* early plant growth of wheat seedlings, and on several biochemical responses related with plant growth, such as peroxidase activity and calcium mobilization.

## MATERIALS AND METHODS

### Biological Material

The strain used in this study was the wild type *A. brasilense* Sp245 (Baldani et al., 1986). The strain was routinely maintained on solid LB medium (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl, 0.186 g/L  $\text{MgSO}_4$ , 0.277 g/L  $\text{CaCl}_2$ , 15 g/L agar).

*Triticum aestivum* seeds, cv Nana F2007, were kindly provided by Dr. Mario González-Chavira (Instituto Nacional

de Investigaciones Forestales, Agrícolas y Pecuarias—Celaya, Guanajuato, México).

## Extraction of Bacterial LPS

Lipopolysaccharide extraction was performed as previously described (Renukadevi et al., 2012), with some modifications. A culture of 50 mL *A. brasilense* was grown for 16 h in LB agar (28°C). Cells were washed (3,200 rpm, 5 min) in phosphate buffered saline (10 mM, pH 7.2). Pelleted cells were then resuspended in 10 mL 50 mM Tris-HCl and 2 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 8.5). The bacterial suspension was mixed with 90% phenol that was preheated to 65°C, and then incubated at 35°C in an orbital shaker (160 rpm) for 15 min, followed by centrifugation at 5,000 rpm for 20 min. The supernatant (water fraction) was used to purify LPS by extensive dialysis against deionized water for 4 days. LPS were concentrated by alcohol precipitation as follow: sodium acetate was added to a final concentration of 0.15 M followed by dropwise addition of ice-cold 96% ethanol to yield a final proportion of 1:4 (v/v). Then, the mixture was incubated for 24 h at −20°C. Pellets containing LPS were collected after centrifugation at 4,000 rpm and then resuspended in distilled water. Final purified LPS were lyophilized and stored at 4°C.

## Azospirillum brasilense Inoculum Preparation

A pre-inoculum was first established taken a colony from a fresh culture and inoculating in 3 mL of liquid LB medium. The culture was incubated for 16 h (~ 1.0 OD<sub>600</sub> nm) at 28 °C, with rotation at 140 rpm. After that, 150 µL of the pre-inoculum was transferred in 50 mL of liquid LB medium and incubated for 16 h (exponential phase) at 28°C, with rotation at 140 rpm. Bacterial cultures were centrifuged at 3,800 g for 12 min at room temperature. The pellet was washed with 0.9% NaCl, centrifuged at 4,300 × g, 10 min, 4°C and resuspended in 0.01 M MgSO<sub>4</sub>, and adjusted using sequential dilutions to yield the desired final concentration of colony-forming units (CFU)/mL for its use.

## Seedlings Treatment

To germination, seeds were first shaking in 1% sodium dodecyl sulfate (SDS) for 3 min, and then surface sterilized with 1% sodium hypochlorite solution for 5 min. Seeds were washed four times with sterile distilled water, 5 min each time, and germinated on distilled water-wetted sterile filter paper in Petri dishes for 3 days in the dark at 28°C. The seedlings were aseptically transferred to assay tubes (15 cm length and 2.5 cm width) where only the roots were immersed in 5 mL liquid Murashige and Skoog (MS) medium (pH 5.7).

Lipopolysaccharide were added to the liquid MS medium at various concentrations (10, 100, 500, and 1,000 µg/mL). Inoculation with *A. brasilense* was performed by adding 10<sup>6</sup> CFU/mL in liquid MS medium. To analyze the role of peroxidase and calcium on plant growth, salicylhydroxamic acid (SHAM), a peroxidase inhibitor, lanthanum chloride (LaCl<sub>3</sub>), a Ca<sup>2+</sup> channel inhibitor, ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), a calcium chelator, and A23187, a calcium ionophore, were used at 50, 1, 100, and 10 µM,

respectively. Chemicals were added alone into MS medium or half hour before the addition of 100 µg/mL LPS. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). Treated seedlings were incubated by 4 days into a growth chamber (Percival Scientific AR-95L) at 22°C with photoperiod of 16 h light, 8 h darkness and light intensity of 100 µmol m<sup>−2</sup> s<sup>−1</sup>. Plant growth parameters were measured as leaf length, root length, number of lateral roots, total fresh weight and dry weight after different treatments.

To *A. brasilense*, inoculum was adjusted to a final concentration of 10<sup>6</sup> colony-forming units (CFU)/mL.

## Total POD Assay

Soluble POD assay was performed according to Svalheim and Robertsen (1990). Seedlings were treated with LPS by 24 h and after that POD activity was assayed. Roots (~ 100 mg) from plants were ground in liquid nitrogen, re-suspended in 10 mM sodium phosphate, pH 6.0, and homogenized by vortex. Samples were centrifuged at 12,000 rpm for 5 min, and the suspension was assayed for peroxidase activity following the formation of tetraguaiacol as an increase in absorbance at 470 nm in a spectrophotometer (Beckman Instruments, Fullerton, CA). Each reaction mixture (1 mL) consisted of 10 µL root extract and 990 µL guaiacol solution containing 0.25% guaiacol (v/v) in 10 mM sodium phosphate buffer pH 6.0 and 0.125% H<sub>2</sub>O<sub>2</sub> (v/v). The reaction was monitored for 1 min. Protein content of extracts was determined by Bradford assay 1976. Bovine serum albumin was used as the standard.

## In vivo Detection of Root POD Activity

Wheat roots growing in MS liquid medium in presence of 100 µg/mL LPS alone or in combination with 50 µM SHAM for 4 days were transferred to solution consisting of 0.1 M Tris-acetate, 0.1 mM 2,6-dichloroindophenol (DCPIP), and 0.9 mM H<sub>2</sub>O<sub>2</sub> at pH 5. After 30 min of incubation, a reddish color was developed, and root images were taken.

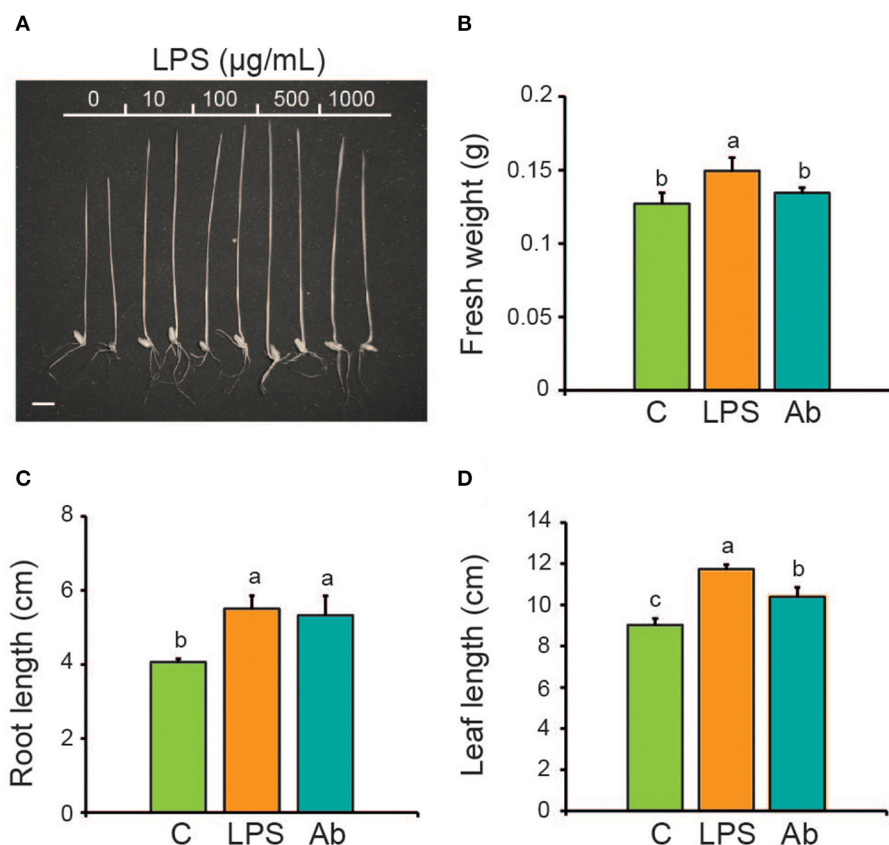
## Data Analysis

Experiments were repeated at least three times and data were expressed as mean ± standard error (SE). Statistical analyses were done with one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests for independent samples. In all cases, the confidence coefficient was set at *P* < 0.05.

# RESULTS

## Effect of Lipopolysaccharides on Plant Growth

The effect of isolated LPS on the growth of wheat seedlings was analyzed. LPS were used at various concentrations (ranging from 10 to 1,000 µg/mL) to treat by 4 days wheat seedlings. The plant growth promotion of the seedlings was concentration-dependent (Figure 1A). Treatment of seedlings with 10–500 µg/mL LPS increased total fresh weight and total dry weight for 57 and 38%, respectively, whereas root length and leaf length were increased for 60 and 31%, respectively relative to untreated seedlings. Although all these concentrations stimulated plant



**FIGURE 1 |** Effect of *A. brasilense* LPS on wheat seedlings growth. LPS were added to seedlings growing in liquid MS medium and after 4 days growth parameters were analyzed and compared with the inoculation of *A. brasilense* cells. **(A)** Image of wheat seedlings treated with increasing LPS concentrations. Barr = 1 cm. **(B)** Total fresh weight. **(C)** Root length. **(D)** Leaf length. C, Control (untreated seedlings); LPS, Lipopolysaccharides; Ab, *Azospirillum brasilense*. LPS in panels **(B–D)** were used at 100 µg/mL. *A. brasilense* was inoculated at  $10^6$  CFU/mL. Data are mean  $\pm$  SE of three independent experiments ( $n = 10$ ). Letters above bars indicate significant differences according to a Duncan test ( $p < 0.05$ ).

growth, this biological activity was consistently observed with LPS at 100 µg/mL. Increasing the LPS concentration above 1,000 µg/mL produced no additional increase in plant growth (Table 1).

The effect of LPS (100 µg/mL) on plant growth was compared with the inoculation of whole cells of *A. brasilense* ( $10^6$  CFU/mL). The inoculation of wheat seedlings increased total fresh weight by  $\sim 44\%$ , and root length and leaf length by  $\sim 26$  and  $20\%$ , respectively, in relation to the non-inoculated control (Figures 1B–D). Thus, LPS shown similar or even better bioactivity to the rhizobacteria to stimulate plant growth.

### Effect of LPS on POD Activity

Wheat seedlings were treated with two concentrations of LPS (10 and 100 µg/mL), and after 4 d of incubation, the POD activity was assayed on roots. Both LPS concentrations were able to increase POD activity (32 and 45% to 10 and 100 µg/mL), with an overall effect of increase observed to a higher concentration (Figure 2A).

Next, we used SHAM, a POD inhibitor, to further assess the effect of the LPS on wheat seedlings. Seedlings were grown in

presence of the inhibitor at 50 µM alone, or in combination with LPS at 100 µg/mL for 4 days. SHAM alone decreased POD activity in the roots by 55 % related to the untreated control. The bacterial LPS failed to increase POD activity in the presence of SHAM (Figure 2B).

Root seedlings treated with LPS (100 µg/mL) and staining with DCPIP to locate *in vivo* POD activity indicated a high increase in the enzyme activity in all root tissues, as well as, a high increase in hair root formation. In control roots, POD activity was mainly detected in the root tips and it was abolished with 50 µM SHAM. The addition of LPS to seedlings treated with SHAM not shown increase in root POD activity (Figure 2C). The plant growth parameters leaf length, root length, and total fresh weight also decreased with SHAM treatment, regardless of whether LPS were added (Figures 2D–F).

### Effect of Calcium on Wheat Responses Stimulated by LPS

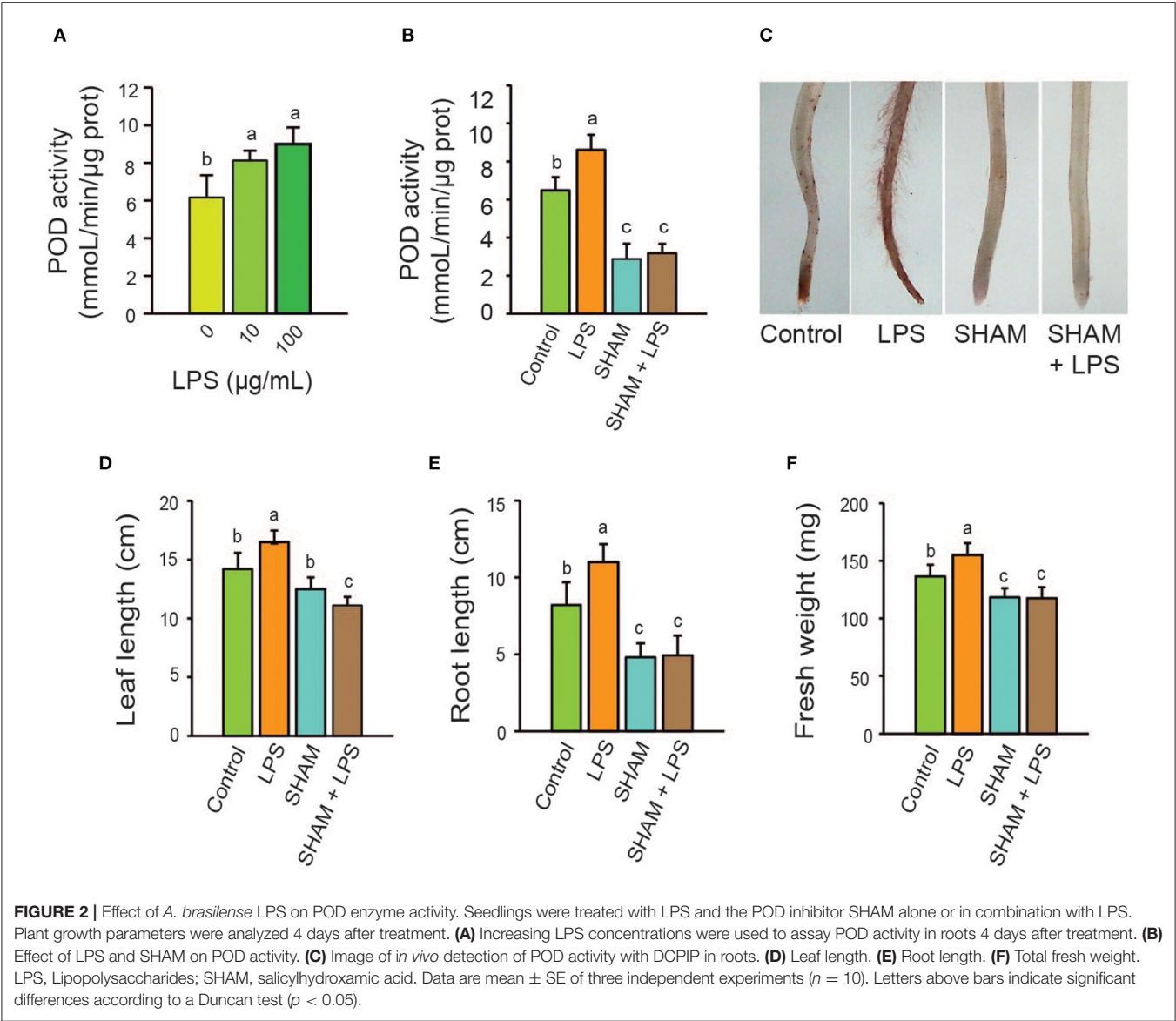
The role of calcium on wheat seedlings growth (total fresh weight, root length, and leaf length) promoted by LPS was analyzed using



TABLE 1 | Effect of *A. brasilense* LPS on wheat seedling growth.

LPS (μg/mL)	FW (g)	DW (g)	RL (cm)	LL (cm)
0	0.074 ± 0.005b	0.029 ± 0.005b	2.8 ± 0.265ab	7.6 ± 0.44c
10	0.117 ± 0.010a	0.040 ± 0.005a	3.8 ± 0.200b	9.3 ± 0.30b
100	0.107 ± 0.008a	0.040 ± 0.004a	4.2 ± 0.128ab	10.1 ± 0.57a
500	0.117 ± 0.007a	0.040 ± 0.005a	4.5 ± 0.163a	10.0 ± 0.53a
1000	0.091 ± 0.007ab	0.031 ± 0.002b	3.8 ± 0.404b	9.6 ± 0.51ab

FW: Fresh weight; DW: Dry weight; RL: Root length; LL: Leaf length. Data are mean ± SE of three independent experiments (n = 10). Letters indicate significant differences according to Duncan test (p < 0.05).



the calcium ionophore A23187 and the calcium channel inhibitor lanthanum chloride. The ionophore A23187 at 10 μM alone stimulated seedling growth at similar levels to those observed with LPS, suggesting that some of the plant growth promoting effects were mediated through Ca<sup>2+</sup> signaling (Table 2). When

the ionophore was added to seedlings in combination with LPS (100 μg/mL) not additional increase in growth was observed compared to untreated control. An increase in POD activity (~32%) in presence of the ionophore, similar to treatment with LPS alone, was observed.



**TABLE 2 |** Effect of A23187, EGTA and LaCl<sub>3</sub> on wheat seedling growth and POD activity.

Treatment	FW (g)	RL (cm)	LL (cm)	POD activity
Control	125.9 ± 17.12a	5.5 ± 1.41b	11.9 ± 0.50b	6.23 ± 1.18b
LPS	135.0 ± 11.03a	7.3 ± 0.94a	14.1 ± 1.05a	8.08 ± 0.77a
A23187 (10 µM)	168.0 ± 19.84a	7.4 ± 0.96a	13.6 ± 0.84a	8.28 ± 0.48a
A23187 (10 µM) + LPS	120.5 ± 08.11bc	6.2 ± 1.17ab	11.6 ± 0.084bc	6.94 ± 0.45bc
EGTA (100 µM)	107.3 ± 14.28b	5.9 ± 1.59ab	11.2 ± 0.90b	4.69 ± 1.39c
EGTA (100 µM) + LPS	96.3 ± 10.63c	4.1 ± 1.37c	8.7 ± 1.31c	3.91 ± 0.72c
LaCl <sub>3</sub> (1 µM)	68.5 ± 10.63c	4.1 ± 0.57bc	6.0 ± 1.25c	3.22 ± 0.65c
LaCl <sub>3</sub> (1 µM) + LPS	36.4 ± 13.50d	3.6 ± 0.62c	3.6 ± 1.20c	2.30 ± 0.37e

FW: Fresh weight; RL: Root length; LL: Leaf length. LPS were added at 100 µg/mL. POD activity is expressed as: mmol/min/µg prot. Data are mean ± SE of three independent experiments (n = 10). Letters indicate significant differences according to Duncan test (p < 0.05).

The addition of A23187 (10 µM) in combination with LPS (100 µg/mL) did not stimulate an additional increase in POD activity.

The Ca<sup>2+</sup> channel inhibitor La<sup>3+</sup> alone (1 µM) inhibited the growth of seedlings. In addition, La<sup>3+</sup> decreased POD activity by 53 % in relation to untreated control. The combination of LaCl<sub>3</sub> (1 µM) with LPS (100 µg/mL) further decreased POD activity by 66%. EGTA use alone or plus LPS decreased at similar levels plant growth and POD activity, but not to degree than with LaCl<sub>3</sub>.

## DISCUSSION

Lipopolysaccharides are molecules consisting of a lipid, a core oligosaccharide, and an O-polysaccharide moiety. The lipid moiety is called lipid A, and is embedded in the outer phospholipid bilayer. Lipid A is linked to a core oligosaccharide via the sugar 3-deoxy-D-manno-2-octulosonate (KDO) (Erbs and Newman, 2003). O-polysaccharide consists of a short series of repeating oligosaccharide units ending in the O-antigen. Variations in LPS structure reside mostly in the O-antigen, commonly used for serotype classification (Luderitz et al., 1971). Characterization of the O-polysaccharide moiety of some *Azospirillum* species LPS has shown that it consists of a polysaccharide with repeating units of that rarely contain more than five residues. Particularly, the polysaccharide of *A. brasilense* Sp245 consist of a linear pentasaccharide containing only D-Rhamnose residues (Fedonenko et al., 2002).

Despite the number of studies addressing the molecular mechanisms of interactions between *Azospirillum* and cereal plants, details about the importance of LPS in growth promotion have yet to be elucidated. The LPS cover almost 80% of the cell surface of Gram-negative bacteria, which likely underlies the critical role of these molecules in bacterial interactions with their eukaryotic hosts (Bedini et al., 2005). To date, most studies on the role of LPS during bacteria-plant interactions have focused on the LPS induction of plant defense responses. LPS from plant pathogenic bacteria function as general elicitors of plant defense responses triggering the production of reactive oxygen species (Meyer et al., 2001; Gerber et al., 2004; Bedini et al., 2005), the expression of pathogenesis-related proteins (Coventry and Dubery, 2001) and the systemic resistance in several plant species

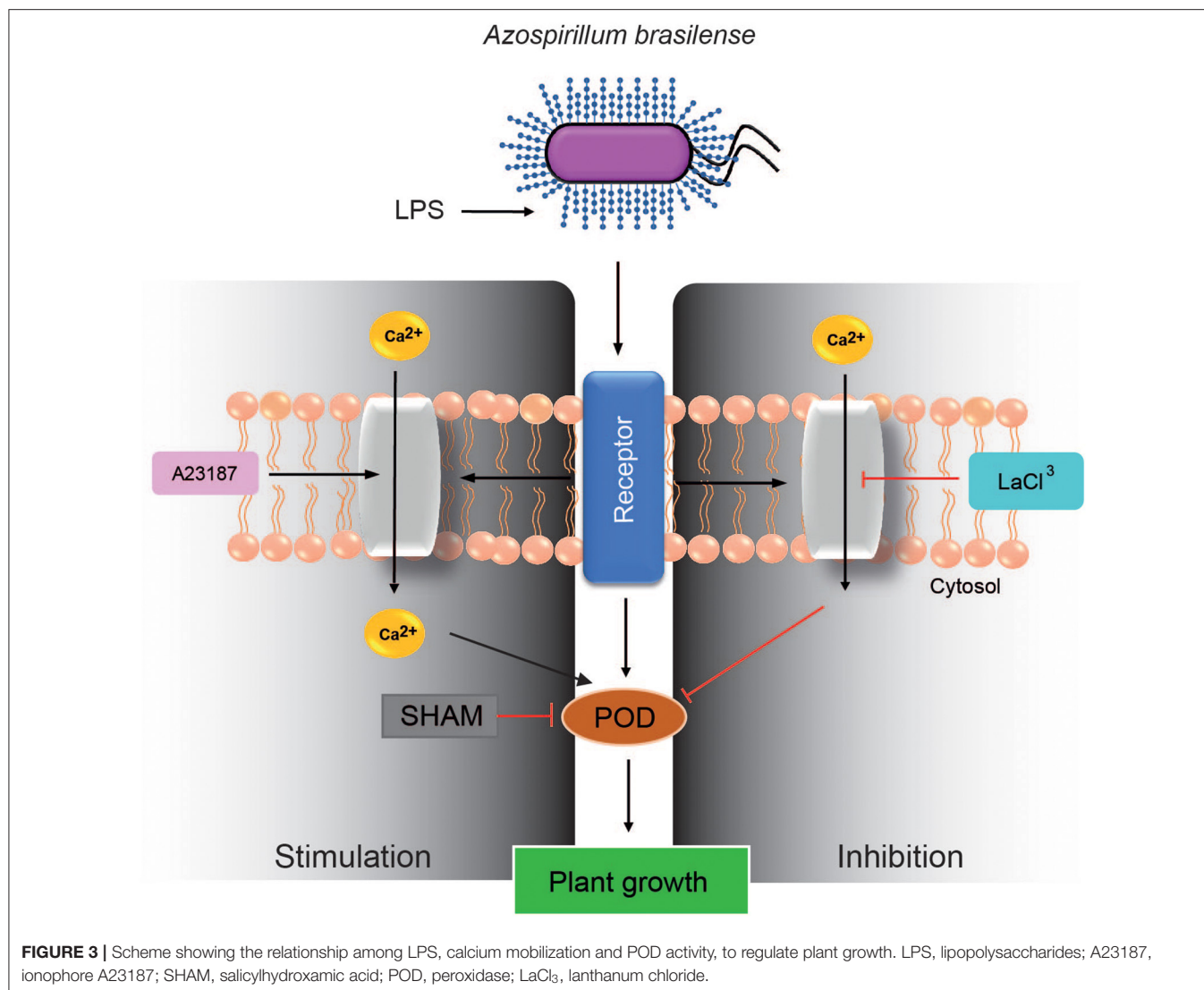
(Leeman et al., 1995; Van Wees et al., 1997; Reitz et al., 2000), among others.

In this study, we showed that LPS molecules isolated from *A. brasilense* directly mediate *in vitro* early wheat seedlings growth, a novel property of rhizobacteria LPS molecules. Also, our study supports previous observations that *A. brasilense* cell surface components, including LPS, are essential to stimulate plant growth (Bahat-Samet et al., 2004; Jofré et al., 2004).

Although the role of rhizobacterial LPS as a key component in the growth-promoting interactions between plants and bacteria awaits additional characterization, Evseeva et al. (2011) reported that treatment of wheat roots seedlings with 10 µg *A. brasilense* Sp245 LPS increased the mitotic index of cells to a degree that was comparable to that produced by inoculation with whole cells, suggesting that LPS-mediated effects on plant growth may be related to their ability to induce cell division.

Analyzing the LPS biochemical action mechanism in plant growth promotion, we found an increase in POD activity. This increase in activity could explain, in part, its effect on promoting plant growth, because SHAM, a POD inhibitor, abolished LPS-induced plant growth. Several plant development processes are regulated by POD enzymes, for example promoting root elongation (Passardi et al., 2006; Cosio et al., 2009), and lateral roots initiation and elongation processes (Mei et al., 2009). It has been reported that PGPR increased plant growth and POD activity in chile and tomato plants (Sharma et al., 2007), thus, both responses are associated during plant growth stimulated by rhizobacteria. Our results suggest that the LPS could be one of the molecules involved in the biological activity from rhizobacteria on plants because they are able to stimulate plant growth and POD activity. The effect of LPS on root morphology was particularly interesting because it caused a high increase in root hair formation concomitant with a high increase in *in vivo* POD activity; these two effects were inhibited by the addition of SHAM, suggesting that the effect of LPS on root morphology is mediated by POD activity.

The role of calcium was analyzed as a candidate molecule to mediate the cellular responses stimulated by LPS. Inhibiting the cellular availability of calcium with either LaCl<sub>3</sub> or EGTA in presence of LPS, the observed effects with LPS on plant growth



and POD activity were inhibited, clearly suggesting that calcium availability is required by LPS to stimulate plant growth. The calcium ionophore A23187 alone increased plant growth, thus supporting the hypothesis that intracellular calcium is needed for LPS biological activity. However, when the calcium ionophore A23187 was used in addition to LPS a decrease in plant growth was observed, perhaps due to an overstimulation of the biological system (Figure 3).

Peroxidase activity is dependent on the presence of calcium ions (Mura et al., 2005a; Pintus et al., 2011) altering its activity via a variety of mechanisms. Some POD enzyme have a calmodulin binding domain (Mura et al., 2005b; Pintus et al., 2011) suggesting that calcium signaling may directly affect POD activity in these enzymes. In general,  $\text{Ca}^{2+}$  ions have been linked to stabilizing POD catalytic activity (Longu et al., 2004), and in the case of horseradish peroxidase, the removal of calcium ions results a considerable decrease in POD activity. Cell wall matrix calcium-dependent binding to pectate enhances apoplastic POD

activities (Shah et al., 2004). In addition, calcium may increase POD secretion in sugar beet (Penel et al., 1984). Thus, LPS might increase POD activity acting on changes in calcium levels stabilizing or stimulating the release of POD enzymes.

Overall, the results suggest that the plant growth promoted by LPS of *A. brasilense* could be mediated by POD activity and calcium mobilization.

In addition, the biological effects of bacterial LPS on plant response could be mediated through dedicated cellular receptors. Receptor to LPS seems to be present in plants, and some candidates have been reported in rice and in *Arabidopsis* (Ranf et al., 2015; Desaki et al., 2018), but the associated signal transduction cascades remain to be better characterized.

The elucidation of the molecular mechanisms implicated in LPS induced plant growth will reveal the relative contributions of these molecules to the plant growth promotion ability of rhizobacteria.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

AAH-E conducted the experiments. EC-M contributed with technical assistance to experimental setup. EV-C and GA discussed the results and revised the manuscript redaction.

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# Use of Plant Growth-Promoting Rhizobacteria in Maize and Sugarcane: Characteristics and Applications

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Free-living bacteria that actively colonize plant roots and provide positive effects on plant development are called plant-growth promoting. Plant growth-promoting bacteria can promote plant growth and use their own metabolism to solubilize phosphates, produce hormones and fix nitrogen, and they can directly affect plant metabolism. PGPR also increase plant absorption of water and nutrients, improving root development and increasing plant enzymatic activity; moreover, PGPR can promote other microorganisms as part of a synergistic effect to improve their effects on plants, promoting plant growth or suppressing pathogens. Many studies have shown several benefits of the use of PGPR in maize and sugarcane crops. These bacteria are an excellent alternative to farmers to reduce chemical fertilization and pesticide input without promoting the environment impact and yield-reducing. The present review is an effort to elucidate the concept of rhizobacteria in the current scenario and their underlying mechanisms of plant growth promotion with recent updates. The latest paradigms of a wide range of applications of these beneficial rhizobacteria in both crops maize and sugarcane have been presented explicitly to garner broad perspectives regarding their functioning and applicability. The results from several studies have shown that the utilization of PGPR in maize and sugarcane is the great alternative to farmers face the challenge the modern agriculture.

**Keywords:** plant-growth-promoting, *Saccharum* spp., *Zea mays*, rhizosphere, yield

## INTRODUCTION

This review focuses on plant growth-promoting rhizobacteria that are beneficial for the plant. Some beneficials do so by promoting plant growth directly, i.e., in the absence of pathogens. Others do this indirectly by protecting the plant against soil-borne diseases, most of which are caused by fungi.

The use of PGPR is one potential way to decrease negative environmental impact resulting from continued use of chemical fertilizers, pesticides and herbicides. This term was first defined by Kloepper and Schroth (1978) to describe soil bacteria that colonize the rhizosphere of plants, growing in, on or around plant tissues that stimulate plant growth by several mechanisms. Since that time, research activities aimed at understanding how these bacteria perform their positive (or negative) effect have steadily increased and many studies have been published on these microorganisms (Vessey, 2003; Lugtenberg and Kamilova, 2009; Perez-Montano et al., 2014).



Increasing crop production to meet the demands of consumer markets and the growing world population relies on the use of a large amount of chemical fertilizers and pesticides, which are often overused in soil (Kumar et al., 2017). The use of chemical fertilizers in crop production provides an average yield increase of approximately 50% compared to production without their use; however, chemical fertilization practices ignore the biological potential of roots or the rhizosphere by increasing nutrient mobilization and acquisition and decreasing the interactions between plants and rhizospheric microorganisms (Meena et al., 2017). Many studies have demonstrated the abilities of plant growth-promoting microorganisms to increase plant nutritional status and reduce the use of pesticides (Aloo et al., 2019).

Plant growth-promoting rhizobacteria is an excellent alternative to farmers face the new challenges of modern agriculture as serious environmental and social problems emerged as a consequence of industrialization of agriculture provoked by necessity to increase a great amount of food to the general population. Currently, it is urgent to maintain high productivity impacting the environment as little as possible (Pérez-Montañón et al., 2013).

There are two mechanisms used by PGPR to promote plant growth. Each mechanism contains several parameters related to plant growth. The direct mechanism contains the parameters production of phytohormones Cassán et al. (2009) such as auxins (Khalid et al., 2004b); siderophores (Yu et al., 2019); phosphorous solubilization Krey et al. (2013), or nitrogen-fixing (Riggs et al., 2001). Indirect mechanism is related to biocontrol, by mean of antagonistic activity against phytopathogenic microorganisms inducing plant systemic resistance responses, interfering in the bacterial quorum sensing (QS) systems, etc. Some reports show PGPR may use more than one of these mechanisms for accomplishing plant growth enhancement (Bashan and Holguin, 1997; Ahmad et al., 2016).

In this review we begin with a description of how bacteria live on the root, which nutrients are available, and how the bacteria colonize the root. Competitive rhizosphere colonization is crucial for many mechanisms of action of plant-beneficial bacteria. Which bacterial traits are important for root colonization when bacteria compete with each other and with other organisms. We describe various mechanisms used by specialized beneficial rhizobacteria to positively influence plant growth. These mechanisms were classified as direct mechanisms and indirect mechanisms. The direct mechanisms approached in this review were phytohormone production, biological nitrogen fixation, phosphorus solubilization, potassium solubilization. The indirect mechanisms approached were production of antibiotics, induced systemic resistance, production of siderophores, rhizoremediation, and stress control and interference with the quorum sensing system. This review brings in **Tables 1, 2** the most important studies related to the use of these bacteria in maize and sugarcane production, respectively. Finally, this review also brings in **Figure 1** that shows the benefits to plants from host-PGPR interactions, **Figure 2**, direct mechanisms that benefit plant growth, and in **Figure 3**, indirect mechanisms that benefit plant growth.

## MICROBES IN THE RHIZOSPHERE

For the plant growth-promoting rhizobacteria colonize the rhizosphere two-step selection process by which the bacterial microbiota of roots is differentiated from the surrounding soil biome are necessary. One potential molecular mechanism underlying the formation of a distinctive rhizosphere microbiota from soil biomes is rhizodeposition. This process refers to intertwined plant developmental and secretory activities in the root system. Rhizodermis cells secrete a wide range of compounds, including organic acid ions, inorganic ions, phytosiderophores, sugars, vitamins, amino acids, purines, and nucleosides, and the root cap produces polysaccharide mucilage (Dakora and Phillips, 2002). Rhizodeposition appears to fuel an initial substrate-driven community shift in the rhizosphere, which converges with host genotype-dependent finetuning of microbiota profiles in the selection of root endophyte assemblages (Bulgarelli et al., 2013). To exert their beneficial effects, bacteria usually must colonize the root surface efficiently (Lugtenberg and Kamilova, 2009). The substrate-driven selection also underlies the establishment of phyllosphere communities but takes place solely at the immediate leaf surface. Both the leaf and root microbiota contain bacteria that provide indirect pathogen protection, but root microbiota members appear to serve additional host functions through the acquisition of nutrients from the soil for plant growth. Thus, the plant microbiota emerges as a fundamental trait that includes mutualism enabled through diverse biochemical mechanisms, as revealed by studies on plant growth-promoting and plant health-promoting bacteria (Bulgarelli et al., 2013).

## MECHANISMS USED BY PGPR

### Direct Mechanisms

The direct action of plant growth-promoting microorganisms involves soil improvements and the production of substances needed for plant growth, which improves fertility by mobilizing soil minerals (Naik et al., 2019). These improvements include the supply of growth regulators and essential minerals such as potassium, phosphorous and (Tabassum et al., 2017).

### Phytohormone Production

Phytohormones are responsible for plant growth development and allow plants to tolerate different stress conditions (Shaterian et al., 2005). Some rhizobacteria are able to produce phytohormones, including cytokinins, auxins, gibberellins, ethylene, and abscisic acid (ABA), which play a role in different growth processes in plants, including cell multiplication, which results in increased cell and root expansion (Glick, 2014; Kaur et al., 2016). However, the production of ABA by rhizobacteria is considered an indirect way of promoting plant growth (Belimov et al., 2014).

### Auxins

Auxins influence many aspects of plant development (Halliday et al., 2009; Grossmann, 2010). The most essential (and well-known) is indole-3-acetic acid (IAA), which is produced by

**TABLE 1** | Bacteria specie, abilities, experiment condition, and results promoted by the application of many plant growth- promoting rhizobacteria in maize crop.

Rhizobacteria/Consortium (maize)	Abilities	Condition	Results	References
<i>Lysinibacillus sphaericus</i> (T19)	BNF and IAA production	Field	Increased productivity	Breedt et al., 2017
<i>A. brasilense</i> Az39, <i>Bradyrhizobium japonicum</i> E109 (individual experiments and consortia)	Phytohormone production	Growth chamber	Increase in promoting seed germination and early seedling development (use of isolated or combined species)	Cassán et al., 2009
<i>B. pumilus</i> S1r1	BNF	Greenhouse	Higher corn cob productivity (up to 30.9%)	Kuan et al., 2016
<i>A. brasilense</i> and <i>P. fluorescens</i>	IAA production and phosphate solubilization	Field	Higher grain yield	Di Salvo et al., 2018
<i>P. fluorescens</i> F113	Nutrient acquisition	Greenhouse	Addition of N, K, Ca, Mg, and Mn equal to 40, 49, 60, 100, and 141%, respectively, in the shoots	Rocha et al., 2019
<i>Enterobacter cloacae</i>	ACC deaminase production	Greenhouse	Increases of 60, 73, 43, 69, 76, and 42%, respectively, in grain production, photosynthetic rate, stomatal conductance, chlorophyll A, total chlorophyll and carotenoids	Danish et al., 2020
<i>B. subtilis</i> and <i>A. brasilense</i>	Phosphate solubilization	Field	Higher grain yield	Pereira et al., 2020
<i>Chryseobacterium</i> sp. NGB-29 and <i>Flavobacterium</i> sp. O NGB-31	BNF and production of large amounts of IAA	Greenhouse	Increased growth parameters	Youseif, 2018
<i>Ralstonia eutropha</i> 1C2 and <i>Chryseobacterium humi</i> ECP37	Zn bioavailability in the soil	Greenhouse	Increased biomass and Zn accumulation and availability in plants	Moreira et al., 2019
<i>Pseudomonas aurantiaca</i> SR1	Production of phytohormones, antibiotics, and siderophores	Field	Increased productivity, length, and shoot and root dry weight	Rosas et al., 2009
<i>B. subtilis</i> 320	Phosphate solubilization and phytohormone production	Field	Increase in productivity and P in the shoots	Lobo et al., 2019
<i>Burkholderia cepacia</i>	Biocontrol and phosphate solubilization	Greenhouse	Increased leaf area, length, and shoot and root dry weight	Zhao et al., 2014
<i>Pseudomonas tolaasii</i> IEXb	Phosphate solubilization	Field	Increase in seedling emergence, shoot length, grain yield, 1,000-grain weight, total dry biomass, and P content in plants	Viruel et al., 2014
<i>Pseudomonas kilonensis</i> F113 and <i>Pseudomonas protegens</i> CHA0	Phosphate solubilization and biocontrol	Field	Increase in leaf yield, height, and length	Alori et al., 2019
<i>Enterobacter cloacae</i> PGLO9	Phosphate solubilization	Greenhouse	Longer root length, shoot length, and increased shoot and root biomass	Verma et al., 2018

different microorganisms and this hormone, in plants, plays an essential role in cell division, fruit development and leaves senescence (McSteen, 2010). IAA stimulates the development of many parts of the plants such as roots, leaves, and flowers (Phillips et al., 2011). In dicotyledons, IAA induces the formation of lateral roots, while in monocotyledons, it induces the formation of adventitious roots (McSteen, 2010).

Plant development is affected by IAA in both favorable and harmful ways and many bacteria have the ability to synthesize IAA, including beneficial bacteria and phytopathogens (Duca et al., 2014). More than 80% of bacteria isolated from the rhizosphere are IAA producer (Patten and Glick, 1996; Khalid et al., 2004a). IAA is responsible by part of the communication and signaling system between plants and rhizospheric bacteria (Spaepen et al., 2007).

The tryptophan is the main precursor for the synthesis of IAA and the addition of this amino acid to culture media

results in increased production in all cases (Spaepen et al., 2007). Tryptophan biosynthesis starts from metabolic nodes in a five-step reaction encoded by *trp* genes (Merino et al., 2008).

The pathways of tryptophan-dependent include indole-3-acetamide, indole-3-pyruvate, indole-3-acetonitrile pathways and tryptamine (Spaepen et al., 2007), although some intermediaries may differ most pathways show similarity to those described in plants (Patten and Glick, 1996; Woodward and Bartel, 2005). IAA synthesis pathways have been identified using several biochemical and genetic methods; however, a small set of genes and enzymes involved in these pathways have been characterized (Spaepen and Vanderleyden, 2011).

The interaction between IAA concentration and plant growth correlation is not linear, and plants should have optimal levels of endogenous IAA for optimal development (Duca et al., 2014). Excessive amounts of IAA can promote harmful effects on plants, decreasing root growth (Duca et al., 2014). Therefore, to

**TABLE 2 |** Bacteria specie, abilities, experiment condition, and results promoted by the application of many plant growth- promoting rhizobacteria in sugarcane.

Rhizobacteria/Consortium (sugarcane)	Abilities	Condition	Results	References
<i>A. brasilense</i> + <i>B. subtilis</i>	Phosphate solubilization	Field	Increased yield, dry matter, total P accumulation, and reduced fertilization by 75%	Rosa et al., 2020
<i>B. pumilus</i>	Production of IAA and enzymes (endoglucanases and xylanases)	Pot	Increase in dry matter and number and diameter of tillers	Santos et al., 2018
<i>B. subtilis</i> (BSSC11) and <i>Bacillus megaterium</i> (BMSE7)	Phosphate solubilization and the production of siderophores, IAA, ammonia, and HCN	Field	Increase in root length, shoot length, and total dry matter	Chandra et al., 2018
<i>P. koreensis</i> and <i>P. entomophila</i>	BNF, production of phytohormones, and biocontrol capacity	Growth chamber	Improvement of plant growth and development	Li et al., 2017
<i>Escherichia</i> sp. (VRE34)	Antagonism to phytopathogens, IAA production, P solubilization, and BNF	Greenhouse	Increase in plant height, stem diameter, and number of leaves	Patel et al., 2019
<i>Burkholderia gladioli</i> TNCSF 021	P solubilization	Pot	Increase in leaf chlorophyll, N content, and total biomass	Muthukumarasamy et al., 2017
<i>Bacillus altitudinis</i> and <i>Bacillus velezensis</i>	Biological control	Greenhouse	Increase in dry weight, surface area, and total root length	Liu et al., 2018
<i>Bacillus xiamenensis</i> PM14	Production of siderophores, IAA, amylase, pectinase, cellulase, chitinase, protease, and ACC deaminase and phosphate solubilization	Greenhouse	Increase in height, fresh weight, length, and root diameter and length	Xia et al., 2020
<i>Azotobacter</i> sp. (AZS3), <i>P. fluorescens</i> (Ps5), and <i>Bacillus</i> sp. (Bc1)	Production of IAA, siderophores and hydrogen cyanide; phosphate solubilization; and antifungal activity	Pot	Increased dry weight of roots and shoots and shoot height	Ahmad et al., 2016
<i>P. fluorescens</i>	Antifungal activity and induced systemic resistance	Field	Improved vegetative germination and productivity	Viswanathan and Samiyappan, 2002
<i>A. brasilense</i>	Nitrogenase activity	Field	Increase in length, diameter, and Brix value	Lopes et al., 2012
<i>B. subtilis</i> NH-160	Production of IAA, phosphate solubilization, and antifungal activity	Greenhouse	Inhibition of red rot infection	Hassan et al., 2010
<i>Azospirillum</i> spp.	Tolerance to water stress	Pot	Increase in root dry matter	Moutia et al., 2010

stimulate plant growth using IAA it must be carefully regulated to avoid inhibitory effects caused by overdosing (Duca et al., 2014). In this context, plants have many mechanisms of neutralization to control excess IAA, such as amino acids production (Sitbon et al., 1992). However, plants are not always able to carry out neutralization and are sometimes harmed by excess IAA (Duca et al., 2014).

*Aeromonas punctata* (Iqbal and Hasnain, 2013), *Azospirillum brasilense* (Camilios-Neto et al., 2014), *Bacillus subtilis* (Tahir et al., 2017), and *Burkholderia phytofirmans* (Poupin et al., 2016) are some bacterial species that efficiently synthesize IAA.

## Cytokinins

Cytokinins are responsible for the formation of shoots, inhibition of root elongation, and the improvement of cell division and root development; cytokinins are also a type of growth regulator (Porcel et al., 2014; Jha and Saraf, 2015). In particular, they are mandatory for the progression of the cell cycle (Schaller et al., 2015). Furthermore, the balance between auxins and cytokinins determines the function of the meristem, the architecture of the root system, the formation of lateral organs of shoots and the development of reproductive organs (Schaller et al., 2015).

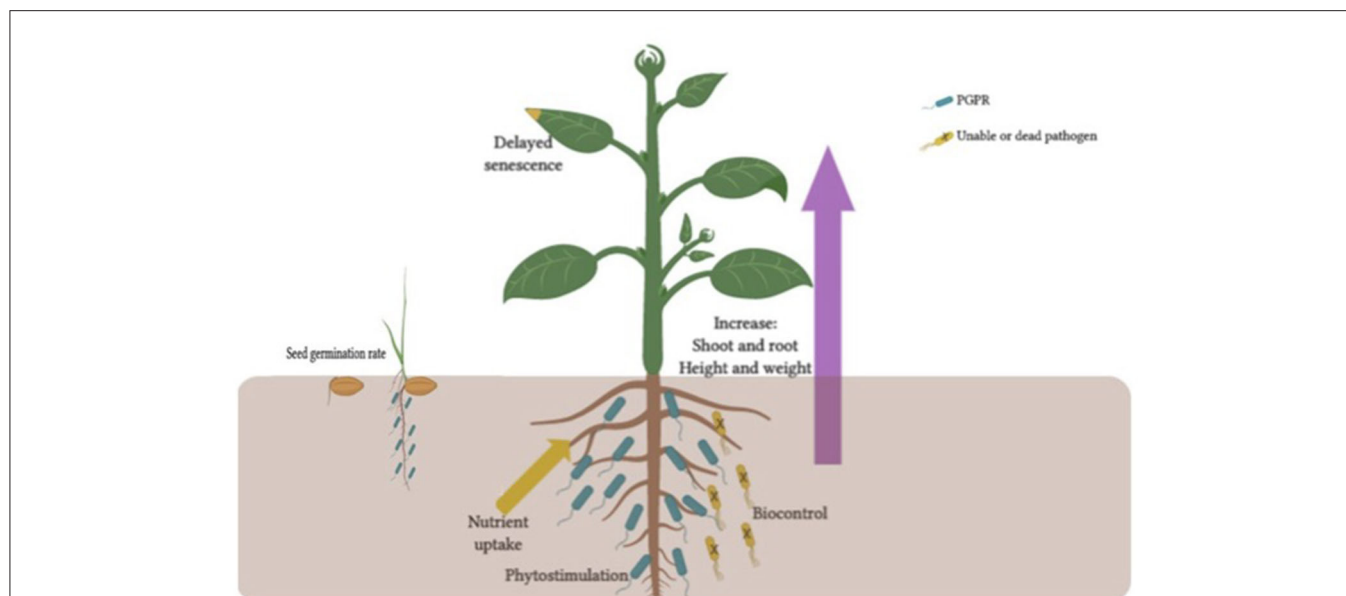
Cytokinins can regulate chloroplast biogenesis and chlorophyll biosynthesis (Cortleven and Schmulling, 2015), and they are related to development of plant resistance to abiotic stressors and biotic (Grosskinsky et al., 2011; O'Brien and Benkova, 2013).

Rhizobacteria can produce several types of cytokines, of which zeatin and kinetin are the most abundant (O'Brien and Benkova, 2013). Rhizobacteria are able to synthesize zeatin in two different ways: directly and indirectly. The direct pathway involves the synthesis of dimethylallyl diphosphate and isopentenyl adenosine monophosphate, while the indirect pathway involves *cis*-zeatin that contains tRNA to release cytokinins (Tabassum et al., 2017).

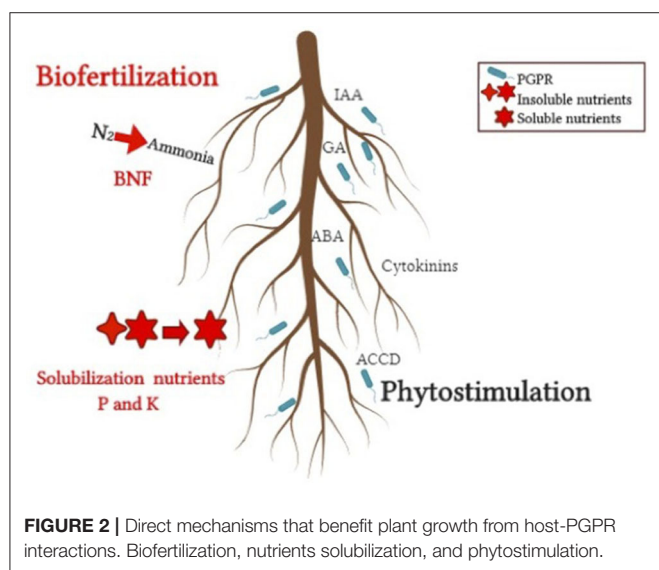
Several *Paenibacillus polymyxa*, *Azotobacter* species., *Rhizobium* species., *B. subtilis*, *Rhodospirillum rubrum*, *Pseudomonas fluorescens*, *Pantoea agglomerans*, strains are capable of producing cytokines (de Salamone et al., 2001; Glick, 2012).

## Gibberellins

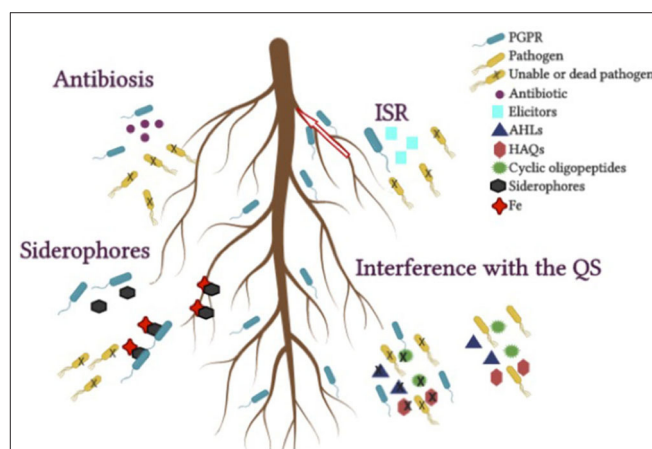
Gibberellins are another essential class of phytohormones released by rhizobacteria; these compounds are responsible for different processes in higher plants, such as seed germination, stem elongation and fruiting, the flowering process (Saleem



**FIGURE 1 |** Benefits to plants from host-PGPR interactions. These benefits have been shown to include in seeds germination rate, root growth, yield, leaf area, chlorophyll content, nutrient uptake, protein content, hydraulic activity, tolerance to abiotic stress, shoot and root weights and heights, bio-control, and delayed senescence.



**FIGURE 2 |** Direct mechanisms that benefit plant growth from host-PGPR interactions. Biofertilization, nutrients solubilization, and phytostimulation.



**FIGURE 3 |** Indirect mechanisms that benefit plant growth from host-PGPR interactions. Antibiosis, siderophores, interference with the quorum sensing (QS), induced systemic resistance (ISR).

et al., 2015). Gibberellins positively regulate cell division and elongation, stimulating the growth of the hypocotyl and stem, and they have a positive effect on root and leaf meristem size (Martínez et al., 2018). Gibberellin can promote xylem increase and shoot growth and can also decrease root growth (Guo et al., 2015; Wang et al., 2015).

Studies have shown that some plants with gibberellin-producing bacteria in their rhizospheres exhibit better growth rates (Poupin et al., 2013; Vacheron et al., 2013). Some gibberellin-producing bacterial species include *Bacillus amyloliquefaciens* (Shahzad et al., 2016), *Enterococcus faecium*

(Wang et al., 2015), *Sphingomonas* spp. (Khan et al., 2014), and *Bacillus pumilus* (Joo et al., 2004).

## Ethylene

At low concentrations, ethylene has been proven to be potentially active in fruit and leaf maturation, seed germination, leaf senescence, flower wilting, flower initiation, root elongation and branching, nodule formation, and leaf abscission (Reid, 1988). At relatively high concentrations, ethylene can be toxic to plants, causing defoliation, root growth inhibition and premature senescence (Vacheron et al., 2013). When plants experience



various stresses, such as infection, flooding, drought, and even the presence of potentially toxic metals, they produce ethylene precursors, that is, 1-aminocyclopropane-1-carboxylate (ACC) (Reid, 1988; Li et al., 2005; Liu et al., 2013).

ACC deaminase (ACCD) is an enzyme synthesized by some rhizobacteria; this enzyme can benefit plants due to its action in reducing ACC levels, transform it into ammonia and  $\alpha$ -ketobutyrate (Belimov et al., 2001). This breakage allows to regulate the adverse effects of excess ethylene, reducing its levels. Therefore, rhizobacteria capable of producing ACCD are beneficial for plants growing under stress conditions, such as high salinity (Mayak et al., 2004) drought (Sandhya et al., 2010), and the presence of potentially toxic metals (Belimov et al., 2001), regulating plant ACC levels and reducing the levels of ethylene to non-toxic levels.

*Azotobacter* spp. (Dubey et al., 2012; Farajzadeh et al., 2012), *Bacillus* spp. (Belimov et al., 2001), and *Pseudomonas* spp. (Sandhya et al., 2010; Kamran et al., 2016) are several species known for their production of ACCD.

## Abscisic Acid

Absciscic acid (ABA) is a phytohormone related in mediating stomatal opening (Mansfield et al., 1990; Maksimov et al., 2011) and also various plant aspects including development and growth in the absence of stress (Cheng et al., 2002). Absciscic acid is a molecule produced by plants, higher fungi, algae, and bacteria (Zeevaart, 1999).

Water stress stimulates high levels of ABA biosynthesis in plants, which causes partial stomatal closure as an adaptive response to conserve water (Dodd, 2007). ABA can also affect the inhibition of seed germination, the of induction of plant senescence and the abscission of fruits and leaves (Munemasa et al., 2015; Sah et al., 2016).

ABA has the abilities to reduce plant growth, even though a certain amount of ABA is required for growth, since this hormone regulates stomatal opening and, therefore, CO<sub>2</sub> absorption and loss of water (Pospisilova, 2003). Some PGPR can reduce ABA levels in host plants and indirectly increase plant growth (Belimov et al., 2014); these positive effects depend on endogenous ABA levels in the host plant (Vacheron et al., 2015).

Many rhizobacteria produce ABA in culture media and mediate the ABA status of plants (Dodd et al., 2010). *Achromobacter xylosoxidans* (Forchetti et al., 2007; Sgroi et al., 2009), *A. brasilense* (Cohen et al., 2009), *Bacillus licheniformis*, *B. subtilis*, *Brevibacterium halotolerans*, and *Pseudomonas putida* (Sgroi et al., 2009) are some of these rhizobacteria.

## Biological Nitrogen Fixation

Nitrogen (N) is the most limiting nutrient for plant development and can be assimilated from the soil in the form of ammonia, nitrate and nitrite (Gopalakrishnan et al., 2017). These N forms are not abundant in most soils, and chemical N fertilizer used in agriculture is often lost because of rain or by mineral leaching of nitrogen fertilizer (Perez-Montano et al., 2014).

Under these circumstances, bacteria play a fundamental role because some bacteria can perform biological nitrogen fixation (BNF) and N-fixing microorganisms are classified

into two groups: symbiotic microorganisms and free-living microorganisms (Gopalakrishnan et al., 2017). BNF is carried out by a specific gene product called *nif*, which, together with other structural genes, participates in protein iron activation, electron transfer biosynthesis of the molybdenum iron cofactor and many other regulatory processes required for enzyme synthesis and activity (Reed et al., 2011).

Bacteria that perform symbiosis, such as *Rhizobium* and *Bradyrhizobium*, act by forming nodules on the roots of plant species such as soybean, pea, peanut, and alfalfa, converting N<sub>2</sub> into ammonia, which can be used by the plant as a source of N (Murray, 2011).

Inside plant cells, rhizobia undergo a differentiation process that generates the specialized form for N fixation—the bacteroid (Olanrewaju et al., 2017). One or more bacteroids then become surrounded by parts of the plant cell membrane to form a so-called symbiosome (Madigan and Martinko, 2006). When the symbiosome is formed do the bacteroids convert atmospheric N through the enzyme nitrogenase into ammonia, in return, the plant provides organic acids (for bacteroids to produce energy) and provides an appropriate microenvironment for the action of nitrogenase, thus establishing a symbiotic relationship with bacteria (Madigan and Martinko, 2006; Olanrewaju et al., 2017).

Free-living bacteria are able to interact with roots; they live close to them so that the nitrogen fixed by these bacteria can be easily absorbed by plants and so that they the bacteria feed on root exudates (amino acids, peptides, proteins, enzymes, vitamins, and hormones) (Tabassum et al., 2017).

Some examples of free-living N-fixing bacteria include *Azotobacter*, *Paenibacillus*, *Burkholderia*, *Bacillus* and *Azospirillum*, *Herbaspirillum* (Huang et al., 2012; Anand et al., 2013; Angus et al., 2013; Habibi et al., 2014; Geddes et al., 2015; Goswami et al., 2016).

## Phosphorus Solubilization

Phosphorus (P) is another essential nutrient for plants and plays an important role in nearly all major metabolic processes, including, signal transduction, energy transfer, respiration, photosynthesis, macromolecular biosynthesis (Anand et al., 2016). Although the P reserve in soils is large, it is present mainly in the form of insoluble compounds that cannot be absorbed by plants, limiting their growth. Plants absorb phosphate only in the form of monobasic (H<sub>2</sub>PO<sub>4</sub><sup>-</sup>) and dibasic ions (HPO<sub>4</sub><sup>-2</sup>) (Perez-Montano et al., 2014).

Microorganisms play an important role in P transformation in the soil, including the P solubilization required for plant growth (Rodriguez and Fraga, 1999).

The ability to solubilize and mineralize P by phosphate-solubilizing bacteria is an important characteristic (Oteino et al., 2015). Several low-molecular-weight organic acids synthesized by various soil bacteria are capable of solubilizing inorganic P (Sharma et al., 2013). Members of the genera *Arthrobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Microbacterium*, *Pseudomonas*, *Erwinia*, *Rhizobium*, *Mesorhizobium*, *Flavobacterium*, *Rhodococcus*, and *Serratia* have the ability to solubilize phosphates (Oteino et al., 2015).



The phosphate solubilization is based on the secretion of organic acids by microorganisms due to sugar metabolism and organisms living in the rhizosphere use sugars from root exudates (Goswami et al., 2014). The acids released by microorganisms act as good chelators of divalent  $\text{Ca}^{2+}$  ions that follow the release of phosphates from insoluble compounds. Many phosphate-solubilizing microorganisms lower the pH of media by secreting organic acids such as acetic acid, lactic acid, malic acid, succinic acid, tartaric acid, gluconic acid, 2-ketogluconic acid, oxalic acid and citric acid (Rodriguez and Fraga, 1999; Patel et al., 2015).

Organic P solubilization is also called organic P mineralization and plays an essential role in the phosphorus cycling of an agricultural system (Khan et al., 2007). P can be released from organic compounds in the soil by enzymes such as non-specific acid phosphatases, phytases, phosphonates and C-P lyases (Sharma et al., 2013).

## Potassium Solubilization

Potassium (K) is the third most important macronutrient required for plant growth and this element plays a vital role in various plant physiological and metabolic processes (Zhao et al., 2001), including photosynthesis (Wang et al., 2012), plant growth, metabolism, assimilation, sugar accumulation, and plant growth and development (Sparks and Huang, 1985). Since more than 90% of K is present in the form of insoluble minerals of rock and silicate, the soluble K concentration is generally very low in soils (Parmar and Sindhu, 2013).

K-solubilizing bacteria, such as *Acidithiobacillus* spp., *Bacillus edaphicus*, *Bacillus mucilaginosus*, *Pseudomonas* spp., *Burkholderia* spp., and *Paenibacillus* spp., have been reported for their action of solubilizing K into assimilable forms from K minerals in the soil (Liu et al., 2012).

As in the case of P solubilization, the main mechanism of K solubilization is the production of organic acids, inorganic acids and protons (the acidolysis mechanism) (Sheng et al., 2008; Parmar and Sindhu, 2013; Maurya et al., 2014; Meena et al., 2015), which are capable of converting insoluble K (mica, muscovite, and feldspar biotite) into soluble forms of K that are easily absorbed by plants (Hu et al., 2006; Mo and Lian, 2011).

Several organic acids involved in the solubilization of insoluble K, tartaric acid, succinic acid,  $\alpha$ -ketogluconic acid, citric acid, and oxalic acid are the most important ones released by K-solubilizing bacteria (Meena et al., 2014).

## Indirect Mechanisms

The indirect action of microorganisms to promote plant growth includes the production of biocontrol agents that inactivate or kill pathogens, providing a healthy environment for plants (Naik et al., 2019).

Antibiosis, competition, production of lytic enzymes (chitinases and glucanases) with the ability to hydrolyze fungal cell walls are considered indirect mechanisms of growth promotion (Bhattacharyya and Jha, 2012). Bacteria can also indirectly improve plant growth by suppressing pathogens and increasing plant innate immunity against pathogens (Tabassum et al., 2017).

## Production of Antibiotics

Antibiotics are low-molecular-weight toxins produced by the bacterial community are able to eliminate or reduce the growth of other microorganisms (Bakker et al., 2013). Both antibiotics and toxins have been identified as being produced by certain rhizospheric bacteria (Nakkeeran et al., 2013). These include amphisines, phenazines, 2-4-diacetylfloroglucinol, pioluteorin, pyrrolnitrin, hydrogen cyanide (HCN), oomycins, polymyxin, circulin, colistin, tensin, tropolone, and cyclic lipopeptides (Maksimov et al., 2011; Pandya and Saraf, 2014; Wani and Khan, 2014; Sherathia et al., 2016).

Bacteria can produce only a single antibiotic substance, while others can secrete several substances (Reimer and Bode, 2014; Majed et al., 2016). The availability of nutrients and environmental stimuli within proximity strongly affect the synthesis of antibiotics (Choudhary et al., 2007).

Rhizobacteria of the genus *Bacillus* are the most important for the production of antibiotics (Jayaprakashvel and Mathivanan, 2011). *B. amyloliquefaciens* and *B. subtilis* are described as producers of a wide variety of antibacterial and antifungal antibiotics, including subtilin, bacillisin, and emicobacillin (Leclerc et al., 2005; Chang et al., 2007).

## Induced Systemic Resistance (ISR)

ISR is described as an enhanced defensive capability of plants in response to various pathogens induced by beneficial microorganisms present in the rhizosphere (Conrath et al., 2015), a phenomenon in which the interaction of some microorganisms with roots results in plant resistance to some pathogenic bacteria, viruses, and fungi (Lugtenberg and Kamilova, 2009). ISR can also be triggered by specific environmental stimuli that lead to the upregulation of plants' innate defenses against biotic challenge and this heightened state of alert allows plants to respond faster and stronger against subsequent attack by pathogens (Van Loon, 1997).

ISR is stimulated by non-pathogenic microorganisms; ISR begins in the roots and extends to the shoots (Solano et al., 2008), initiates the defense mechanisms of plants and protects unexposed parts of plants against future pathogenic attacks by microorganisms and insects. This defense response depends on the signaling of ethylene and jasmonic acid in the plant (van Loon, 2007; Pieterse et al., 2012).

ISR has been reported to be one of the mechanisms by which PGPR can reduce the occurrence of some plant diseases, modulating the physical and biochemical properties of host plants and consequently promoting plant growth (Pieterse et al., 2014).

Some of the defense mechanisms produced via ISR in plants include reinforcement of cell walls (Ahn et al., 2007), production of secondary metabolites (Choudhary et al., 2007) and accumulation of defense-related enzymes, such as chitinases, glucanases, peroxidase, phenylalanine ammonia-lyase and polyphenol oxidase (Bhattacharyya and Jha, 2012).

*P. fluorescens* (Pieterse et al., 1996), *Burkholderia phytofirmans* (Compant et al., 2005), *B. pumilus* (Benhamou et al., 1996), *Bacillus cereus* (Conn et al., 2008), *Rhizobium leguminosarum*, *P.*

*putida* and *Serratia marcescens* (Bhattacharyya and Jha, 2012) are examples of rhizobacteria that undergo ISR.

## Production of Siderophores

Iron, which is one of the most abundant elements on Earth, is not available for direct assimilation by plants and microorganisms because, in nature, it occurs mainly as  $\text{Fe}^{3+}$  and is generally present in the form of insoluble hydroxides and oxy-hydroxides (Rajkumar et al., 2010).

To obtain iron for their growth and development, some bacteria synthesize low-molecular-weight iron-chelating molecules called siderophores (Shaikh and Sayyed, 2015; Mhlongo et al., 2018). Siderophore-producing bacteria can stimulate plant growth directly, improving plant Fe nutrition, or indirectly, inhibiting the activity of plant pathogens in the rhizosphere, which in turn limits their Fe availability (Ma et al., 2011).

Pathogen suppression caused by the production of siderophores occurs by restricting the survival of pathogens by inhibiting iron nutrition by chelating available iron (Chaiharun et al., 2009). In other words, solubilization and the competitive acquisition of iron under limiting conditions reduces the availability of iron to other soil inhabitants, subsequently limiting their growth (Haas and Defago, 2005).

In addition to iron, there is evidence indicating that siderophores form stable compounds with other potentially toxic metals, such as Al, Cd, Cu, Pb, and Zn (Gururani et al., 2013). This phenomenon is advantageous for alleviating plant stress caused by potentially toxic metals present in polluted soils Ahemad and Kibret (2014) and is not solely due to the increased availability of mineral nutrients for plants (Babu et al., 2013).

Bacterial siderophores can be classified into four main classes based on the type of ligand and basic characteristics of the functional groups associated with iron. The main classes include catecholates, carboxylates, and hydroxamates (Crowley, 2006).

Several bacterial species are capable of producing siderophores, including, *Azospirillum* (Banik et al., 2016), *Dickeya* (Sandy and Butler, 2011), *Klebsiella* (Zhang et al., 2017; Bailey et al., 2018), *Nocardia* (Hoshino et al., 2011; Soutar and Stavriniades, 2018), *Pantoea* (Burbank et al., 2015), *Pseudomonas* (Baune et al., 2017; Deori et al., 2018; Pourbabaee et al., 2018), *Azotobacter* (Romero-Perdomo et al., 2017), *Paenibacillus* (Liu et al., 2017), *Bacillus* (Kesaulya et al., 2018; Pourbabaee et al., 2018), *Serratia* (Lee et al., 2017) and *Streptomyces* (Schutze et al., 2015; Gáll et al., 2016; Goudjal et al., 2016).

## Rhizoremediation and Stress Control

Plants are often exposed to various environmental stresses, and plant growth is sometimes inhibited by a large number of biotic (insects, bacteria, fungi, and viruses) and abiotic (radiation, salinity, temperature, flood, drought, and contaminants,) stresses, resulting in highly negative impacts on survival and plant biomass (Islam et al., 2016).

Phytoremediation uses plants and microorganisms to remove, destroy or scavenge toxic metals from contaminated environments in an efficient and economical way (Ma et al., 2011).

Most potential metals are toxic to plants, but bacteria are capable of neutralizing metal toxicity, linking it to negatively charged functional groups throughout the cell wall, which provides interactions with positive ions, in particular metal ions—a phenomenon called metal biosorption (Syed and Chinthala, 2015). Microbial species with considerable resistance to metals showed immobilization of toxic metals or reducing its concentration when added to contaminated soils by reducing their toxicity to the plant or crop (Wani and Khan, 2014).

## Interference With the Quorum Sensing System

Many bacteria rely on chemical communication to recognize the environment and retrieve information about population density. Consequently, multiple molecules are released, which synchronize the expression of genes, coordinate behavior through a process called quorum sensing (QS) and determine the relationships with eukaryotic species (Ortiz-Castro and Lopez-Bucio, 2019). QS is considered a social trait of bacteria (Whiteley et al., 2017).

Communication between cells is mediated by small molecules of diffusible signals called self-inducers (Fuqua et al., 1994). Generally, signaling mediated by self-inducers occurs at high population densities because microorganisms act in communities where there are advantages for the entire population of cells, simulating a multicellular organism (Ganin et al., 2009; Bai and Rai, 2011).

While N-acyl-homoserine-lactones (AHLs) and occasionally 4-hydroxy-2-alkylquinolones (HAQs) are often found in gram-negative bacteria, gram-positive bacteria mainly use cyclic oligopeptides. AHLs are the most common self-inducing molecules that regulate the expression of genes involved in the production of virulence factors or biofilm formation in various plant pathogens (Quiñones et al., 2005). Many plants are capable of producing molecules that specifically interfere with QS systems of bacteria associated with plants, and in any case, depending on whether the bacterium is detected as a pathogen or as a beneficial microorganism, the molecule improves or inhibits QS-regulated phenotypes (Pérez-Montaña et al., 2013).

## PGPR Used in Maize

Maize (*Zea mays* L.), which is a member of the Poaceae family, is one of the most important cereal crop species in the world and serves as a staple food for many populations (Rouf Shah et al., 2016). According to the International Grains Council (2019), global maize consumption is expected to reach new peaks in the coming years (projection until 2024), and the use of maize for animal feed is expected to increase during the same period.

Maize is among the three most important crop species in the world, providing almost half of the daily energy to organisms in Africa and the Americas (FAOSTAT Food Balance Sheets, 2020). The demand for maize in response to growing populations will require marked increases in the production, sustainability, and resilience of maize-based agricultural systems (Shiferaw et al., 2011).

To maintain increases in maize productivity, an increase in the amount of fertilizers will be necessary, resulting in

both an increase in production costs and a greater negative impact on the environment. Many beneficial effects of plant growth-promoting rhizobacteria on crop growth and yield have been well-documented. Breedts et al. (2017) found an increase yield ranging from 24 to 34% using *Paenibacillus alvei*, *B. safensis*, *B. pumilus*, and *Brevundimonas vesicularis*. Cassán et al. (2009) assessed the effect of the mixture of *A. brasilense* with *Bradyrhizobium japonicum* and verified the increase of seeds germination rate, and early development. Kuan et al. (2016) reported that plant growth-promoting bacteria may provide a biological alternative to fix atmospheric N<sub>2</sub> and delay N remobilization in maize plant to increase crop yield based on an understanding that plant-N remobilization is directly correlated to its plant senescence promoting high ear up to 30.9% with reduced fertilizer-N input. Di Salvo et al. (2018) reported that PGPR used as inoculants of cereal crops including maize can improve their growth and grain yield. The crops responses to inoculation are complex because are defined by plant-microorganisms interactions, many of them still unknown. Thus, it is necessary to improve the knowledge about the microbial ecology of the rhizosphere of crops under different agricultural practices.

Several bacteria that have the ability to produce IAA and have positive effects on shoot and root weight and nutrient uptake on maize plants. Besides, activities like phosphorus solubilization, or even other non-evaluated PGPR traits that stimulate plant growth (Lobo et al., 2019).

The bioprotective role of PGPR on maize crops has also been studied. The toxigenic fungus *Fusarium* is one of the significant genera associated with maize. Some PGPR such as *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* were able to protect maize against *Fusarium verticillioides* when applied in the form of seed coatings (Pereira et al., 2011). Interestingly, some PGPR species have appeared to promote plant growth by acting as both biofertilizers and as biocontrol agents. For instance, strains of *B. cepacia* have been observed with biocontrol characteristics against *Fusarium* spp. Simultaneously, they can also stimulate the growth of maize under iron-poor conditions via siderophore production (Bevivino et al., 1998).

## PGPR Used in Sugarcane

Sugarcane (a hybrid of *Saccharum* species) is one of the oldest and most valuable crop species in the world due to the vast benefits associated with its industrial use. Sugarcane is grown in tropical and subtropical regions (Chhabra et al., 2016) and is mainly used to supply raw materials to the sugar industry for the production of sugar (Zhao and Li, 2015). In addition, sugarcane also has global importance due to the benefits associated with the production of biofuel and biogas (Hoang et al., 2015).

New technologies developed in all production sectors have allowed extending the period of sugarcane planting and, consequently, more efficient use of labor and inputs, thus increasing the sustainability and competitiveness of the sugar and alcohol industries (Oliveira et al., 2012).

Among the various techniques available, PGPR stand out for their environmental and economic gains, which can reduce the amount of fertilizers needed, optimizing sugarcane production.

One of the most limitations for sugarcane production is the poor and inadequate fertile soil which fail to meet the nutritional and growth requirements and hence unable to achieve high production (Caione et al., 2015). Phosphorus (P) is the most critical element with high interaction with soil Raj and Antil (2011) required in a small amount by sugarcane in comparison of N and K, but still plays an essential role in the development of tillering and root system and greatly influence the longevity (Kingston, 2013).

The low availability of phosphorus is due to low P in the source material, clay absorption and its precipitation with oxides and hydroxides of iron and aluminum (Caione et al., 2015). As consequence of it, a great amount of P fertilizers is applied in sugarcane production promoting high production cost. Furthermore, phosphate fertilizers are produced by rock phosphate and the phosphate source worldwide are not renewable (Sattari et al., 2012).

Around 10–30% of the phosphorus fertilizer applied in the first year is absorbed by the roots of cane crop whereas an intensive amount accumulates in the soil as fixed P, not available to plants (Syers et al., 2008). Therefore, is urgent to find alternatives to reduce the use of phosphate fertilizers. Since every day looking to a more sustainable agriculture combined with increase in yield and economically practicable.

The use of plant growth-promoting rhizobacteria (PGPR) is a promising alternative in sugarcane production with low environmental impact to increase the efficiency of the use of mineral fertilizers including phosphate, providing high cost-effective yields (Spolaor et al., 2016).

Several field and greenhouse studies with phosphate fertilizer in sugarcane (Calheiros et al., 2012; Caione et al., 2015; Albuquerque et al., 2016; Borges et al., 2019).

Rosa et al. (2020) evaluated the effect of inoculation with three PGPR species and five P doses in sugarcane and reported that the inoculation can play a fundamental role in cultivation, generating great benefits to the crop and saving fertilizers cost for the producers. These results revealed a combination of *Azospirillum brasilense* and *Bacillus subtilis* allied to the low cost of P<sub>2</sub>O<sub>5</sub> was the best fertilizers management in the sugarcane which is meaningful production practice of sugarcane.

Santos et al. (2018) reported that the use of *B. subtilis* together with by-products can improve soil fertility parameters and decrease adverse effects associated with vinasse fertilization, in addition to providing shoot and root growth and providing collective synergy for high yield of sugarcane production with environmental benefits. Moura et al. (2018) have shown that the use of *Azospirillum* in sugarcane crop improved root system leading to better water and nutrient uptake that in turn may influence yield positively. This report showed that the significant interaction of cultivar x water regime x *Azospirillum* inoculation suggests a complex interplay of these factors, likewise, involving indigenous plant auxin pool. Li et al. (2017) isolated *Pseudomonas* sp associated with sugarcane rhizosphere and verified useful activity the isolate such as phosphate solubilization, siderophore production, ACC deaminase activity, and IAA production, as well as N<sub>2</sub> fixing activity and diseases management. These features are measured



as important PGP traits and have been found to be effective in improving the growth and nitrogen content of sugarcane plants. The association of PGPR in sugarcane production may be an eminent development biofertilizer application, for sustainable crops production, in reducing environmental pollution and in biological agri-business. (Muthukumarasamy et al., 2017) verified that the association between diazotrophic P and K solubilizing *Rosneathales terrae* and *Burkholderia gladioli* with sugarcane were able to increase the leaf chlorophyll, N content and total biomass and encourage the farmers to use PGPR to improve N, K, and K availability in the soil.

## CONCLUSIONS AND PERSPECTIVES

This review has focused on a heterogeneous group of microorganisms found in the rhizosphere. These microorganisms live in association with roots, can stimulate plant growth and can reduce the incidence of diseases. Among the large number of PGPR, the most studied genera include *Azospirillum*, *Bacillus*, and *Pseudomonas*. The important role that PGPR play in agriculture is proven by the large number of

publications on this topic to date. The exact mechanisms used by PGPR are not yet fully known, although some characteristics of these bacteria can be used to promote plant development. In addition, for these growth-promoting characteristics to have an effect on plants, bacteria need to be rhizosphere competent and must be able to survive in rhizospheric soil, where communities can be affected by a large number of factors, such as soil characteristics, plant genotype, and agricultural practices, that together determine the presence and predominance of certain microbial groups. Growth promotion of maize and sugarcane could be optimized with appropriate combinations of PGPR, environmental conditions and plant genotypes. In this sense, additional efforts must be made in the development of good inoculants and production systems that allow reducing the amount of chemical fertilizers and insecticides used to increase soil fertility and crop productivity.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Temporal Dynamics of Rhizobacteria Found in Pequin Pepper, Soybean, and Orange Trees Growing in a Semi-arid Ecosystem

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Harsh environmental conditions in drylands force plants and their associated microbial communities to adapt to abiotic stresses. In semi-arid environments, climatic conditions and poor agricultural management have a strong impact on plant yield and thus, enhancing soil fertility by means of beneficial microorganisms such as plant growth-promoting rhizobacteria (PGPR) has been proposed as part of sustainable agricultural management. As drylands will increase due to climate change, studying microbial community dynamics of crops under such conditions is crucial as it might favor rhizobacteria adapted to drought. While the microbiome of many native dryland crops has been characterized, the microbial community composition from non-native crops under semi-arid environmental conditions is understudied. Thus, the aim of this study was to characterize the bacterial community associated with the roots of three crops with different growth cycles, cultivated in the same semi-arid environment, to understand their microbial community composition during the season with the highest temperature in northeast Mexico. We performed high throughput sequencing of the V3-V4 region of the 16S rRNA gene from root samples of Pequin pepper, soybean and orange trees. Classified taxa were evaluated according to crop, sampling time and climatological parameters. Our findings revealed that changes in temporal dynamics of microbial communities correlate with environmental temperature. Moreover, the microbial community of pepper was more diverse and differed from that of soybean and orange. Regarding PGPR, 47.6% of the genera were shared among crops with a high relative abundance of *Bacillus*, but we also detected crop-specific microbial associations where *Serratia* was specific to orange trees and *Rhodobacter* to pepper. When analyzing PGPR in correlation to climatological parameters, *Bacillus* was found to thrive under lower precipitation rates, higher temperatures and higher evaporation rates in pepper and orange. In contrast, some PGPR commonly used in commercial biofertilizers such as *Rhizobium* and *Azospirillum* were affected by high temperatures. This study provides a better understanding of the rhizobacterial assemblies of economically relevant crops grown under a semi-arid environment.

**Keywords:** dryland, plant growth promoting rhizobacteria (PGPR), Mexico, sustainable agriculture, biofertilizers, 16S rRNA-based metagenomic analysis

## INTRODUCTION

Drylands represent around 41% of the total Earth's land surface, being mostly composed by semi-arid regions (Huang et al., 2016) which are characterized by adverse environmental conditions that urge plants and other organisms to adapt in order to alleviate abiotic stress (Soussi et al., 2016). In drylands, plants are prone to experience high temperature, drought, high salinity and osmotic stress. Surviving these climatic conditions requires plants to adapt and acclimate, overall impacting crop yield (Vimal et al., 2017) and consequently, affecting the global production of cereals, oil crops, fruits and vegetables, and experiencing hundreds of billion-dollar losses (Dwivedi et al., 2018). In this environment, plant's growth is limited due to low nutrient availability caused by decreased primary productivity, high nutrient immobilization and loss by soil erosion (Cui et al., 2019).

Additionally, interactions of climate change and human activities have a strong impact on semi-arid locations. In recent years the intensive practices of modern agriculture have brought awareness of their environmental impact, for instance in greenhouse gas emissions and nutrient leaching. Besides, climate change has contributed to an expansion of the drylands all over the world with semi-arid regions having the largest growth (Huang et al., 2016). More than ever, finding sustainable alternatives to fertilize and protect plants against phytopathogens while increasing food production is extremely important (Ferreira et al., 2019). Therefore, practices that aim at enhancing soil fertility and at the same time maintain crop yield by supporting diverse microbiota from the rhizosphere have been proposed (Hartman et al., 2018).

The rhizosphere, which is rich in nutrients from root exudates, represents a stable and protected interface with the adjacent soil. Its microbiota is composed of bacteria and other microorganisms that collectively regulate ecosystem processes and play key roles in nutrient cycling, i.e., by decomposing organic matter as well as transforming and fixing soil nutrients (Hartman et al., 2018; Singh et al., 2019). Understanding rhizosphere communities is not trivial, as they are affected by interactions between soil types, plant species and their growth stages (Smalla et al., 2001; Xu et al., 2009; Philippot et al., 2013; Yuan et al., 2015; Qiao et al., 2017). In addition, an increasing amount of research has shown that in drylands the composition of bacterial assemblies is favored by drought adaptation to alleviate plant stress (Soussi et al., 2016). The root microbiome in these conditions is especially sensitive to disturbances. This aspect has been considered key to make drylands particularly prone to desertification caused by poor management and climatic events (Taketani et al., 2017).

Beneficial microorganisms like plant growth promoting rhizobacteria (PGPR) can be used as biofertilizer by working as nutrient solubilizers, but also strengtheners, biostimulants, and biopesticides (Mahnert et al., 2018; Wang et al., 2020). Bacteria classified as PGPR present at least one trait that will result in an increase of plant growth, e.g., nitrogen fixation, enhancing resistance to abiotic or biotic stress, or phytohormone production (Hayat et al., 2012; Gopalakrishnan et al., 2015; Lyngvi et al., 2016; Nehra et al., 2016; Zaeferian and Rezvani,

2016). PGPR have been identified in cereals (rice, wheat, barley, and maize) and important agricultural crops (soybean, potato, pepper, sugar cane, coffee, tea, and grapevine) (Numan et al., 2018). Moreover, some PGPR genera such as *Pseudomonas*, *Rhizobium*, *Azospirillum*, *Azotobacter*, *Burkholderia*, *Bacillus*, and *Serratia*, are present in large-scale commercial biofertilizers (Ramakrishna et al., 2019).

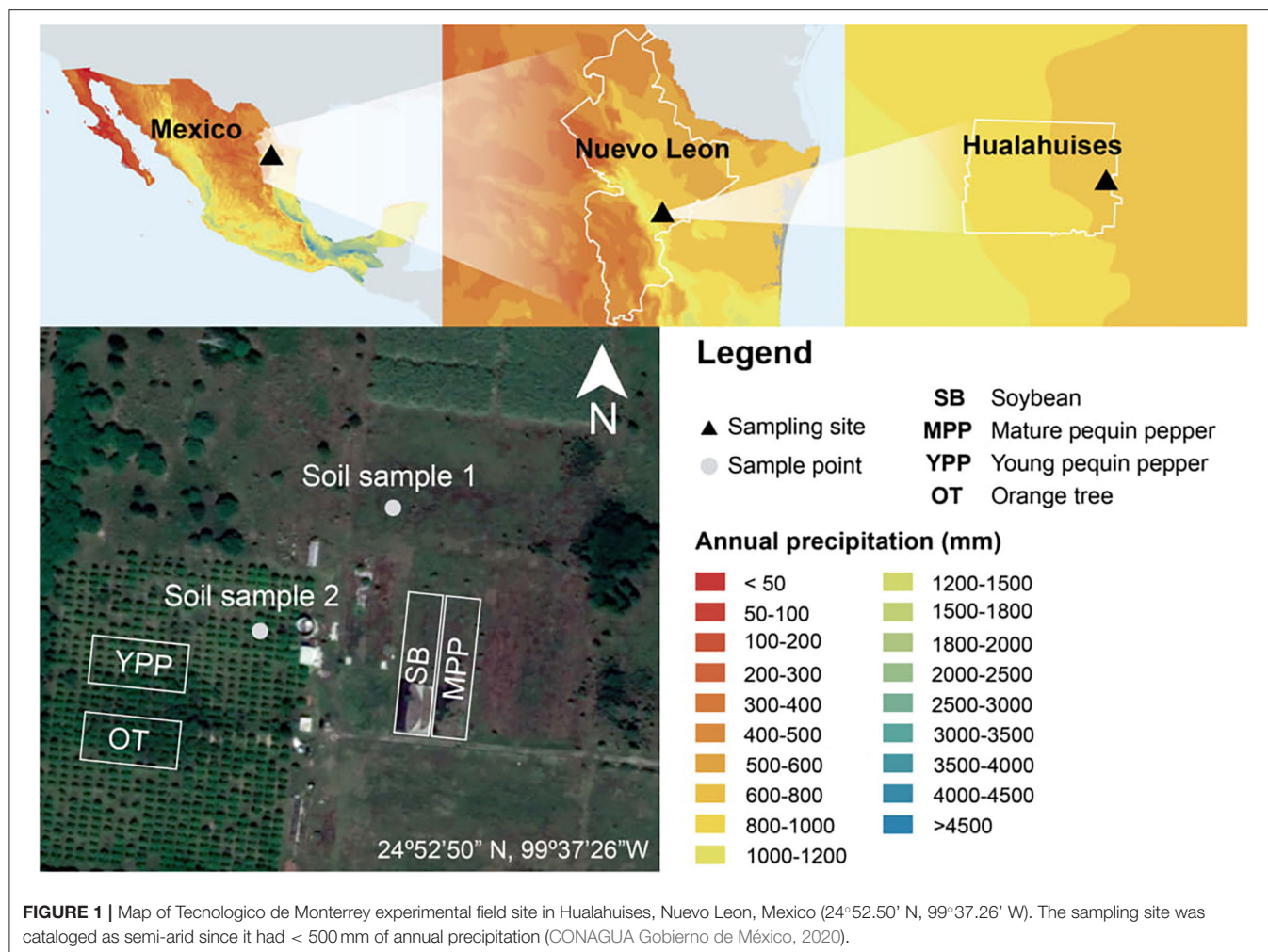
Insights on the impact of soil moisture on wheat rhizosphere microbiome in a semi-arid ecosystem have identified significant differences in specific taxa classified as PGPR (Mavrodi et al., 2018). However, current studies that evaluate plant root microbiome on drylands are mainly limited to native crops (Coleman-Derr et al., 2016; Fonseca-García et al., 2016; Dastogeer et al., 2018; Khan et al., 2020) and do not focus on introduced species that may require growth enhancers for their production. Hence, to produce biofertilizers specific for crops cultivated in drylands it is necessary to conduct studies of microbiota dynamics in field conditions for each crop.

The aim of this study is to characterize the rhizosphere bacterial community of three crops with different cycles growing under the same field conditions on a semi-arid ecosystem. A perennial crop of Pequin pepper (*Capsicum annuum* var. *glabriusculum*) and annual crops of soybean (*Glycine max* L.) and orange trees (*Citrus sinensis*) were analyzed to identify crop specific rhizobacteria and the effects of climatic conditions on microbial communities. Identified taxa were evaluated according to sampling time and crop type. The PGPR community was analyzed in correlation with climatological parameters characteristic of drylands. To our knowledge, this is the first study that evaluates the microbiome dynamics from non-native plants with different growth cycles in a semi-arid environment.

## MATERIALS AND METHODS

### Site Selection, Sampling, and DNA Extraction

Pequin pepper, soybean and orange trees were sampled from the Tecnológico de Monterrey experimental field site in Hualahuises, Nuevo Leon, Mexico (24°52.50' N, 99°37.26' W). This site has been described by the Mexican Environmental and Natural Resources Secretary as a semi-arid location based on Penman's aridity index. The sampling was done in May, July and September 2017. The sites planted with soybean and pepper were in close proximity (10 m apart), whereas the orange trees and young peppers were planted ~50 m from these crops (Figure 1). Two composite soil samples were taken each at 0–30 cm depth and were analyzed by the ISO certificated company Fertilab (Guanajuato, Mexico). The soils are of a medium clay-loam texture with pH 8.1–8.5 and only slight differences were found among the sites regarding soil characteristics (Supplementary Table 1). Lateral roots were collected at a depth of 10 cm from the ground and at least 10 cm of root-length. For Pequin pepper, plants with different ages, young (2 months old) and mature (1 year old), were sampled; for the orange trees, peripheral roots under the tree canopy were taken from approximately a radial length of 2 m, details from each sampling



time are resumed in **Table 1**. The collected roots were kept in Whirl-Pak bags at 4°C for transportation. Roots were cleaned using a sieve, washed with tap water and 96% ethanol, clean roots were cut in 1 cm long pieces and stored at −20°C.

In total, 33 samples (four biological replicates) were selected for DNA extraction performed by the FastDNA Spin Kit for Soil, according to the standard protocol, with the lysing matrix type A, but with one extra ¼ inch ceramic bead to assure complete rupture of the roots (Senés-Guerrero et al., 2014). DNA was stored at −20°C until sequencing library preparation.

### PCR, Library Preparation, and Sequencing

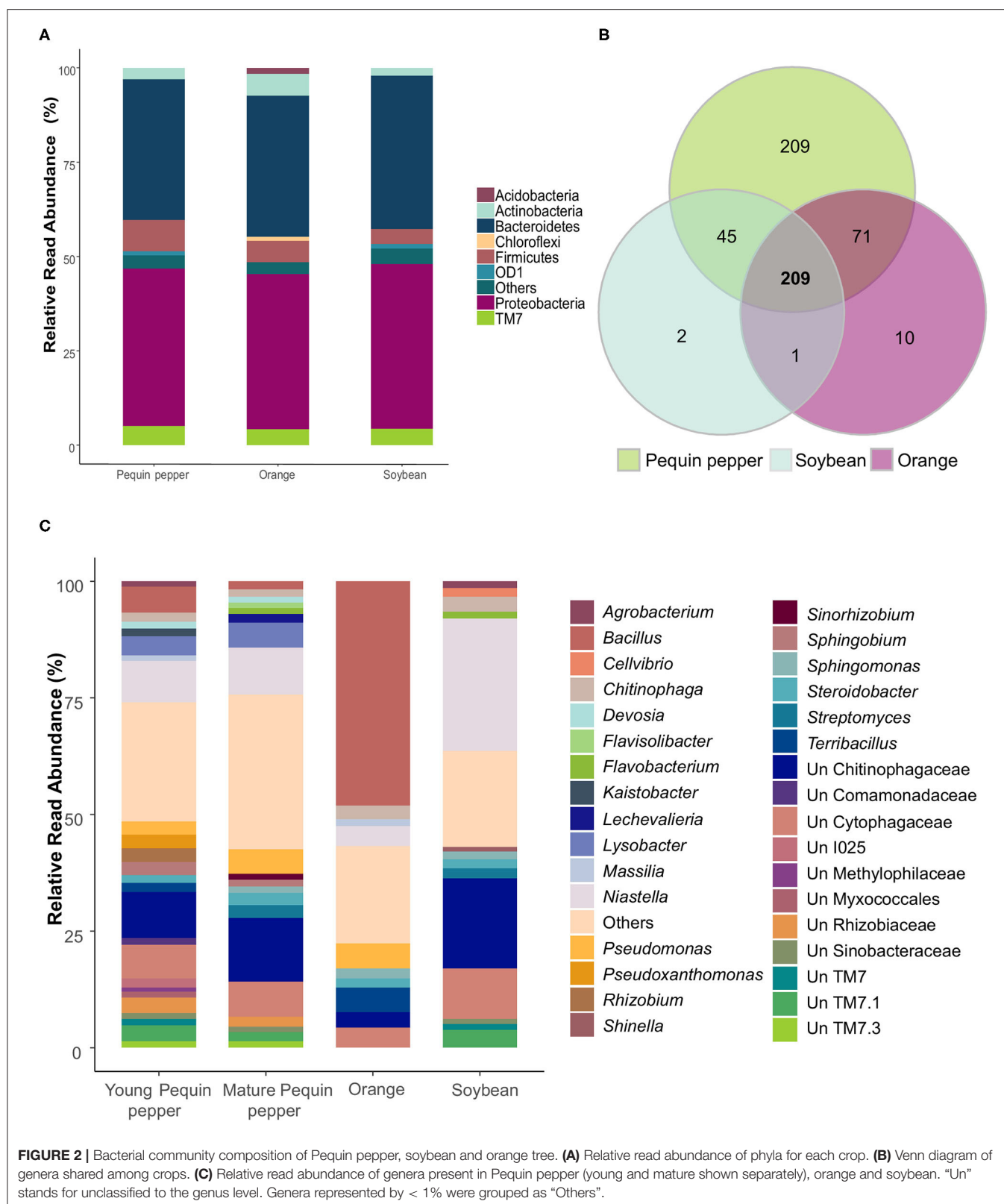
Amplification of the variable regions V3-V4 (~550 bp, including indexes and adapters) from the 16S rRNA gene was performed using the primers Bakt\_341F (5'-CCTACGGGNGGCWGCAG-3') and Bakt\_805R (5'-GACTACHVGGGTATCTAATCC-3'), following the Illumina protocol for 16S Metagenomic Sequencing Library Preparation. Each PCR was performed in triplicate per sample and visualized in a 2% agarose gel. Nextera v2 indexes (Illumina, San Diego, CA, USA) were attached to pooled PCR products according to the afore mentioned protocol. Amplicon clean-up was done using AMPure XP

**TABLE 1 |** Sampling details for Pequin pepper, soybean, and orange tree at each sampling period.

Sampling time	Crop	Age	Growth stage
May	Pequin pepper	2 months	Vegetative
		12 months	Fruiting
	Soybean	2 months	Vegetative
July	Orange	12 years	Mature
	Pequin pepper	4 months	Fruiting
		14 months	Fruiting
	Soybean	4 months	Flowering and fruiting
September	Orange	12 years and 2 months	Mature
	Pequin pepper	6 months	Fruiting (ripening fruits)
		16 months	Fruiting
	Soybean	6 months	Senescent
	Orange	12 years and 4 months	Mature

beads and indexed PCR products were observed in a 2% agarose gel.

The Qubit® dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for library

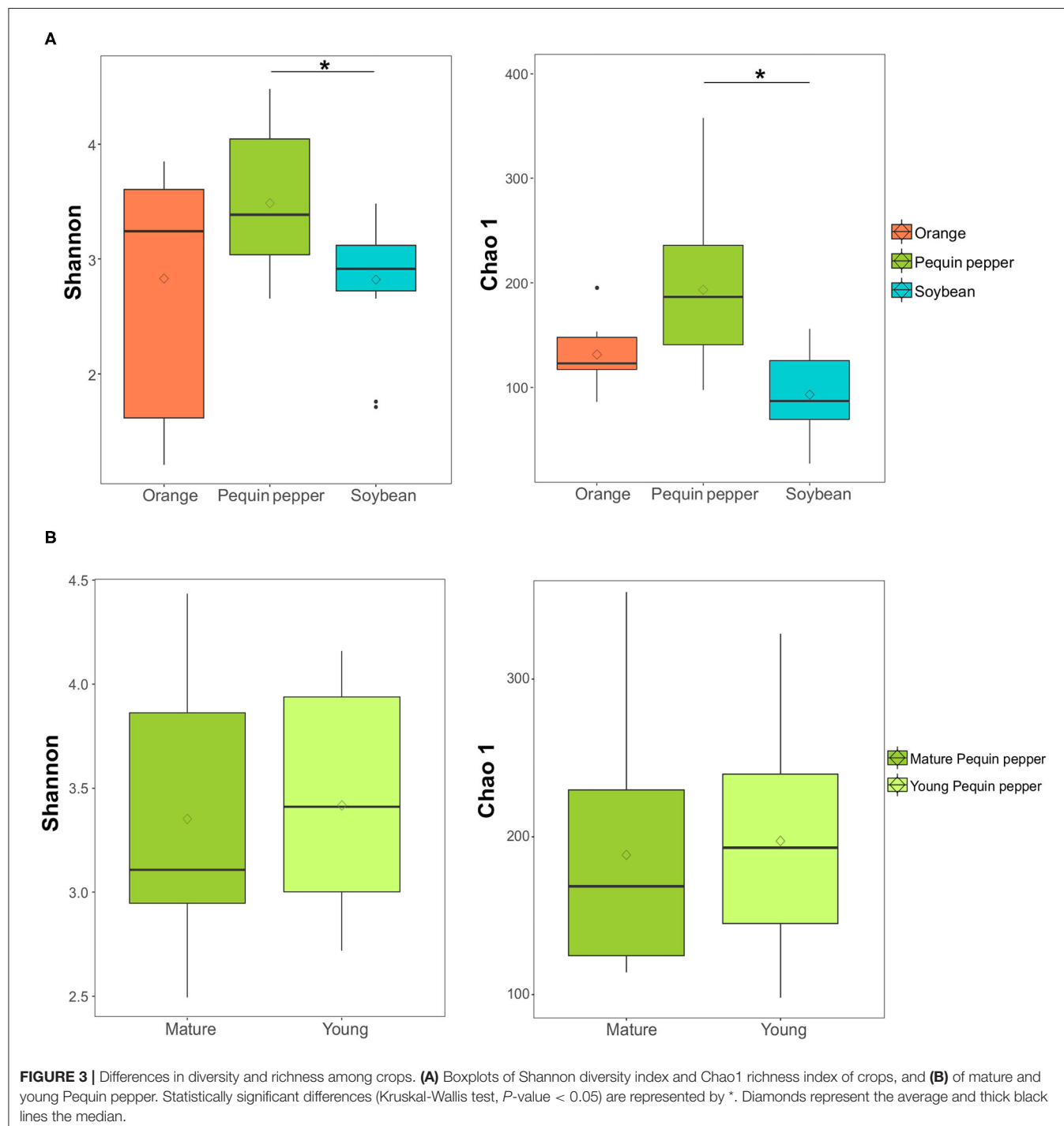




quantification in a Qubit 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA). Samples were pooled at a concentration of 6.3 pM and loaded together with 30% Phix control into an Illumina® MiSeq sequencer using the MiSeq Reagent Kit v3 (Illumina, San Diego, CA, USA) for paired-end sequencing ( $2 \times 300$  bp) carried out at the sequencing facilities of Tecnológico de Monterrey, Campus Monterrey.

## Bioinformatic and Statistical Analyses

Raw reads were used as input for QIIME2 version 2019.4 (Bolyen et al., 2019) and a standard pipeline for 16S rDNA amplicon bioinformatics analysis was followed. Briefly, forward and reverse reads were joined and filtered according to Q score prior to denoising with Deblur, obtaining a table with samples grouped in Amplicon Sequence Variants (ASVs). To assign taxonomy, a classifier was constructed using 99% similarity



and the GreenGenes database version 13.4. The classifier was trained using the primers and the length of the samples. Taxa bar plots were generated to assign the corresponding taxonomy to the ASV table. Files of phyla and genera were downloaded from view.qiime2.org in CSV format to continue further analysis.

Results were analyzed using RStudio (version 1.1.463; R Core Team, 2019). Rarefaction curves were constructed per sample. Diversity and richness indexes were computed with the vegan package (Oksanen et al., 2019) rarefied to the median read abundance, the samples that had less reads than the median were kept rarefied to the maximum. To analyze temporal dynamics, data was normalized with the DESeq2 package and used as input for perMANOVA, principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) representing the distribution with 95% interval ellipses and eliminating the genera that were present in less than 10% of the samples. Statistical non-parametric analyses were done using the vegan package and plotted with the package ggplot2 (Wickham, 2009). Unclassified genera were denoted by “Un” and the previous taxonomic level to which they were classified. For relative read abundance, taxa bar plots and presence-absence plots were generated where pepper samples were grouped in young and mature plants. Genera with relative read abundance <1% were classified as “Others.” Genera known to promote plant growth were selected as PGPR genera. Additionally, a CIRCOS plot of the PGPR genera was constructed using the package circlize (Gu et al., 2014), relating the presence of PGPR genera to each crop.

To understand the effects of climatic conditions, Mexico's Water National Commission datasets were used to construct boxplots comparing rainfall, evaporation rate, maximal and minimal temperature of the year from which sampling was done (2017) to historic data from 1998 to 2018 (CONAGUA Gobierno de México, 2020). The climatic variables were measured at a climatological station located at 2 km from the experimental field. These climatological parameters were used to analyze their correlation with PGPR genera using Pearson's correlation with the corrplot package (Wei and Simko, 2017). Historic climatic data was also analyzed from 1998 to 2018 to classify the sampling site as semi-arid.

## RESULTS

### Rhizobacteria Community Composition of Pequin Pepper, Soybean, and Orange Trees

As a first approach to describe temporal dynamics of rhizobacteria of Pequin pepper, soybean and orange trees, we characterized their root endophytic bacterial community at three sampling times in a single field site under semi-arid environmental conditions. Taxonomic classification was assigned at two distinct levels, phylum and genus (Figure 2). Regarding the most abundant phyla, crop microbial composition was highly similar among samples (Figure 2A), where the most abundant phyla were Proteobacteria ranging from 41.1 to 43.6% relative read abundance and Bacteroidetes from 37.2 to 40.7%.

**TABLE 2 |** perMANOVA for the factors crop and sampling time.

	Df	Sum Sq.	Mean Sq.	F model	R <sup>2</sup>	P-value
Crop	2	1.2189	0.60946	8.6100	0.22080	0.001
Time	2	1.0705	0.53525	7.5616	0.19391	0.001
Crop: time	4	0.6829	0.17072	2.4118	0.12370	0.001
Residuals	36	2.5483	0.07079		0.46159	
Total	44	5.5206			1.00000	

Among the tested crops, orange was the only one that possessed *Acidobacteria* and *Chloroflexi* in an abundance higher than 1%.

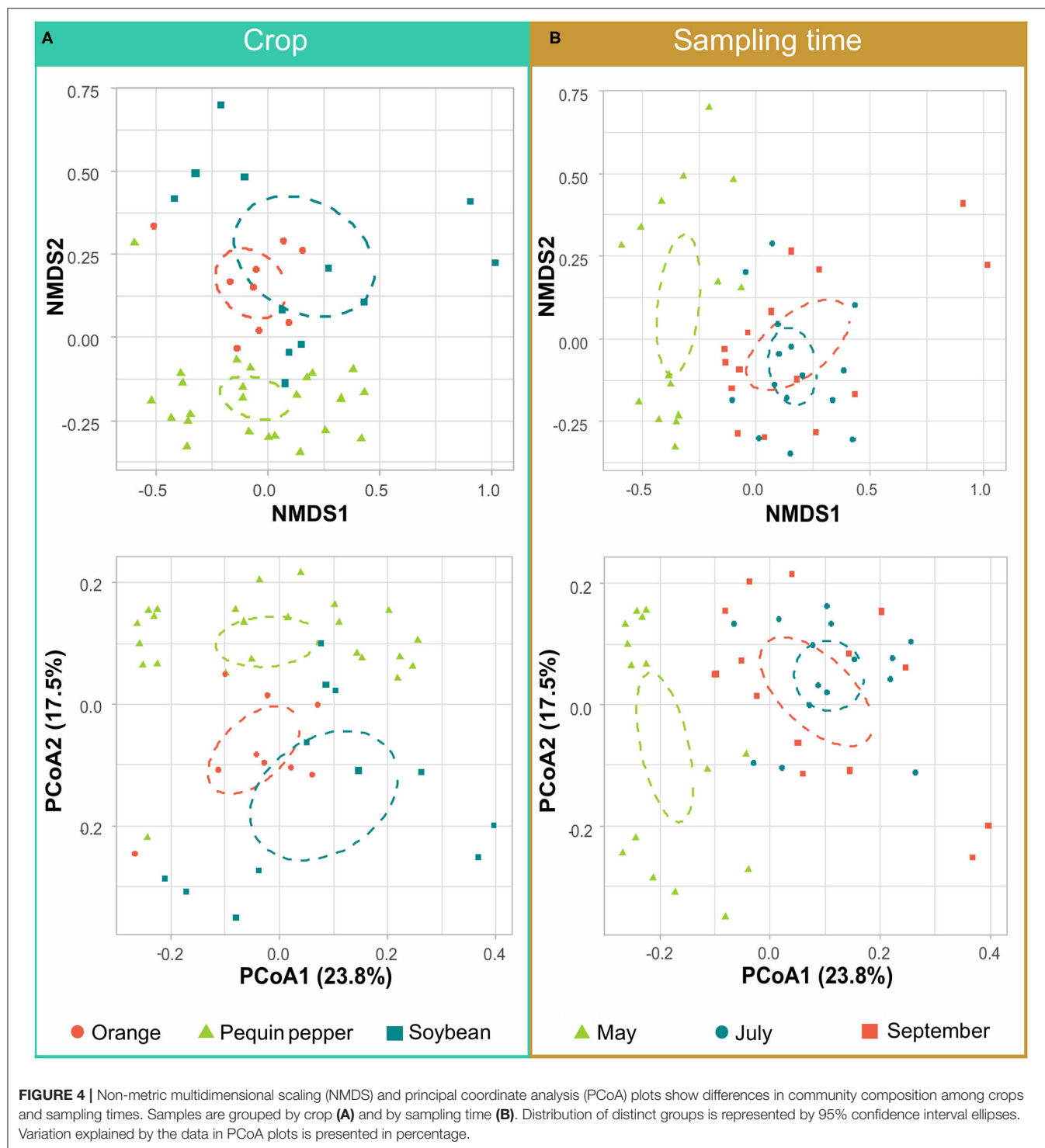
At the genus level, the Venn diagram showed a total of 547 genera. Five hundred thirty-four genera were identified in Pequin pepper, 291 in orange tree and 257 in soybean roots (Figure 2B). Differences in shared genera among crops revealed that the Pequin pepper had 209 specific genera, absent in the other crops, while soybean and orange had only 2 and 10 specific genera, respectively. The three crops shared 209 genera among them. Young and mature Pequin pepper samples were grouped as they did not significantly differ in microbial community composition (Supplementary Figure 2, Supplementary Table 2). They shared 45.2% of the total amount of genera that represented 55.8 and 57.1% of the relative abundance of all genera, for young and mature plants, respectively. Soybean samples had a lower abundance of unassigned genera of 17.6% compared to pepper that ranged from 43.9 to 44.5% and soybean with 46.1% (Figure 2C). In addition, orange trees and soybean possessed fewer genera with a predominance of *Bacillus* (48.1%) in orange and *Niastella* (28.3%) in soybean.

Additionally, to compare crop bacterial assemblies, as well as the two ages of pepper plants, diversity and richness at the genus level was calculated using the Shannon diversity index and Chao1 richness index. Pequin pepper showed a higher diversity and richness of the microbial root endosphere community compared to soybean but not to orange ( $P < 0.05$ ) (Figure 3A); there were no significant differences between young and mature Pequin pepper (Figure 3B).

### Crop Specificity and Temporal Dynamics of Rhizobacteria

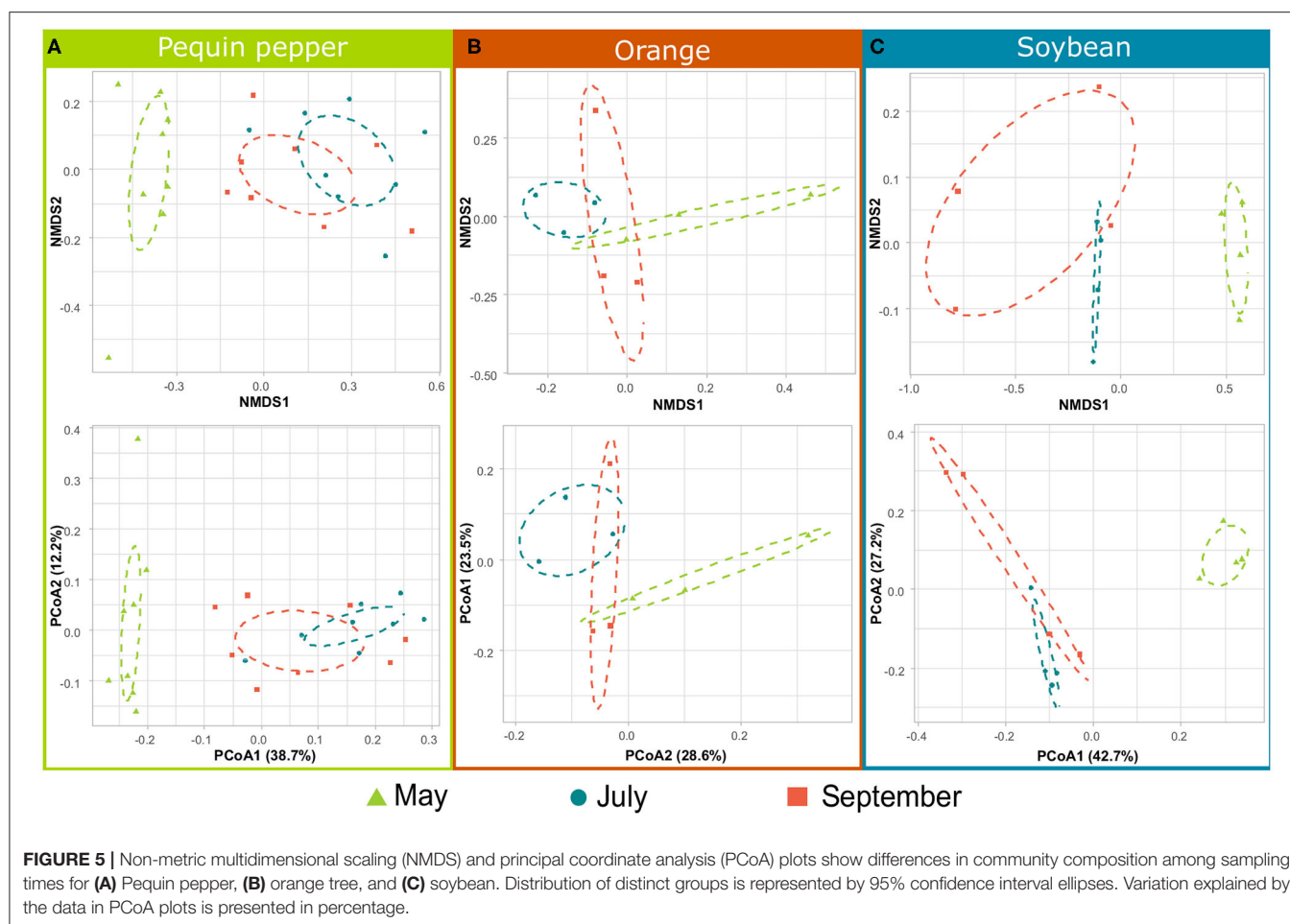
Microbial community differences among crops, sampling times and the interaction between these parameters were shown to be statistically significant when analyzing results at the genus level (Table 2). Furthermore, these dissimilarities in microbial communities were visualized by performing NMDS and PCoAs analyses of crop and sampling time (Figure 4) and sampling time for each crop (Figure 5). Both methods yielded similar results, where rhizobacteria community composition of Pequin pepper was significantly different from that of orange and soybean (Figure 4A).

Regarding temporal dynamics, May samples were significantly different from July and September (Figure 4B). In the same way, differences between sampling times of individual crops resulted in May microbial communities being more dissimilar



for Pequin pepper and soybean (Figures 5A,C). However, in the case of the orange trees all sampling times were similar (Figure 5B). Concerning climatological parameters during sampling, May exhibited in average lower minimum temperature ( $20.0 \pm 3.2^{\circ}\text{C}$ ) compared to July and September (Supplementary Figure 3). July exhibited the highest average

maximum evaporation rate ( $5.9 \pm 2.0\text{ mm}$ ; daily average) and lowest rainfall ( $46.1\text{ mm}$ ; accumulated for the month), whereas September had the highest average rainfall ( $180.9\text{ mm}$ ; accumulated for the month) and a maximum temperature ( $35.2 \pm 3.1^{\circ}\text{C}$ ) very similar to May ( $34.4 \pm 3.1^{\circ}\text{C}$ ) and July ( $36.7 \pm 2.9^{\circ}\text{C}$ ).



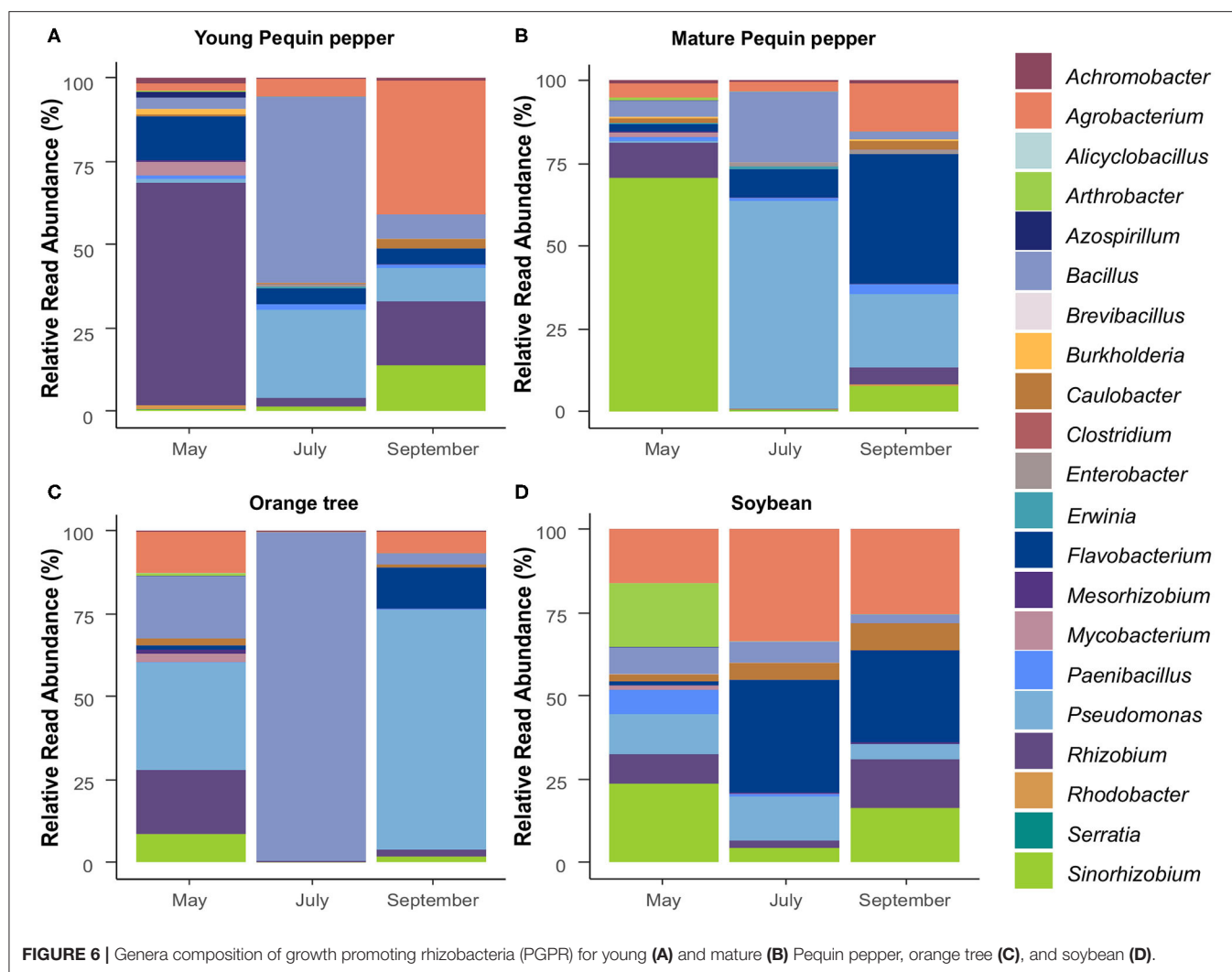
## Plant Growth Promoting Rhizobacteria Community by Crop and Sampling Time

Among the 547 identified genera in this study, 21 (3.84%) represented genera known to play roles as PGPR. Of these, 11 PGPR genera were found in all crops. *Bacillus*, *Pseudomonas*, and *Agrobacterium* were highly abundant in all crops, with variations in abundance depending on sampling time (Figure 6). Soybean exhibited the most stable PGPR community, where microbial component proportions slightly changed with time (Figure 6D). Contrary, Pequin pepper and orange tree showed strong changes in abundance of various genera such as *Rhizobium*, *Bacillus*, *Pseudomonas* and *Sinorhizobium* (Figures 6A–C). There are similarities among crops such as the presence of *Agrobacterium* and *Bacillus* in all samples at all sampling times. However, in contrast to global genera distribution, PGPR genera showed differences between young and mature Pequin peppers through sampling times (Figures 6A,B). For instance, the most abundant genus in May is *Rhizobium* (66.5%) in young Pequin pepper, whereas in mature Pequin pepper is *Sinorhizobium* (70.8%). *Bacillus* was found in larger quantity in July for all samples of Pequin pepper and orange, in the latter representing up to 99.1% of the PGPR community (Figure 6C). This tendency, however, is not observed in soybean samples, where *Bacillus* has the

highest relative abundance in May with 8.21%. For soybean, there was not a high dominance of a single genus at any sampling point, where *Agrobacterium*, *Flavobacterium*, and *Sinorhizobium* presented high abundances (Figure 6D). The number of samples in which each PGPR genus was identified revealed that a few genera were only observed in specific crops, e.g., *Serratia* in orange, *Azospirillum* in young Pequin pepper and *Rhodobacter* in young and mature Pequin pepper (Figure 7A). Consequently, we found few PGPR genera specific to one crop and sampling time, such as *Serratia*, *Azospirillum* and *Alicyclobacillus* (Figure 7B).

Since PGPR genera showed differential distributions among sampling times, their correlation with climatological parameters characteristic of drylands (i.e., rainfall, evaporation rate and temperature) was tested using Pearson's correlation (Figure 8). Overall, young Pequin pepper had the highest amount of PGPR genera influenced by climatological parameters, *Mycobacterium*, *Arthrobacter*, *Mesorhizobium*, *Rhizobium*, *Azospirillum*, and *Burkholderia* were significantly affected by an increase in temperature. This trend was similar in mature Pequin pepper for *Mycobacterium*, *Rhizobium* and, specific to this crop, in *Sinorhizobium*. Also, in soybean *Arthrobacter* presence was affected by high temperatures. For orange trees, *Bacillus* was significantly correlated to all climatological parameters, affected





**FIGURE 6 |** Genera composition of growth promoting rhizobacteria (PGPR) for young (A) and mature (B) Pequin pepper, orange tree (C), and soybean (D).

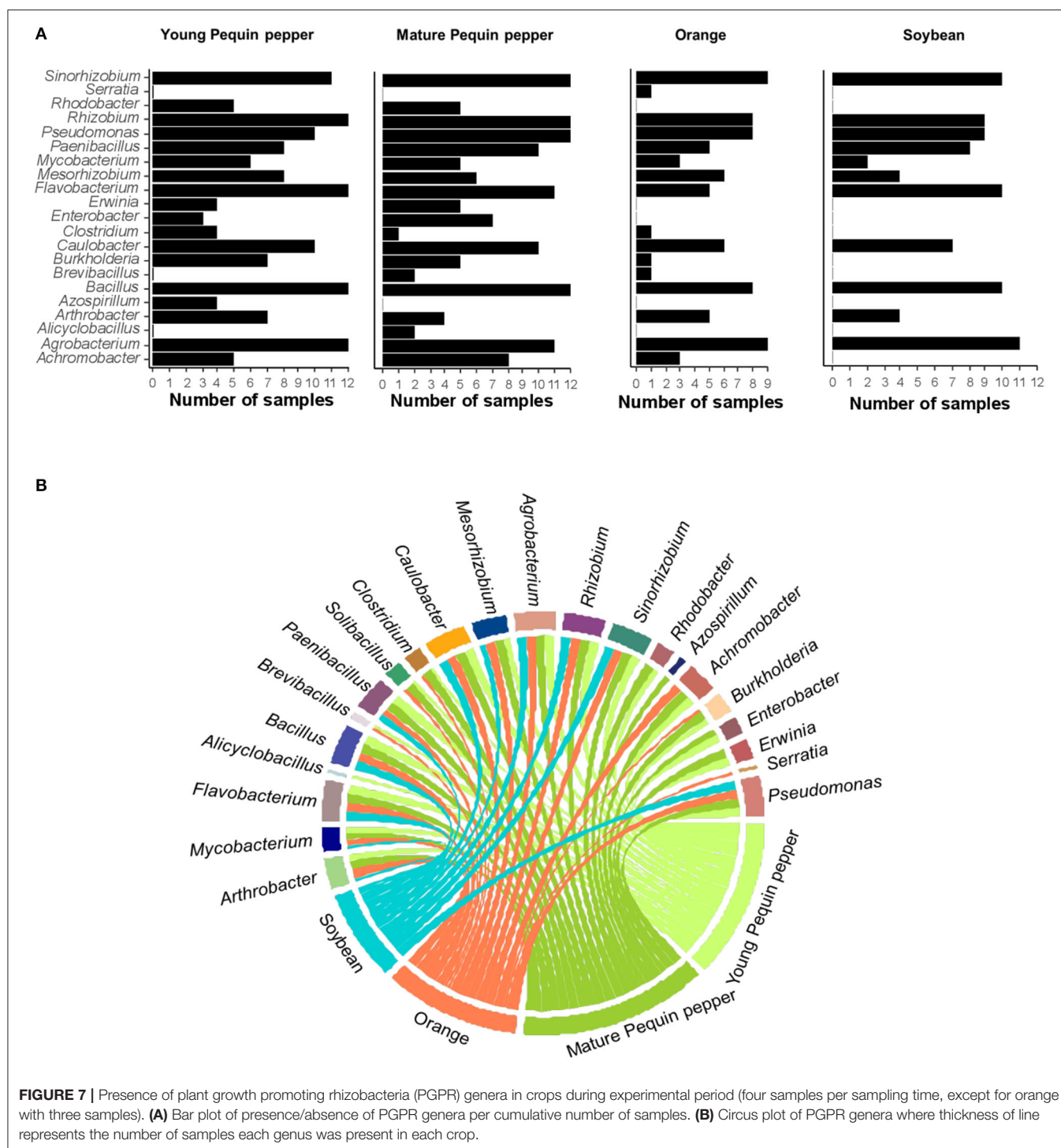
by high rainfall and increasing its abundance as evaporation rate and temperature increased.

## DISCUSSION

For semi-arid ecosystems, it has been shown that bacterial assemblies of adapted plants have a reduced diversity and are strongly influenced by climatological parameters (Taketani et al., 2017). In places with soil aridity and drought, rhizosphere microbiota is driven by plant drought stress alleviation and/or adaptation to environmental conditions (Cherni et al., 2019; see Jansson and Hofmockel, 2019 for a review). For example, Acidobacteria was shown to decrease in abundance as aridity increases, while Chloroflexi and Proteobacteria showed the opposite behavior (Maestre et al., 2015). Here, we also observed this pattern except for Chloroflexi, which we only found in an abundance of around 1% in orange tree roots and which is known for multiple adaptation mechanisms to harsh environments (Lacap et al., 2011). Moreover, Proteobacteria and Firmicutes were reported as the most abundant phyla under drought

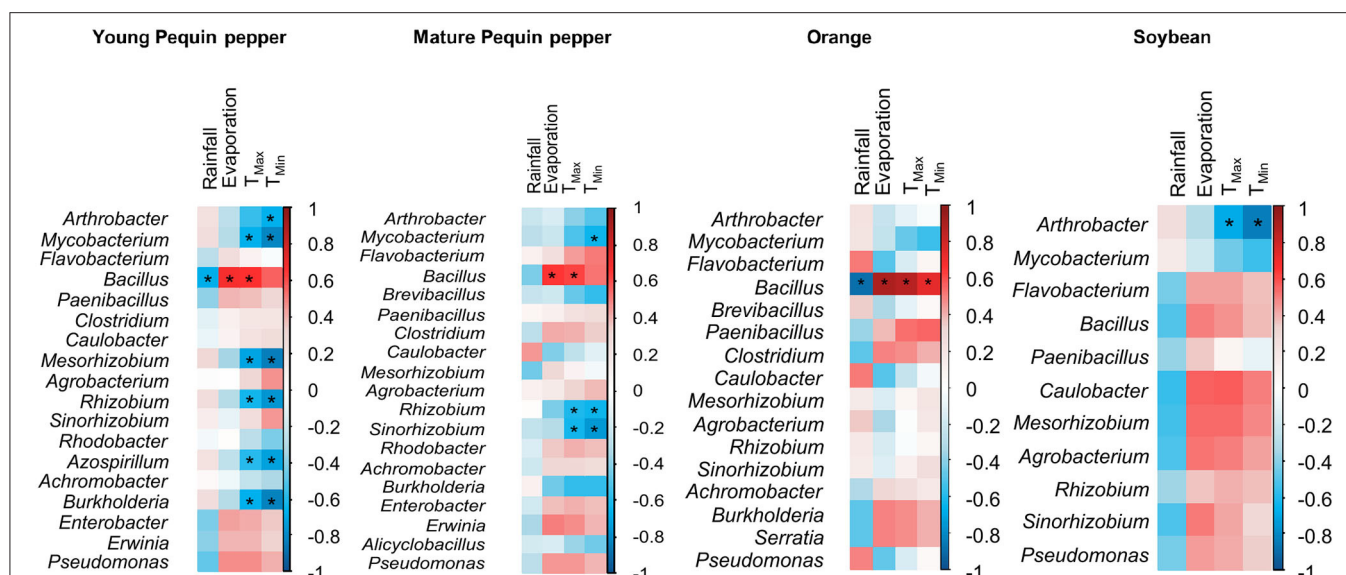
stress for orange trees (Cherni et al., 2019) but we observed a high abundance of Bacteroidetes, instead of Firmicutes, and Proteobacteria in all three crops. Contrary to the climatic conditions in our study, it was reported that both Firmicutes and Bacteroidetes increased in abundance under heavy rain and low temperatures (Štřovíček et al., 2017). Also, a higher abundance in Bacteroidetes has been shown to be the result of rich organic root exudates composed of sugar, amino and organic acids (Sánchez-Cañizares et al., 2017).

For pepper plants (*Capsicum annuum* L.) cultivated in arid ecosystems, it was found that the root endosphere is colonized by a restricted community of bacteria compared to the rhizosphere and adjacent soil (Marasco et al., 2012), but no information is available on whether pepper plant-age affects the microbial communities. Interestingly, no statistical difference was found when analyzing young and mature Pequin pepper root bacteria at the genus level, which is in contrast to some other plants (e.g., rice) where endophytic microbiota communities are characteristic of younger plants when exposed to drought stress (Edwards et al., 2018). In soybean, the rhizosphere



community has been reported as a subset of the bulk soil bacterial assembly with some microbial groups being associated with the rhizosphere, such as bacteria belonging to the phyla Chloroflexi, Proteobacteria, Acidobacteria, and Bacteroidetes. These associations are related to plant needs of nutrient uptake and growth enhancement, impacting the diversity of the root microbiome (Mendes et al., 2014).

The dynamics of bacterial assemblies can be related to climatic stressors and plant development (Cao et al., 2019). Bacterial communities in drylands are affected significantly by soil aridity index (evaporation rate/rainfall), total water holding capacity, total organic carbon, total nitrogen and pH (Wang et al., 2017). Here, results indicate that significant differences are due to crop specificity and climatic conditions, most pronounced



**FIGURE 8 |** Plant growth promoting rhizobacteria (PGPR) correlated with climatological parameters. Correlation matrix was constructed using Pearson lineal correlation method. “\*\*\*”: significant correlation ( $P \leq 0.05$ ). Red and blue color represents positive and negative correlation, respectively. +1 means a perfect positive correlation and -1 means a perfect negative correlation. Rainfall, Evaporation, T<sub>max</sub> and T<sub>min</sub> are the climatic variables measured at the climatological station located at 2 km from the experimental field during 2017.

in May. Regarding climatic conditions, even though July had the harshest environmental circumstances due to the lowest average rainfall and highest temperature and evaporation rate, we hypothesize that significant differences in May root microbiome could have been the result of low or nearly lacking rainfall in the previous 4 months (Supplementary Figure 3). In contrast to July, higher rainfall and a lower evaporation rate occur in September. In leguminous trees, a higher diversity was observed during rainy season compared to the dry season, even though the taxa abundance distribution remained consistent (Taketani et al., 2017). Indeed, microbial response to rain is shown to be dependent not only on its intensity but also on ambient temperature (Št'oviček et al., 2017). This may indicate that changes in microbial communities may be observed after September and October, the only 2 months where rainfall increases.

With respect to crop specificity, pepper is reported to be more sensitive to water stress, requiring a specific bacteria consortium to reduce water deprivation such as high abundances of *Bacillus* spp. and *Klebsiella* spp., known to improve drought stress (Marasco et al., 2012). For orange trees, *Bacillus* was the most abundant genus at this time, whereas *Bacillus* has generally been reported as a dominant genus of the endophytic communities in *Citrus* (Wu and Srivastava, 2012). In the case of soybean, sampling times corresponded to plant developmental stages (i.e., vegetative, flowering and fruiting, and senescent). It has been previously recognized that for soybean the variation in rhizosphere bacterial communities is more influenced by plant growth than by environmental factors (Sugiyama, 2019), which may be reflected by the young soybean microbial composition differing from that of mature or senescent plants, in this study.

In comparison, the orange tree root microbiome remained more static, which is in accordance to previous reports indicating that tree-microbiome interactions differ from annual plants, since trees need decades to grow and strongly depend on the nutrients from the soil (Colin et al., 2017).

Adaptation to the harsh environment of drylands leads to changes in root bacterial assemblies, increasing the presence of PGPR (Dimkpa et al., 2009). When analyzing the correlation of climatological parameters and PGPR genera, *Bacillus* was found to increase its relative abundance under high temperature and evaporation rates as well as under low rainfall. In this matter, it has been previously reported that *Bacillus* species enhance resistance to drought stress in various plants (Dimkpa et al., 2009) by inducing systemic resistance and creating a primed physiological state that may cause increased tolerance against abiotic stress (Chakraborty et al., 2006). PGPR strains development for field application is often hampered by the difficulty of bacteria survival under harsh environmental conditions, including temperature (Kaushal and Wani, 2016). Indeed, *Arthrobacter*, *Azospirillum*, *Burkholderia*, *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* were shown to be affected by high temperature (Huang et al., 2017). *Burkholderia* spp. have been reported to reduce evapotranspiration under drought stress and improve survival of maize (Huang et al., 2017). *Azospirillum*, *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* species have nitrogen fixing activity, form symbiosis with plants and promote their growth (Antoun and Kloepper, 2001; Zarea, 2017). While *Azotobacter* spp. also can fix nitrogen in a non-symbiotic way, other plant growth promoting traits like phytohormone production and phosphorous solubilization are responsible of promoting plant growth

(Antoun and Kloepper, 2001; El\_Komy et al., 2020). *Arthrobacter* spp. have caused mild biotic stress in pepper plants while increasing plant biomass and induced osmotic stress relief in a controlled environment (Sziderics et al., 2007). PGPR genera susceptibility to high temperature is problematic since drylands are extremely prone to present heat stress. Since young Pequin pepper plants showed more PGPR genera affected by high temperatures, our results indicate that they presented PGPR more susceptible to climatic conditions than mature plants. Consistently, it has been reported that the root microbiota community presents high dynamics during the vegetative plant growth phase and stabilizes in composition during the remaining plant life cycle (Edwards et al., 2018); once well-established, the root associated bacteria assembly is structurally more robust (Dombrowski et al., 2017).

In conclusion, pepper root bacterial community dynamics in drylands may be explained by environmental stresses such as long periods of drought, short seasons with rainfall and high temperature and evaporation rates. While these environmental conditions can possibly shape bacterial population in pepper (perennial), for soybean, which is an annual plant, growth stages may be more significant than climatic conditions. For orange trees, the microbial assemblies were more robust. Overall, the presence of *Bacillus* was correlated to a decrease in rainfall and increase of temperature and evaporation. Our results revealed significant preferential crop-microbe associations, as well as different profiles of PGPR genera affected by rainfall, evaporation rate and temperature. Additionally, several drought stress resistance mediating bacteria may be susceptible to high temperature. Together, these findings suggest that dynamics of root bacterial assemblies are influenced by crop type and semi-arid climatic conditions. Moreover, they provide a better understanding of the suitability of commonly used PGPR genera to promote plant growth on drylands. Further efforts are needed to evaluate potential PGPR candidates for biofertilizers under these conditions.

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## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA657454>, PRJNA657454.

## AUTHOR CONTRIBUTIONS

AMD-G performed bioinformatic and statistical analyses. AS and CS-G conceived the research project. AP, JIF-R, and CS-G collected field samples, constructed the library for Illumina sequencing, and sequenced the samples. MSG-H performed statistical analyses. All authors wrote and revised the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.602283/full#supplementary-material>

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# Coffee Microbiota and Its Potential Use in Sustainable Crop Management. A Review

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Intensive coffee production is accompanied by several environmental issues, including soil degradation, biodiversity loss, and pollution due to the wide use of agrochemical inputs and wastes generated by processing. In addition, climate change is expected to decrease the suitability of cultivated areas while potentially increasing the distribution and impact of pests and diseases. In this context, the coffee microbiota has been increasingly studied over the past decades in order to improve the sustainability of the coffee production. Therefore, coffee associated microorganisms have been isolated and characterized in order to highlight their useful characteristics and study their potential use as sustainable alternatives to agrochemical inputs. Indeed, several microorganisms (including bacteria and fungi) are able to display plant growth-promoting capacities and/or biocontrol abilities toward coffee pests and diseases. Despite that numerous studies emphasized the potential of coffee-associated microorganisms under controlled environments, the present review highlights the lack of confirmation of such beneficial effects under field conditions. Nowadays, next-generation sequencing technologies allow to study coffee associated microorganisms with a metabarcoding/metagenomic approach. This strategy, which does not require cultivating microorganisms, now provides a deeper insight in the coffee-associated microbial communities and their implication not only in the coffee plant fitness but also in the quality of the final product. The present review aims at (i) providing an extensive description of coffee microbiota diversity both at the farming and processing levels, (ii) identifying the “coffee core microbiota,” (iii) making an overview of microbiota ability to promote coffee plant growth and to control its pests and diseases, and (iv) highlighting the microbiota potential to improve coffee quality and waste management sustainability.

**Keywords:** wastes and by-products management, quality, biocontrol agent, plant growth promoting agents, core microbiota, coffee microbiota

## INTRODUCTION

The coffee tree is a perennial plant belonging to the Rubiaceae family. The *Coffea* genus consists of c.a. one hundred species, but only *Coffea arabica*, *C. canephora*, and *C. liberica* are used for beverage production, the two formers representing around 70 and 30% of the world production, respectively (Davis et al., 2006; Vieira et al., 2006). *Coffea arabica* is native from the Ethiopian's highlands, between 1,300 and 2,000 m above the sea level, whereas the origin of *C. canephora* is more dispersed across the African tropical areas below 1,000 m (Wintgens, 2004). Coffee is the second most consumed beverage after water and the most traded tropical agricultural commodity (Mussatto et al., 2011; FAO, 2018). Around 25 million smallholder producers, especially in developing countries, rely on the coffee sector for their livelihood (FAO, 2018; ICO, 2019a). By order of importance, the main producing countries in 2018/2019 are Brazil, Vietnam, Colombia, Indonesia, Honduras, Mexico, Guatemala, and Ivory Coast (ICO, 2019b). Although the production and consumption have followed fairly parallel increasing trends over the past 50 years (FAO, 2015), the coffee market is periodically characterized by an oversupply in years of optimal environmental conditions due to innovation in cultivation techniques and planting material leading to a price decrease trend (Ponte, 2002; Bitzer et al., 2008). Farmers that rely on a perennial crop such coffee for their livelihood cannot either easily change their land use or anticipate their income. Therefore, it is difficult for them to adapt to fluctuations in market and environmental conditions (Amamo, 2014; Schroth and Ruf, 2014).

In order to increase yield, the modernization of the coffee production has heavily engaged in the use of new varieties, the reduction of shade, and the increase of plant density and agrochemical inputs (Perfecto et al., 1996). Nowadays, coffee management strategies fall along an intensity continuum ranging from natural or managed forests with coffee plants grown under tree canopy, to trees artificially planted to provide shade up to open sunlight plantation (Moguel and Toledo, 1999; Rice, 1999; Gobbi, 2000; Jezeer et al., 2018; Otero-Jiménez et al., 2018). Nevertheless, the intensive coffee systems result in serious environmental contamination due to excessive use of inputs (DaMatta, 2004), higher soil degradation (Ataroff and Monasterio, 1997) and are linked with a loss of biodiversity compared to traditional coffee systems (Perfecto et al., 1996; Guillemot et al., 2018). After harvest, coffee cherries undergo several processing steps aiming at removing all the external part of the fruit in order to reduce the water content to a level compatible with storage. To do so, three different processing techniques (dry, semi-wet, and wet) are implemented depending on the species, the country, and the farm size (Brando, 2004; Cleves, 2004; Schwan et al., 2012). However, coffee processing generates several by-products and wastes that can represent source of environmental pollution (Chanakya and De Alwis, 2004; Haddis and Devi, 2008; Beyene et al., 2012; Awoke et al., 2016). Finally, the coffee crop is already facing the climate change. This will decrease the suitability of the cultivated areas (Bunn et al., 2015; Ovalle-Rivera et al., 2015) and potentially increase

the distribution and the impact of pests and diseases (Ghini et al., 2008; Jaramillo et al., 2011; Groenen, 2018).

The adverse effects of coffee cultivation and processing on the environment highlight the importance of developing sustainable solutions in order to maintain growers' livelihood while limiting the environmental impact in the climate change context. Thus, the benefit of smart agronomical systems, such as agroforestry, are increasingly highlighted (Méndez et al., 2010; De Beenhouwer et al., 2013; Vaast et al., 2016; Sauvadet et al., 2019; Gomes et al., 2020). Moreover, some high-technology microbial inputs (biofertilizers and biopesticides) are prone to increase the performances of such systems (reviewed in Singh et al., 2016a,b). Indeed, it is now well-documented that some of the microorganisms interacting with plants are directly beneficial by promoting their growth or indirectly by acting as antagonist of their pathogens (Compant et al., 2005; Olanrewaju et al., 2017). Therefore, to address the challenges associated with a sustainable crop management, research focused on the plant-associated microbes has been increasingly developed during the last decades (Berg et al., 2016; Compant et al., 2019; Arif et al., 2020). Nowadays, there is a shift in the ways of understanding the relationships between macroorganisms and microorganisms leading to the "holobiont" concept (Rosenberg and Zilber-Rosenberg, 2013; Bordenstein and Theis, 2015). According to this concept, the plants can be considered as superorganisms composed by the plant and associated microorganisms, the latter acting as an entire component of the host fitness by playing a role in the mineral nutrition, hormone balance, and adaptation capacity to biotic and abiotic stresses (Lemanceau et al., 2017; Simon et al., 2019). The development in this research area that describes the microbial communities has been accompanied with the use of specific terms such as "microbiome" and "microbiota" whose definitions are still debated (Marchesi and Ravel, 2015; Berg et al., 2020). In the present review, the term microbiota refers to all microorganisms interacting in a specific environment while the term microbiome encompasses their structural elements, molecules (e.g., DNA, metabolites) as well as the environmental conditions associated with the microbiota as initially described by Whipps et al. (1988) and clarified by Berg et al. (2020).

The use of microbes in the coffee farming and industry is still poorly exploited despite its potential capacity to reduce the amount of chemical inputs, improve coffee quality, and increase the farmer income through sustainable certifications (Mithöfer et al., 2017). Moreover, engineering the plant microbiome/microbiota and taking it into account in the new plant breeding strategies could represent some promising approaches to sustainably maintain the productivity (Nogales et al., 2016; Orozco-Mosqueda et al., 2018; Arif et al., 2020). The present review intends to (i) describe the diversity of the microorganisms that make up the coffee microbiota by focusing on archaea, bacteria, and fungi, (ii) summarize the current knowledge on the use of microorganisms to promote the coffee plant growth as well as to control the pests and diseases, and (iii) give an overview of their potential incorporation in the coffee processing and by-products management.



## STRATEGIES USED TO STUDY THE COFFEE MICROBIOTA

The first mention of the microorganisms associated with coffee plants dates from the nineteenth century and the description of the arbuscular mycorrhizal fungi (AMF) colonizing the roots of *C. arabica* and *C. liberica* (Janse, 1897). Since then, two major approaches have been used to describe the coffee microbiota diversity in combination with numerous identification strategies allowing to identify microorganisms at varying taxonomic levels from the highest (e.g., kingdom and phylum) to the lowest (e.g., genus and species).

The first one is the culture-dependent approach involving the isolation and the purification of the microorganisms. In that case, some basic morphological identifications using staining and microscopy were frequently employed to identify mycorrhizal species (Caldeira et al., 1983; Bertolini et al., 2020) or filamentous fungi (Mislivec et al., 1983; Casas-Junco et al., 2018). The morphology was often combined with standard biochemical tests such as those analyzing carbon sources utilization and enzymatic assays to identify bacteria (Pederson and Breed, 1946; Teshome et al., 2017) and fungi including yeasts (Agate and Bhat, 1966; Ranjini and Raja, 2019). Some more complex biochemical tests were sometimes applied to confirm the microorganisms' identity such as the multilocus enzyme electrophoresis (MLEE) in the case of nitrogen-fixing (N-fixing) bacteria (Jimenez-Salgado et al., 1997; Fuentes-Ramírez et al., 2001), the fatty acid methyl esters gas chromatography (FAME-GC) for bacterial isolates (Vega et al., 2005; Silva et al., 2012; Miguel et al., 2013), and the matrix-assisted laser desorption ionization–time of flight–mass spectrometry (MALDI-TOF-MS) for several bacteria and yeasts (Martins et al., 2020).

Regarding the molecular-based methods, the DNA–DNA reassociation study was one of the first molecular methods employed by bacterial taxonomists to describe the relatedness between bacterial species since the 1960s (Goris et al., 2007). Up to now, it is still the gold standard to identify new species as well as to discriminate bacterial isolates at the lowest taxonomic levels such as species and strain (Stackebrandt and Goebel, 1994; Janda and Abbott, 2007; Lagier et al., 2015). This method was successfully employed to describe some N-fixing bacterial species associated with coffee (Jimenez-Salgado et al., 1997; Estrada-De Los Santos et al., 2001). With the development of the first-generation sequencing technologies, DNA sequence comparisons contributed in an unprecedented manner to the number of identified microbial species (Rossi-Tamisier et al., 2015; Franco-Duarte et al., 2019).

The amplification and sequencing of simple genetic markers such as the rDNA gene repeats like the 16S rDNA of bacteria, as well as the 18S or 26S/28S rDNA and the ITS of fungi, have been extensively used (Sakiyama et al., 2001; Masoud et al., 2004; Oliveira et al., 2013; Prates Júnior et al., 2019; Martins et al., 2020). Sometimes, some housekeeping genes like those coding the  $\beta$ -tubulin (Samson et al., 2004; De Almeida et al., 2019) and TEF-1 $\alpha$  factor (Mulaw et al., 2010, 2013) were sequenced for fungal identifications. The combination of several

sequences in the multilocus sequence typing was also used to increase the reliability of the identification (Peterson et al., 2005). More recently, the whole-genome sequencing using the next-generation sequencing (NGS) technology was also employed to sequence the genome of a lactic acid bacterial strain of *Pediococcus acidilactici* isolated during the coffee fermentation (Muynarsk et al., 2019).

The second methodology does not require cultivation of the microorganisms. During its early development, it consisted in pooling DNA extractions, amplifying some DNA markers regions, and then sequencing them after some separation techniques such as the denaturing gradient gel electrophoresis (DGGE) or the cloning of single sequences. This procedure was used to study the endophytes in coffee cherries (Oliveira et al., 2013) and AMF (endophytic symbiotic fungi), colonizing the roots (Mahdhi et al., 2017) as well as the bacteria and fungi present during different coffee processing techniques (Vilela et al., 2010; Feng et al., 2016).

Nowadays, the culturable-independent strategy is increasingly used. The development of the NGS technologies also allows performing metabarcoding analyses involving the amplification and sequencing of specific marker genes to identify a whole community in an environmental DNA sample without the need of cloning or separation steps (Santos et al., 2020). For example, De Beenhouwer et al. (2015a,b) were among the first to use NGS in order to highlight the differences of AMF communities across a gradient of coffee management intensity. Then, two metabarcoding studies, describing the bacterial inhabitants of the coffee rhizosphere under organic or conventional cropping, were also performed (Caldwell et al., 2015; Rodríguez et al., 2020). In a recent work, Lamelas et al. (2020) examined the bacterial communities present in the *C. arabica* rhizosphere, in parasitic root-knot nematodes (females and eggs) as well as in healthy and nematode-infected coffee roots in order to determine the specific microbial assemblages correlated with the infection by *Meloidogyne enterolobii* and *M. paranaensis*. In another recent study, a metabarcoding analysis also highlighted the influence of edaphic and topographical factors on the bacterial and fungal communities associated with both rhizosphere and cherries of *C. arabica* (Veloso et al., 2020). Other authors also studied the fungi associated with *C. arabica* leaves infected by *Hemileia vastatrix*, the causal agent of the coffee leaf rust (CLR), with the aim to identify some potential mycoparasites (James et al., 2016). Recently, Fulthorpe et al. (2020) investigated both fungal and bacterial endophytes in *C. arabica* roots across a climatic gradient (temperature and humidity) in full sun and agroforestry cropping systems in Costa Rica and Nicaragua. Furthermore, the metabarcoding approach was also used to study the microbial (bacteria and fungi) communities linked with several postharvest processing steps and their impacts on coffee quality (De Bruyn et al., 2017; De Oliveira Junqueira et al., 2019; Zhang et al., 2019b; Elhalis et al., 2020a,b). Finally, the NGS approach involving the random sequencing of the fragmented DNA extract (shotgun sequencing) now allows to study the microbial diversity and to predict associated genes' function. This technique was used to perform a metagenomic analysis and to decipher the functional

characteristics of the microbial communities found during *C. arabica* bean fermentation (Zhang et al., 2019a).

As usual, it is important to highlight that each approach displays its own strengths and weaknesses. On the one hand, the culture-dependent strategy allows isolating the microorganisms and further characterizing their biochemical and functional traits. However, it is laborious and time consuming with a limited capacity to cover the whole diversity of microorganisms because it is dependent of many parameters such as the culture media employed. Indeed, the concept of “unculturable microorganisms” was highlighted in the early twentieth century with the finding that there was far less colony able to grow on the medium than the number of cells observed by microscopy (Amann, 1911 in Ghosh and Bhadury, 2019). Nevertheless, this limit can now be bypassed with the use of various culture media leading to the development of the “culturomics” (Lagier et al., 2012).

On the other hand, the culture-independent approach is more labor/cost effective in studying the diversity of microorganisms as it allows identifying the uncultivable ones. This strategy can also picture the relative abundance of the microorganisms in metabarcoding studies and the potential function of associated genes when the metagenomic strategy is used. Despite that a bias can be introduced by the DNA extraction step when studying the microbial relative abundance, the introduction of an artificial community (mock) and the improvement of the DNA extraction protocols can help to standardize the results (Berg et al., 2020). Another constraint is the difficulty to reach the lowest taxonomic levels because of the limited amplicons length with the second-generation sequencers (Johnson et al., 2019; De Corato, 2020; Santos et al., 2020). Indeed, most of the metabarcoding studies related to coffee microorganisms' diversity were performed with second-generation sequencing platforms (Roche 454 and Illumina MiSeq) that allow sequencing only a part (usually hypervariable regions) of markers such as the 16S rDNA for bacteria and 18S rDNA for fungi or only smaller markers such as the ITS for fungi (Caldwell et al., 2015; De Beenhouwer et al., 2015a,b; De Bruyn et al., 2017; De Oliveira Junqueira et al., 2019; Zhang et al., 2019a,b; Elhalis et al., 2020a,b; Fulthorpe et al., 2020; Lamelas et al., 2020; Rodríguez et al., 2020; Veloso et al., 2020). Moreover, it has already been demonstrated for bacteria that the partial sequence does not achieve the taxonomic resolution obtained with the full-length 16S rDNA (Johnson et al., 2019). By contrast, the last technologies (third and fourth generations) now allow generating longer sequences compared to the advent of NGS, but this is done at the expense of the quality due to a higher sequencing error rate (Kulski, 2016; De Corato, 2020). Thus, James et al. (2016) were the only ones to study the coffee microbiota using a third-generation sequencing platform (PacBio) and concluded that the error rate remained very low. However, the full capacity of the platform remained unexploited as they sequenced only the ITS1-5.8S-ITS2 region of rDNA (<1 kb). Finally, it is important to have in mind that the data generated during metabarcoding/metagenomic analyses need a both statistical and bioinformatical treatment and the algorithms used still need to be improved (Ghosh and Bhadury, 2019).

To conclude, it is worth noting that both culture-dependent and independent approaches remain complementary. In other words, it is of a great interest to decipher the microbial diversity through metabarcoding/metagenomic analyses because this allows a better understanding of the interactions between coffee and microorganisms. Furthermore, the microbial diversity is a relevant indicator of environmental changes. However, it is necessary to isolate the microorganisms (e.g., to screen beneficial capacities and also to develop some biotechnological applications); hence, more efforts are certainly required to develop the culturomics approaches with the coffee microbiota as it has already been established to characterize the human (Lagier et al., 2018) and plant microbiota (Sarhan et al., 2019).

## COFFEE MICROBIOTA DIVERSITY

The main objective of the present review is to make an extensive survey of the literature describing the microbiota associated with coffee plants, including mainly the archaeal, bacterial, and fungal kingdoms. The keywords used for database search were Coffee, *Coffea*, microbiome, microbiota, archaea, bacteria, fungi, yeast, endophytes, epiphyte, rhizosphere, plant growth promotion, PGPR, PGPB, PGPF, and PGPM (plant growth promoting rhizobacteria, bacteria, fungi, and microorganisms, respectively), biocontrol, BCA (biocontrol agent), sustainable, biological, postharvest, processing, fermentation, wastes, and by-products. The databases screened were PubMed, Google Scholar, Web of Science, and SciELO. In total, 234 publications were found with identifications at least at the genus level and a well-defined origin of the microorganisms (rhizosphere, episphere, endosphere or associated with the cherries, beans, and wastes during the postharvest processes). The full detailed dataset describing the microorganisms' origin (continent, country, *Coffea* species, the type of colonization, the plant organs, the type of postharvest processing), the identification strategies, the thresholds used to filter the identifications, the accession numbers (when available), and all the other analyses described in the scientific articles and their potential applications are provided in the **Supplementary Table 1**.

Then, the microbiota was further divided into two principal components. The first one is the indigenous microbiota composed of the microorganisms living in close association with the coffee plants, in the soil at the vicinity of the roots (rhizosphere), at the surface (episphere), and inside the plant tissues (endosphere). The second component is related to the postharvest microbiota that encompasses all the microorganisms associated with the coffee cherries postharvest processing and its by-products including the fermentation, the drying steps, and the wastes (husks, pulps, and wastewater).

The indigenous and the postharvest coffee microbiota have been relatively equally studied with 115 and 127 publications, respectively (**Table 1**). To the best of our knowledge, the overall coffee microbiota is covering 22 phyla, encompassing 129 orders, 607 genera, and 923 species mainly belonging to the bacterial and fungal kingdoms. Indeed, only two archaeal phyla (including four orders and five genera without any species

**TABLE 1** | Overview of the coffee microbiota with the numbers of phyla, orders, genera, species, and citations for the three kingdoms (archaea, bacteria, and fungi) constituting the coffee microbiota (indigenous and postharvest).

Microbiota	Kingdom	Phylum	Order	Genus	Species	No. of citations
Indigenous	Archaea	2	4	5	0	2
	Bacteria	9	43	152	174	50
	Fungi (AMF;yeasts)	4 (1;2)	53 (4;6)	248 (24;10)	380 (126;24)	72 (38;9)
	Total number	15	100	405	554	115
Postharvest	Bacteria	14	59	227	176	51
	Fungi (yeasts)	4 (2)	34 (10)	119 (51)	270 (117)	105 (47)
	Total	18	93	346	446	127
Total	Archaea	2	4	5	0	2
	Bacteria	15	67	279	265	97
	Fungi (AMF;yeasts)	5 (1;2)	58 (4;11)	315 (24;53)	610 (126;133)	172 (38;54)
	Total number	22	129	607	923	234

For the fungal kingdom, the detailed numbers of arbuscular mycorrhizal fungi (AMF) and yeasts are indicated between brackets.

identification) have been described (**Supplementary Table 1**). This is not surprising since archaea were discovered relatively recently in the microbiology history (Woese et al., 1978) and remain quite difficult to cultivate (Song et al., 2019).

It is worth noting that our survey is a qualitative description of the coffee microbiota diversity. However, we tried to (i) give an insight into the relative abundances of the microorganisms based on their occurrence frequency in the literature and (ii) compare these results with the relative abundances identified through metabarcoding studies (when available).

## The Indigenous Coffee Microbiota

Many biotic and abiotic factors influence the plant microbiota such as the soil physical–chemical characteristics (Fierer, 2017; Tkacz et al., 2020), the plant compartment (rhizosphere, episphere, and endosphere), and the organ studied (Compant et al., 2019; Berg et al., 2020; Tkacz et al., 2020). Moreover, the plant genotype is also believed to influence the microbial community (Patel et al., 2015; Mina et al., 2020). However, it is worth noting that most of the microorganisms associated with coffee have been described in *C. arabica* across 170 scientific articles while the remaining studies referred to *C. canephora*, *C. liberica*, and *C. congensis* or did not specified the *Coffea* species studied (**Supplementary Table 1**). Thereby, due to the multifactorial influence on the microbiota, we decided to split the indigenous microbiota in the following plant compartments, namely, the rhizosphere, the episphere, and the endosphere.

## The Coffee Rhizospheric Microbiota

The soil represents an underestimated reservoir of microbial diversity for which a large part never been cultivated (Mendes et al., 2013). Plants are able to influence the diversity of microorganisms in their rhizosphere and to potentially select from the soil the beneficial ones through the production of root exudates (Hartmann et al., 2008; Mendes et al., 2013). As attested by the increasing use of the term “plant growth-promoting

rhizobacteria” since its formulation by Kloepper and Schroth (1978), the rhizosphere microorganisms represent a well-known source of plant beneficial microorganisms able to improve the acquisition of nutrients as well as the resistance to biotic and abiotic stresses (Avis et al., 2008; De Zelicourt et al., 2013; Meena et al., 2017).

In the present review, we recorded 34 publications related to the coffee rhizospheric microbiota. Thirty-one used a culture-dependent approach, and only three studies employed a metabarcoding approach to describe the microorganisms in the coffee rhizosphere (Caldwell et al., 2015; Lamelas et al., 2020; Rodríguez et al., 2020). Based on this survey, we recorded that the rhizosphere microbiota diversity covers 12 phyla, 40 orders, 98 genera, and 58 species with as the most studied kingdoms the bacteria and fungi across 30 and 8 articles (**Table 2**).

The bacterial diversity is composed of eight phyla, 31 orders, 81 genera, and 42 species. The most encountered and diversified phylum is the Proteobacteria with 45 genera and 22 species described, with the *Pseudomonas* being the most diversified and common genus with 11 species identified across 14 publications. All studies that used the NGS to describe the coffee rhizosphere prokaryotic abundance and diversity also reported the dominance of the Proteobacteria phylum and the *Pseudomonas* genus (Caldwell et al., 2015; Lamelas et al., 2020; Rodríguez et al., 2020). The remaining diversity is distributed among the Acidobacteria, Actinobacteria, Bacteroidetes, Firmicutes, and Verrucomicrobia phyla.

The fungal diversity comprises three phyla, eight orders, 16 genera, and 16 species, the Ascomycota phylum being the most reported. Indeed, all the eight publications dealing with fungi in the coffee rhizosphere described some members of this phylum with 14 genera and 16 species identified. The most commonly identified genera belonging to this phylum are by number of citations *Penicillium* (6), *Aspergillus* (5), *Fusarium* (4), and *Trichoderma* (3). Even though there is no metabarcoding analysis

**TABLE 2 |** Rhizospheric archaea, bacteria, and fungi diversity including phyla, orders, and genera, as well as the numbers of species identified and citations.

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Archaea	Thaumarchaeota	Nitrososphaerales	<i>Nitrososphaera</i>	0	1
Bacteria	Acidobacteria	Acidobacteriales	<i>Edaphobacter</i>	0	1
		Bryobacterales	<i>Bryobacter</i>	0	1
			<i>Candidatus Solibacter</i>	0	1
			<i>Solibacter</i>	0	1
				0	1
	Actinobacteria	Acidothermales	<i>Acidothermus</i>	0	1
		Corynebacteriales	<i>Gordonia</i>	1	2
			<i>Mycobacterium</i>	0	1
			<i>Rhodococcus</i>	1	1
				0	1
		Micrococcales	<i>Agromyces</i>	0	1
			<i>Arthrobacter</i>	0	1
			<i>Kocuria</i>	0	1
			<i>Leifsonia</i>	1	1
			<i>Micrococcus</i>	0	1
			<i>Phycococcus</i>	0	1
			<i>Salinibacterium</i>	0	1
			<i>Salinispora</i>	0	1
			<i>Aeromicrobium</i>	0	1
			<i>Streptomyces</i>	0	1
	Bacteroidetes	Chitinophagales	<i>Filimonas</i>	0	1
		Flavobacteriales	<i>Chryseobacterium</i>	0	3
			<i>Flavobacterium</i>	0	4
		Sphingobacteriales	<i>Pedobacter</i>	0	1
			<i>Sphingobacterium</i>	0	1
				0	1
		Firmicutes	<i>Alicyclobacillus</i>	0	1
			<i>Ammoniphilus</i>	0	1
			<i>Bacillus</i>	16	12
			<i>Brevibacillus</i>	0	1
			<i>Lysinibacillus</i>	0	1
			<i>Paenibacillus</i>	0	2
			<i>Pasteuria</i>	1	1
			<i>Terribacillus</i>	0	1
	Nitrospirae	Nitrospirales	<i>Nitrospira</i>	0	1
	Planctomycetes	Planctomycetales	<i>Planctomyces</i>	0	1
	Proteobacteria	Aeromonadales	<i>Aeromonas</i>	0	2
			<i>Achromobacter</i>	0	2
			<i>Acidovorax</i>	0	1
		Burkholderiales	<i>Alcaligenes</i>	0	2
			<i>Burkholderia</i>	1	7
			<i>Comamonas</i>	0	1
			<i>Janthinobacterium</i>	0	1
			<i>Rhodoferax</i>	0	1
			<i>Variovorax</i>	0	1
			<i>Caulobacter</i>	0	1
			<i>Phenylobacterium</i>	0	1
		Cellvibrionales	<i>Cellvibrio</i>	0	1
		Chromatiales	<i>Rheinheimera</i>	0	1
		Enterobacteriales	<i>Citrobacter</i>	0	1
			<i>Enterobacter</i>	1	2
			<i>Erwinia</i>	1	2

(Continued)



TABLE 2 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Proteobacteria	Enterobacterales	<i>Serratia</i>	2	4
			<i>Shinella</i>	0	1
		Neisseriales	<i>Chromobacterium</i>	0	2
		Nevskiales	<i>Steroidobacter</i>	0	2
		Pasteurellales	<i>Pasteurella</i>	0	2
		Pseudomonadales	<i>Acinetobacter</i>	0	3
			<i>Azotobacter</i>	1	6
			<i>Chryseomonas</i>	0	2
			<i>Pseudomonas</i>	11	14
		Rhizobiales	<i>Afipia</i>	0	1
			<i>Agrobacterium</i>	0	3
			<i>Beijerinckia</i>	0	1
			<i>Bradyrhizobium</i>	0	2
			<i>Devosia</i>	0	1
			<i>Kaistia</i>	0	1
			<i>Mesorhizobium</i>	0	1
			<i>Methylobacter</i>	0	1
			<i>Ochrobactrum</i>	1	2
			<i>Pedomicrobium</i>	0	1
			<i>Pseudolabrys</i>	0	1
			<i>Rhodoplanes</i>	0	2
		Rhodospirillales	<i>Acetobacter</i>	0	1
			<i>Azospirillum</i>	0	2
			<i>Gluconacetobacter</i>	3	3
		Sphingomonadales	<i>Kaistobacter</i>	0	1
			<i>Sphingobium</i>	0	1
			<i>Sphingomonas</i>	0	2
		Vibrionales	<i>Vibrio</i>	0	2
		Xanthomonadales	<i>Stenotrophomonas</i>	1	5
	Verrucomicrobia	Chthoniobacterales	<i>Candidatus Udaeobacter</i>	0	1
			<i>Candidatus Xiphinematobacter</i>	0	1
			<i>Luteolibacter</i>	0	1
Fungi	Ascomycota	Verrucomicrobiales		0	1
		Chaetothyriales	<i>Cladophialophora</i>	0	1
		Cladosporiales	<i>Cladosporium</i>	0	2
		Eurotiales	<i>Aspergillus</i>	1	5
			<i>Paecilomyces</i>	1	1
			<i>Penicillium</i>	2	6
		Hypocreales	<i>Acremonium</i>	0	2
			<i>Aschersonia</i>	0	1
			<i>Cylindrocarpon</i>	2	2
			<i>Fusarium</i>	0	4
			<i>Trichoderma</i>	10	3
		Sordariales	<i>Chaetomium</i>	0	2
			<i>Humicola</i>	0	1
	Basidiomycota	Cantharellales	<i>Rhizoctonia</i>	0	1
	Mucoromycota	Mucorales	<i>Mucor</i>	0	1
(Yeasts)	Ascomycota	Saccharomycetales	<i>Candida</i>	0	1
			<i>Saccharomyces</i>	0	1
Archaea	1	1	1	0	1
Bacteria	8	31	81	42	30
Fungi (Yeasts)	3 (1)	8 (1)	16 (2)	16 (0)	8 (1)
Total number	12	40	98	58	34

of the fungal community in the coffee rhizosphere, it has already been reported for several other plant species that the rhizosphere is dominated by fungi from the Ascomycota phylum (Wang et al., 2017; Qiao et al., 2019; Schöps et al., 2020; Tkacz et al., 2020). By contrast, the other fungal phyla are underrepresented with only two isolates belonging to the Basidiomycota and Mucoromycota.

### The Coffee Epiphytic Microbiota

In general, the plant epiphytic microbiota is composed of a high diversity of microorganisms able to attach and live on the surface of the above and belowground plant tissues (Vorholt, 2012; Newman and Cragg, 2020). This microhabitat requires a specific adaptation of the microorganisms to tolerate the particular environment at the surface of the plant tissues especially the leaves (Vorholt, 2012; Vandenkoornhuyse et al., 2015). Furthermore, the episphere is somehow considered as a boundary for the microorganisms limiting their establishment as endophytes (Vandenkoornhuyse et al., 2015).

Despite the fact that the episphere represents a source of plant beneficial microorganisms and bioactive compounds (Vandenkoornhuyse et al., 2015; Newman and Cragg, 2020), it is the least studied compartment of the indigenous coffee microbiota with only 19 articles (Table 3). All the reviewed studies used a culture-dependent approach to isolate the microorganisms at the surface of several coffee tissues, mainly from leaves (Vélez and Rosillo, 1995; Haddad et al., 2014) and cherries (Agate and Bhat, 1966; Compri et al., 2016), but also from roots (Velmourougane et al., 2000; Teshome et al., 2017) and stems (Velmourougane et al., 2000; Waller and Masaba, 2006). Two metabarcoding studies could have pictures the epiphytic communities at the surface of coffee leaves and cherries; however, the authors extracted DNA from the crushed organs making impossible to discriminate epiphytes from endophytes (James et al., 2016; Veloso et al., 2020) (Supplementary Table 1).

The coffee epiphytic diversity is composed of seven phyla, 25 orders, 52 genera, and 42 species (Table 3). The fungal kingdom is the most studied, encompassing 3 phyla, 15 orders, 34 genera, and 25 species. The most cited fungal phylum is the Ascomycota followed by the Basidiomycota and Mucoromycota and by citation number the genera *Fusarium* (6), *Penicillium* (5), and *Aspergillus* (4). Regarding the bacterial kingdom, it includes four phyla 10 orders, 18 genera, and 17 species, the Proteobacteria phylum being the most cited and diversified with 12 genera, 13 species and the *Pseudomonas* as the most commonly isolated genus.

### The Coffee Endophytic Microbiota

Endophytic microorganisms are characterized by their capacity to colonize the internal part of the plant tissues without causing any negative symptoms to their host (Wilson, 1995; Hyde and Soyong, 2008). The endophytic lifestyle is therefore characterized by microorganisms spending only a part up to their entire life cycle within the plant tissues (Hardoim et al., 2015). It is worth noting that some endophytes can be vertically transmitted while other are characterized by diverse colonization patterns (Saikkonen et al., 2004; Hardoim et al., 2015; Frank et al., 2017). It is also believed that the internal colonization capacity allows

endophytes to be less affected by soil condition fluctuations and by competition with other microorganisms (Santoyo et al., 2016). In addition, endophytes were reported to display plethora of activities that can be beneficial for the plants (Afzal et al., 2019; White et al., 2019; Yan et al., 2019).

It must be stressed that more than half of the studies related to coffee endophytes focused on AMF, which represent a particular class of endophytic symbiotic fungi belonging to the Mucoromycotina phylum and the Glomeromycotina sub-phylum (Spatafora et al., 2016). They are qualified of mutualistic obligate symbionts colonizing the plant roots while the external mycelium is foraging the soil to transfer some water and inorganic compounds (phosphorus, nitrogen, and other essential nutrients) to their host in exchange of a carbon source (Genre et al., 2020). Furthermore, their beneficial effects not only on the plant nutrition but also on their tolerance to biotic and abiotic stresses are now well-documented (Chen et al., 2018; Begum et al., 2019).

The endosphere is the most studied compartment of the indigenous coffee microbiota. We reviewed the content of 71 publications dealing with coffee endophytes, among which 65 employed a culture-dependent method to isolate bacterial and fungal endophytes from various *C. arabica*, *C. canephora*, and *C. liberica* tissues including cherries (Sakiyama et al., 2001; Vega et al., 2008; Miguel et al., 2013), leaves (Santamaría and Bayman, 2005; Bongiorno et al., 2016), roots (Raviraja et al., 1996; Jimenez-Salgado et al., 1997; Vega et al., 2006; Hoang et al., 2020; Duong et al., 2021), seeds (Vega et al., 2006; Duong et al., 2021), and stems (Vega et al., 2005, 2010). Basic culture-independent strategies were used in three studies to identify archaea, bacteria, and fungi including AMF inside the *C. arabica* roots and cherries (Oliveira et al., 2013; Mahdhi et al., 2017; Prates Júnior et al., 2019). Finally, three metabarcoding studies were also performed to study the AMF, endophytic bacteria, and fungi associated with *C. arabica* roots across some management and environmental gradients (De Beenhouwer et al., 2015a,b; Fulthorpe et al., 2020).

The coffee endophytic microbiota encompasses 12 phyla, 70 orders, 241 genera, and 350 species (Table 4). Fungi are the most studied microorganisms with a total 55 articles (38 related to AMF), followed by bacteria and archaea with 18 and 1 studies, respectively. The fungal kingdom is composed of four phyla, 39 orders, 149 genera, and 253 species. In terms of citations for the filamentous fungi and the yeasts, the Ascomycota phylum is by far the most studied with 18 citations, followed by the Basidiomycota (4), the Cryptomycota (1), and the Mucoromycota (1). This is also the case in terms of richness with 102 genera and 88 species belonging to the Ascomycota while the Basidiomycota phylum is represented by only 21 genera with two species identified. Finally, only one genus is reported for both the Cryptomycota and the Mucoromycota phyla with no species identified. In the Ascomycota phylum, the most cited genera by number of citations are *Cladosporium* (9), *Colletotrichum* (9), *Aspergillus* (6), *Penicillium* (6), *Fusarium* (6), and *Trichoderma* (6). It is noteworthy that Fulthorpe et al. (2020) also reported using a metabarcoding approach these genera in coffee roots from all the sites that they studied across a gradient of temperature and humidity. In the same study,

**TABLE 3 |** Epiphytic bacteria and fungi diversity including phyla, orders, and genera, as well as the numbers of species identified and citations.

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Actinobacteria	Micrococcales	<i>Curtobacterium</i>	1	1
			<i>Kocuria</i>	1	1
		Streptomycetales	<i>Streptomyces</i>	0	1
			<i>Flavobacterium</i>	0	2
	Bacteroidetes	Flavobacteriales	<i>Flavobacterium</i>	0	2
	Firmicutes	Bacillales	<i>Bacillus</i>	2	4
		Lactobacillales	<i>Streptococcus</i>	0	1
	Proteobacteria	Burkholderiales	<i>Burkholderia</i>	2	2
			<i>Cedecea</i>	1	1
		Enterobacterales	<i>Citrobacter</i>	1	1
			<i>Enterobacter</i>	2	2
			<i>Pantoea</i>	1	1
			<i>Proteus</i>	0	1
			<i>Serratia</i>	1	1
			<i>Yersinia</i>	1	1
		Pseudomonadales	<i>Pseudomonas</i>	1	5
		Rhodospirillales	<i>Gluconacetobacter</i>	2	1
		Xanthomonadales	<i>Stenotrophomonas</i>	1	1
			<i>Xanthomonas</i>	0	1
Fungi	Ascomycota	Botryosphaerales	<i>Botryosphaeria</i>	0	1
			<i>Guignardia</i>	1	1
		Cladosporiales	<i>Cladosporium</i>	1	2
			<i>Phomopsis</i>	0	1
		Eurotiales	<i>Aspergillus</i>	3	4
			<i>Penicillium</i>	0	5
		Glomerellales	<i>Colletotrichum</i>	3	2
			<i>Beauveria</i>	1	1
		Hypocreales	<i>Calcarisporium</i>	2	2
			<i>Cylindrocarpon</i>	0	1
			<i>Fusarium</i>	1	6
			<i>Lecanicillium</i>	0	1
			<i>Simplicillium</i>	1	2
			<i>Trichoderma</i>	0	2
			<i>Verticillium</i>	1	3
			<i>Sporothrix</i>	1	2
		Pleosporales	<i>Alternaria</i>	1	1
			<i>Bipolaris</i>	0	1
			<i>Drechslera</i>	0	1
			<i>Epicoccum</i>	0	1
			<i>Exserohilum</i>	0	1
			<i>Phoma</i>	0	1
		Trichosphaerales	<i>Nigrospora</i>	0	2
		Xylariales	<i>Pestalotia</i>	0	1
			<i>Xylaria</i>	0	1
	Basidiomycota	Cantharellales	<i>Rhizoctonia</i>	0	1
	Mucoromycota	Mucorales	<i>Mucor</i>	0	3
			<i>Rhizopus</i>	0	1
(Yeasts)	Ascomycota	Pleosporales	<i>Torula</i>	0	1
		Saccharomycetales	<i>Candida</i>	4	2
			<i>Saccharomyces</i>	3	3

(Continued)

TABLE 3 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
(Yeasts)	Ascomycota	Saccharomycetales	<i>Torulopsis</i>	1	1
		Schizosaccharomycetales	<i>Schizosaccharomyces</i>	0	1
	Basidiomycota	Sporidiobolales	<i>Rhodotorula</i>	1	2
Bacteria	4	10	18	17	9
Fungi (Yeasts)	3	15	34	25	13
	(2)	(4)	(6)	(9)	(4)
Total	7	25	52	42	19

these authors also highlighted that the genera *Cladosporium* and *Penicillium* represented more than 40 and 10% of all the fungal sequences, respectively. When focusing only on AMF, the most cited genera are *Acaulospora* (34), *Gigaspora* (30), *Glomus* (29), *Claroideoglomus* (18), *Rhizophagus* (18), *Scutellospora* (15), *Ambispora* (13), *Funneliformis* (12), *Sclerocystis* (11), *Paraglomus* (11), *Archaeospora* (10), and *Entrophospora* (10). The genera *Glomus* and *Acaulospora* are also the most diversified with 26 and 23 different species, respectively (see also **Supplementary Table 2** for the complete list of species). Moreover, several metabarcoding studies also reported as dominant AMF associated with coffee the genera *Glomus*, *Acaulospora*, and *Archaeospora* under various management and environmental conditions (De Beenhouwer et al., 2015a,b; Fulthorpe et al., 2020).

Finally, the coffee endophytic bacteria diversity is composed of seven phyla, 28 orders, 88 genera, and 134 species. The most common bacterial phyla are the Firmicutes, Proteobacteria, and Actinobacteria with 15, 15, and 12 mentions in the literature, respectively. These phyla are also the most diversified with 42 genera and 61 species for the Proteobacteria, 28 genera and 39 species for the Actinobacteria, and 8 genera and 31 species for the Firmicutes. The most encountered genera in terms of number of citations are *Bacillus* (14), *Enterobacter* (8), *Cedecea* (6), *Paenibacillus* (6), *Pseudomonas* (6), and *Pantoea* (5). In addition, the authors of the only metabarcoding analysis dealing with endophytic bacteria also reported as dominant the genera *Pantoea*, *Enterobacter*, and *Pseudomonas* with a relative abundance of 17, 12, and 4% of all the bacterial sequences obtained from all the studied locations across a climatic gradient (Fulthorpe et al., 2020).

## The Microbiota Associated With Coffee Postharvest Processes

Before discussing the microbiota associated with postharvest treatments, it is important to have in mind the various processes commonly used in coffee (reviewed in Brando, 2004; Cleves, 2004; Schwan et al., 2012). Independently of the *Coffea* species, all postharvest techniques aim to remove all the external part of the cherries (exocarp, mesocarp, and endocarp) in order to produce green coffee beans to be commercialized. To do so, dry, semi-wet, and wet processes are implemented. The first one consists in drying the whole cherries and to mechanically remove the external parts (hulling) to obtain the green coffee

beans. The two other methods involve the removal of the exocarp (skin) and a part of the mesocarp (pulp) of the fresh cherries leaving the beans with a remaining part of the mesocarp (mucilage). In the semidry process, the beans are then dried before the hulling (dry fermentation) while in the wet processing the mucilage layer is removed by a fermentation step in tanks (wet fermentation) before being dried and hulled. In the final steps, the coffee endocarp (“parchment” or “husk”) is removed to obtain the green coffee bean enveloped in its spermoderm (“silverskin”). Whatever the method used, it is well-known that the microorganisms play important roles during the postharvest processes, especially by degrading the mucilage layer during the fermentation (Agate and Bhat, 1966) but also by influencing either positively (Elhalis et al., 2020a) or negatively (Ndayambaje et al., 2019) the organoleptic quality of the final product as well as its safety with respect to the presence of mycotoxins (Urbano et al., 2001).

These are the reasons why the postharvest microbiota has been extensively studied in order to describe the diversity of microorganisms associated with the dry (Pasin et al., 2011; Evangelista et al., 2014) the semidry (Van Pee and Castelein, 1971; Silva et al., 2013) and the wet processes (Pederson and Breed, 1946; De Oliveira Junqueira et al., 2019). Furthermore, microorganisms associated with the beans during the storage (Mislivec et al., 1983; Ndayambaje et al., 2019) and with the wastes and by-products were also studied (Aquihuatl et al., 1988; Pires et al., 2017; Oumer and Abate, 2018). These researches were conducted in order to better understand the role of the microbial component of the coffee processing and to develop some biotechnological applications to improve coffee quality as well as the wastes management sustainability.

Among the 127 publications related to the microbiota present after the harvest, most of them were performed using basic culture-dependent and independent methods. However, seven studies used NGS to perform some metabarcoding (one metagenomic) analyses of the bacterial and fungal communities associated with coffee fermentation (De Bruyn et al., 2017; De Carvalho Neto et al., 2018; De Oliveira Junqueira et al., 2019; Zhang et al., 2019a,b; Elhalis et al., 2020a,b).

Based on the survey of these studies, we can notice that the coffee postharvest microbiota is constituted by 18 phyla, 93 orders, 346 genera, and 446 species belonging to the bacterial and fungal kingdoms (**Table 5**). The fungi (including yeast) are the most studied microorganisms associated with the coffee



**TABLE 4 |** Endophytic archaea bacteria and fungi diversity including phyla, orders, and genera, as well as the numbers of species identified and citations.

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Archaea	Euryarchaeota	Halobacteriales	<i>Halobacterium</i>	0	1
			<i>Halococcus</i>	0	1
		Haloferacales	<i>Haloferax</i>	0	1
		Methanobacteriales	<i>Methanobrevibacter</i>	0	1
Bacteria	Acidobacteria	Acidobacteriales	<i>Acidipila</i>	0	1
			<i>Acidobacterium</i>	0	1
			<i>Edaphobacter</i>	0	1
			<i>Granulicella</i>	0	1
	Actinobacteria	Corynebacteriales	<i>Corynebacterium</i>	1	1
			<i>Gordonia</i>	0	1
			<i>Mycobacterium</i>	3	3
			<i>Mycolicibacterium</i>	1	1
			<i>Nocardia</i>	5	2
			<i>Rhodococcus</i>	1	1
		Frankiales	<i>Frankia</i>	0	1
		Micrococcales	<i>Arthrobacter</i>	2	2
			<i>Brachybacterium</i>	1	1
			<i>Brevibacterium</i>	1	1
			<i>Cellulomonas</i>	3	2
			<i>Clavibacter</i>	1	2
			<i>Curtobacterium</i>	3	2
			<i>Humibacter</i>	0	1
			<i>Janibacter</i>	1	1
			<i>Kocuria</i>	4	4
			<i>Leifsonia</i>	1	1
			<i>Microbacterium</i>	1	4
			<i>Micrococcus</i>	4	2
			<i>Sinomonas</i>	2	1
		Nakamurellales	<i>Nakamurella</i>	0	1
		Pseudonocardiales	<i>Amycolatopsis</i>	0	1
			<i>Kutzneria</i>	0	1
			<i>Lechevalieria</i>	1	1
		Solirubrobacterales	<i>Solirubrobacter</i>	0	1
		Streptomycetales	<i>Kitasatospora</i>	2	1
			<i>Streptomyces</i>	1	2
		Streptosporangiales	<i>Actinoallomurus</i>	0	1
	Bacteroidetes	Cytophagales	<i>Cytophaga</i>	1	1
		Flavobacteriales	<i>Chryseobacterium</i>	2	2
	Chloroflexi	Ktedonobacterales	<i>Ktedonobacter</i>	0	1
			<i>Thermosporothrix</i>	0	1
	Firmicutes	Bacillales	<i>Bacillus</i>	23	14
			<i>Brevibacillus</i>	1	3
			<i>Lysinibacillus</i>	1	1
			<i>Ornithinibacillus</i>	0	1
			<i>Paenibacillus</i>	4	6
			<i>Staphylococcus</i>	1	2
			<i>Virgibacillus</i>	0	1
			<i>Lactobacillus</i>	1	1
		Lactobacillales	<i>Lactobacillus</i>	1	1
		Gemmatales	<i>Gemmata</i>	0	1
		Pirellulales	<i>Blastopirellula</i>	0	1

(Continued)

TABLE 4 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Proteobacteria	Aeromonadales	<i>Aeromonas</i>	1	1
			<i>Alcaligenes</i>	1	1
			<i>Burkholderia</i>	7	4
			<i>Caballeronia</i>	1	1
			<i>Comamonas</i>	1	1
			<i>Herbaspirillum</i>	1	1
			<i>Hydrogenophaga</i>	1	1
			<i>Janthinobacterium</i>	0	1
			<i>Pandoraea</i>	1	2
			<i>Paraburkholderia</i>	2	1
			<i>Ralstonia</i>	0	1
			<i>Variovorax</i>	1	1
		Enterobacterales	<i>Cedecea</i>	1	6
			<i>Citrobacter</i>	0	2
			<i>Enterobacter</i>	4	8
			<i>Erwinia</i>	0	2
			<i>Escherichia</i>	3	4
			<i>Klebsiella</i>	4	3
			<i>Kluyvera</i>	1	2
			<i>Pantoea</i>	2	5
			<i>Pectobacterium</i>	1	1
			<i>Salmonella</i>	2	2
			<i>Serratia</i>	1	2
			<i>Shigella</i>	1	1
		Neisseriales	<i>Chromobacterium</i>	0	1
		Nevskiales	<i>Steroidobacter</i>	0	1
		Nitrosomonadales	<i>Nitrosovibrio</i>	0	1
		Pseudomonadales	<i>Acinetobacter</i>	4	4
			<i>Pseudomonas</i>	9	6
		Rhizobiales	<i>Agrobacterium</i>	1	1
			<i>Bradyrhizobium</i>	1	2
			<i>Methylobacterium</i>	2	2
			<i>Ochrobactrum</i>	0	2
			<i>Rhizobium</i>	2	2
		Rhodobacterales	<i>Paracoccus</i>	1	1
		Rhodospirillales	<i>Azospirillum</i>	0	1
			<i>Gluconacetobacter</i>	1	1
			<i>Saccharibacter</i>	0	1
		Sphingomonadales	<i>Sphingobium</i>	1	1
			<i>Sphingomonas</i>	0	1
		Xanthomonadales	<i>Luteibacter</i>	1	1
			<i>Stenotrophomonas</i>	1	3
Fungi	Ascomycota	Botryosphaerales	<i>Botryosphaeria</i>	0	2
			<i>Diplodia</i>	0	1
			<i>Guignardia</i>	2	4
			<i>Lasiodiplodia</i>	1	1
			<i>Macrophomina</i>	0	2
			<i>Microdiplodia</i>	0	1
			<i>Codinaeopsis</i>	0	1
		Chaetosphaerales	<i>Coniosporium</i>	0	1

(Continued)

TABLE 4 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Fungi	Ascomycota	Chaetothyriales	<i>Exophiala</i>	0	1
			<i>Knufia</i>	0	1
		Cladosporiales	<i>Cladosporium</i>	5	9
		Diaporthales	<i>Diaporthe</i>	2	2
			<i>Ophiognomonia</i>	0	1
			<i>Phomopsis</i>	2	5
		Dothideales	<i>Aureobasidium</i>	1	1
		Eurotiales	<i>Aspergillus</i>	9	6
			<i>Emericella</i>	1	1
			<i>Neosartorya</i>	0	1
			<i>Paecilomyces</i>	2	5
			<i>Penicillium</i>	17	6
			<i>Talaromyces</i>	1	1
		Glomerellales	<i>Brunneochlamydosporium</i>	0	1
			<i>Chordomyces</i>	0	1
			<i>Colletotrichum</i>	5	9
			<i>Glomerella</i>	2	3
			<i>Musidium</i>	0	1
			<i>Plectosphaerella</i>	0	2
			<i>Cryptosporiopsis</i>	1	1
		Helotiales	<i>Acremonium</i>	1	2
			<i>Beauveria</i>	2	5
		Hypocreales	<i>Bionectria</i>	0	1
			<i>Clonostachys</i>	2	3
			<i>Cylindrocarpon</i>	0	1
			<i>Engyodontium</i>	0	1
			<i>Fusarium</i>	3	6
			<i>Isaria</i>	0	1
			<i>Lecanicillium</i>	1	1
			<i>Myrothecium</i>	1	1
			<i>Sarocladium</i>	1	1
			<i>Trichoderma</i>	6	6
			<i>Verticillium</i>	0	1
			<i>Xenomyrothecium</i>	0	1
		Incertae_sedis	<i>Phyllosticta</i>	1	1
			<i>Triscelophorus</i>	3	1
		Magnaporthales	<i>Pseudohalonestria</i>	1	1
		Microascales	<i>Microascus</i>	0	1
			<i>Parascedosporium</i>	0	1
			<i>Petriella</i>	0	1
			<i>Pseudallescheria</i>	1	1
			<i>Acrodontium</i>	0	1
		Mycosphaerellales	<i>Cercospora</i>	0	2
			<i>Mycocentrospora</i>	0	1
			<i>Mycosphaerella</i>	0	4
			<i>Staninwardia</i>	0	1
			<i>Lacazia</i>	1	1
		Onygenales	<i>Conoplea</i>	0	1
		Pezizales	<i>Acrocallymma</i>	0	1
		Pleosporales	<i>Alternaria</i>	2	3
			<i>Ascochyta</i>	0	1

(Continued)

TABLE 4 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Fungi	Ascomycota	Pleosporales	<i>Bipolaris</i>	0	1
			<i>Camarosporium</i>	0	1
			<i>Crassiparies</i>	1	1
			<i>Dokmaia</i>	0	1
			<i>Drechslera</i>	1	1
			<i>Epicoccum</i>	1	2
			<i>Leptosphaeria</i>	0	1
			<i>Leptosphaerulina</i>	0	1
			<i>Microsphaeropsis</i>	0	1
			<i>Neodidymella</i>	0	1
			<i>Neopyrenochaeta</i>	0	1
			<i>Neosetophoma</i>	0	1
			<i>Nigrograna</i>	0	1
			<i>Paraconiothyrium</i>	0	2
			<i>Paraphaeosphaeria</i>	0	1
			<i>Periconia</i>	0	1
			<i>Phaeosphaeria</i>	0	1
			<i>Phoma</i>	3	3
			<i>Rhizopycnis</i>	0	2
			<i>Roussoella</i>	0	1
			<i>Stagonospora</i>	0	1
			<i>Stagonosporopsis</i>	0	2
		Sordariales	<i>Chaetomium</i>	0	1
			<i>Lunulospora</i>	1	1
		Trichosphaerales	<i>Khuskia</i>	1	1
			<i>Nigrospora</i>	0	1
		Xylariales	<i>Biscogniauxia</i>	0	1
			<i>Daldinia</i>	0	1
			<i>Hansfordia</i>	0	1
			<i>Hypoxylon</i>	0	2
			<i>Idriella</i>	0	1
			<i>Leptosillia</i>	0	1
			<i>Libertella</i>	0	1
			<i>Lopadostoma</i>	0	1
			<i>Muscodor</i>	1	1
			<i>Nemania</i>	0	1
			<i>Nodulisporium</i>	1	2
			<i>Pestalotia</i>	0	1
			<i>Pestalotiopsis</i>	1	3
			<i>Phialemoniopsis</i>	0	1
			<i>Pseudobeltrania</i>	0	1
			<i>Rosellinia</i>	0	1
			<i>Xylaria</i>	0	5
	Basidiomycota	Agaricales	<i>Clitocybe</i>	0	1
			<i>Marasmius</i>	0	1
			<i>Mycena</i>	0	1
			<i>Schizophyllum</i>	0	2
		Auriculariales	<i>Exidiopsis</i>	0	1
		Entylomatales	<i>Tilletiopsis</i>	0	1
		Exobasidiales	<i>Meira</i>	0	1
		Hymenochaetales	<i>Fuscoporia</i>	0	1

(Continued)



**TABLE 4 | Continued**

Kingdom	Phylum	Order	Genus	No. of species	No. of citations			
Fungi	Basidiomycota	Incertae_sedis	<i>Peniophora</i>	0	1			
		Microstromatales	<i>Jaminalaea</i>	0	1			
		Polyporales	<i>Irpex</i>	0	1			
			<i>Phlebiopsis</i>	0	1			
			<i>Trametes</i>	0	1			
			Russulales	<i>Stereum</i>	0	1		
			Tilletiales	<i>Tilletia</i>	0	1		
		Trechisporales	<i>Sistotremastrum</i>	1	1			
			<i>Trechispora</i>	0	1			
		Ustilaginales	<i>Pseudozyma</i>	0	1			
		Cryptomycota	Rozellida	<i>Paramicrosporidium</i>	0	1		
			Mucoromycota	Mucorales	<i>Gongronella</i>	0	1	
		(AMF)	Mucoromycota	Archaeosporales	<i>Ambispora</i>	6	13	
					<i>Archaeospora</i>	3	10	
			Diversisporales	<i>Acaulospora</i>	23	34		
			<i>Cetraspora</i>	4	7			
			<i>Dentiscutata</i>	4	9			
			<i>Diversispora</i>	3	4			
			<i>Entrophospora</i>	1	10			
			<i>Gigaspora</i>	5	30			
			<i>Otospora</i>	1	1			
			<i>Pacispora</i>	1	1			
			<i>Racocetra</i>	3	5			
			<i>Redeckera</i>	1	1			
			<i>Scutellospora</i>	5	15			
			<i>Sieverdingia</i>	1	4			
			<i>Claroideoglomus</i>	5	18			
			<i>Dominikia</i>	1	1			
			<i>Funnelliformis</i>	8	12			
			<i>Glomus</i>	26	29			
			<i>Oehlia</i>	1	3			
			<i>Rhizoglomus</i>	1	2			
			<i>Rhizophagus</i>	9	18			
			<i>Sclerocystis</i>	6	11			
			<i>Septoglomus</i>	3	5			
			(Yeasts)	Basidiomycota	Paraglomerales	<i>Paraglomus</i>	5	11
					Sporidiobolales	<i>Rhodotorula</i>	1	1
						<i>Sporobolomyces</i>	0	1
					Tremellales	<i>Cryptococcus</i>	0	2
					Archaea	1	3	4
			Bacteria	7	28	88	134	18
Fungi	4	39	149	216	55			
(AMF;Yeasts)	(1;1)	(4;2)	(24;3)	(126;1)	(38;3)			
Total	12	70	241	350	71			

postharvest steps with 105 references. This kingdom includes four phyla, 34 orders, 119 genera, and 270 species. In terms of citations, the most encountered fungal phyla are the Ascomycota (105), Mucoromycota (23), and Basidiomycota (19) and at the genus level the *Aspergillus* (67), *Penicillium* (45), *Fusarium* (37),

*Cladosporium* (29), *Mucor* (16), and *Rhizopus* (16) focusing on filamentous fungi. Indeed, the presence of filamentous fungi has been extensively studied due to the presence of mycotoxins such the ochratoxins and aflatoxins (Joosten et al., 2001; Rezende et al., 2013). It is worth noting that all these toxigenic fungi

**TABLE 5 |** Postharvest bacteria and fungi diversity including phyla, orders, and genera, as well as the numbers of species identified and citations.

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Acidobacteria	Acidobacteriales	<i>Koribacter</i>	0	1
	Actinobacteria	Actinomycetales	<i>Actinomyces</i>	0	1
		Actinopolysporales	<i>Actinopolyspora</i>	0	1
		Bifidobacteriales	<i>Bifidobacterium</i>	0	1
		Corynebacteriales	<i>Corynebacterium</i>	2	3
			<i>Mycobacterium</i>	0	1
			<i>Nocardia</i>	2	1
			<i>Rhodococcus</i>	1	3
			<i>Williamsia</i>	0	1
		Geodermatophilales	<i>Geodermatophilus</i>	0	1
	Incertae_sedis		<i>Actinobacterium</i>	0	1
	Kineosporiales		<i>Kineococcus</i>	0	1
			<i>Kineosporia</i>	0	1
	Micrococcales		<i>Arsenicicoccus</i>	0	1
			<i>Arthrobacter</i>	4	7
			<i>Brachybacterium</i>	0	2
			<i>Brevibacterium</i>	0	3
			<i>Cellulomonas</i>	0	2
			<i>Cellulosimicrobium</i>	2	3
			<i>Cryocolla</i>	0	1
			<i>Curtobacterium</i>	1	3
			<i>Dermabacter</i>	0	1
			<i>Kocuria</i>	0	2
			<i>Lysinimonas</i>	1	1
			<i>Microbacterium</i>	5	4
			<i>Micrococcus</i>	2	2
			<i>Pseudoclavibacter</i>	0	1
			<i>Rathayibacter</i>	0	1
			<i>Rothia</i>	0	1
			<i>Salana</i>	0	1
			<i>Salinibacterium</i>	0	1
			<i>Terracoccus</i>	0	1
	Micromonosporales		<i>Actinoplanes</i>	0	1
			<i>Pilimelia</i>	0	1
	Propionibacteriales		<i>Aeromicrobium</i>	0	1
			<i>Nocardioides</i>	0	2
	Pseudonocardiales		<i>Actinomycetospora</i>	0	1
			<i>Pseudonocardia</i>	0	2
	Solirubrobacterales		<i>Patulibacter</i>	0	1
	Streptomycetales		<i>Streptomyces</i>	1	4
	Streptosporangiales		<i>Actinoallomurus</i>	0	1
			<i>Sphaerisporangium</i>	0	1
	Armatimonadetes	Fimbrimonadales	<i>Fimbrimonas</i>	0	2
			<i>Bacteroides</i>	0	1
Bacteroidetes	Bacteroidales		<i>Dysgonomonas</i>	0	1
			<i>Paludibacter</i>	0	1
			<i>Parabacteroides</i>	0	1
			<i>Prevotella</i>	0	2
			<i>Chitinophaga</i>	0	1
	Chitinophagales		<i>Flavisolibacter</i>	0	1
			<i>Niabella</i>	0	1
			<i>Sediminibacterium</i>	0	1
			<i>Segetibacter</i>	0	1
			<i>Dyadobacter</i>	0	1
	Cytophagales		<i>Emticicia</i>	0	1
			<i>Hymenobacter</i>	0	2
			<i>Larkinella</i>	0	1
			<i>Leadbetterella</i>	0	1

(Continued)

**TABLE 5 |** Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Bacteroidetes	Cytophagales	<i>Rudanella</i>	0	1
			<i>Spirosoma</i>	0	2
		Flavobacteriales	<i>Blattabacterium</i>	0	1
			<i>Capnocytophaga</i>	0	1
			<i>Chryseobacterium</i>	3	4
			<i>Flavobacterium</i>	1	4
			<i>Fluvicola</i>	0	1
			<i>Wautersiella</i>	0	1
		Sphingobacteriales	<i>Olivibacter</i>	0	1
			<i>Pedobacter</i>	0	2
			<i>Sphingobacterium</i>	1	3
	Chlamydiae	Parachlamydiales	<i>Protochlamydia</i>	0	1
			<i>Rhabdochlamydia</i>	0	1
	Cyanobacteria	Nostocales	<i>Anabaena</i>	0	1
		Oscillatoriales	<i>Arthrospira</i>	0	2
		Spirulinales	<i>Halospirulina</i>	0	1
	Deinococcus-Thermus	Deinococcales	<i>Deinococcus</i>	0	1
	Firmicutes	Bacillales	<i>Truepera</i>	0	1
			<i>Alicyclobacillus</i>	0	1
			<i>Ammoniphilus</i>	0	1
			<i>Bacillus</i>	9	21
			<i>Brevibacillus</i>	1	1
			<i>Brochothrix</i>	0	1
			<i>Domibacillus</i>	0	1
			<i>Exiguobacterium</i>	0	1
			<i>Kurthia</i>	0	1
			<i>Lysinibacillus</i>	0	2
			<i>Paenibacillus</i>	1	3
			<i>Puillimonas</i>	0	1
			<i>Rummeliibacillus</i>	0	1
			<i>Saccharibacillus</i>	0	2
			<i>Staphylococcus</i>	3	4
		Clostridiales	<i>Blautia</i>	0	1
			<i>Clostridium</i>	0	5
			<i>Coprococcus</i>	0	1
			<i>Dorea</i>	0	1
			<i>Faecalibacterium</i>	0	1
		Erysipelotrichales	<i>Oscillospira</i>	0	1
			<i>Ruminococcus</i>	0	1
			<i>Turicibacter</i>	0	1
			<i>Lactobacillales</i>		
			<i>Enterococcus</i>	2	8
			<i>Fructobacillus</i>	0	3
			<i>Lactobacillus</i>	6	18
			<i>Lactococcus</i>	2	10
			<i>Leuconostoc</i>	5	20
			<i>Oenococcus</i>	0	1
			<i>Pediococcus</i>	2	6
			<i>Streptococcus</i>	2	2
			<i>Weissella</i>	4	9
	Fusobacteria	Fusobacteriales	<i>Fusobacterium</i>	0	1
	Gemmatimonadetes	Gemmatimonadales	<i>Gemmatimonas</i>	0	1
	Nitrospirae	Nitrospirales	<i>Nitrospira</i>	0	1
	Planctomycetes	Gemmatales	<i>Gemmata</i>	0	1
		Planctomycetales	<i>Planctomyces</i>	0	2
Proteobacteria	Aeromonadales		<i>Aeromonas</i>	1	3
			<i>Idiomarina</i>	0	1
			<i>Marinobacter</i>	0	1
	Bdellovibrionales		<i>Bdellovibrio</i>	0	2
			<i>Achromobacter</i>	0	1

(Continued)

TABLE 5 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Proteobacteria	Burkholderiales	<i>Burkholderia</i>	1	3
			<i>Comamonas</i>	0	2
			<i>Hylemonella</i>	0	1
			<i>Janthinobacterium</i>	1	3
			<i>Lautropia</i>	0	1
			<i>Paucibacter</i>	0	1
			<i>Pigmentiphaga</i>	0	1
			<i>Polaromonas</i>	0	1
			<i>Polynucleobacter</i>	0	1
			<i>Rubrivivax</i>	0	1
		Campylobacterales	<i>Arcobacter</i>	0	1
			<i>Campylobacter</i>	0	1
		Cardiobacteriales	<i>Cardiobacterium</i>	0	1
		Caulobacterales	<i>Brevundimonas</i>	0	1
			<i>Phenylobacterium</i>	0	2
		Cellvibrionales	<i>Cellvibrio</i>	0	1
		Enterobacterales	<i>Bandoniozyma</i>	2	1
			<i>Buttiauxella</i>	1	1
			<i>Cedecea</i>	0	1
			<i>Citrobacter</i>	3	8
			<i>Cronobacter</i>	1	1
			<i>Enterobacter</i>	9	18
			<i>Erwinia</i>	5	11
			<i>Escherichia</i>	2	7
			<i>Ewingella</i>	1	1
			<i>Hafnia</i>	1	3
			<i>Klebsiella</i>	5	16
			<i>Kluyvera</i>	2	1
			<i>Kosakonia</i>	1	1
			<i>Leminorella</i>	1	1
			<i>Pantoea</i>	8	12
			<i>Pectobacterium</i>	0	2
			<i>Plesiomonas</i>	0	1
			<i>Proteus</i>	2	3
			<i>Rahnella</i>	1	2
			<i>Raoultella</i>	0	1
			<i>Rosenbergiella</i>	0	1
			<i>Salmonella</i>	3	6
			<i>Serratia</i>	4	11
			<i>Shigella</i>	1	1
			<i>Tatumella</i>	2	6
			<i>Trabulsiella</i>	0	1
			<i>Yersinia</i>	2	4
		Legionellales	<i>Legionella</i>	0	1
		Methylococcales	<i>Crenothrix</i>	0	1
		Myxococcales	<i>Nannocystis</i>	0	1
		Neisseriales	<i>Chromobacterium</i>	1	1
			<i>Eikenella</i>	0	1
			<i>Neisseria</i>	0	1
		Nevskiales	<i>Steroidobacter</i>	0	1
		Nitrosomonadales	<i>Thiobacillus</i>	0	1
		Pasteurellales	<i>Aggregatibacter</i>	0	1
			<i>Pasteurella</i>	1	1
		Pseudomonadales	<i>Acinetobacter</i>	4	10
			<i>Moraxella</i>	1	2
			<i>Pseudomonas</i>	18	17
			<i>Agrobacterium</i>	1	3
		Rhizobiales	<i>Alsobacter</i>	0	1
			<i>Aminobacter</i>	0	1
			<i>Beijerinckia</i>	0	2

(Continued)

TABLE 5 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Bacteria	Proteobacteria	Rhizobiales	<i>Bosea</i>	0	1
			<i>Devosia</i>	0	1
			<i>Hyphomicrobium</i>	0	1
			<i>Kaistia</i>	0	1
			<i>Labrys</i>	0	1
			<i>Mesorhizobium</i>	0	1
			<i>Methylobacterium</i>	0	5
			<i>Methylocella</i>	0	1
			<i>Methylocystis</i>	0	1
			<i>Methylopila</i>	0	1
			<i>Methylosinus</i>	0	1
			<i>Mycoplana</i>	0	2
			<i>Neorhizobium</i>	0	2
			<i>Ochrobactrum</i>	1	4
			<i>Parvibaculum</i>	0	2
			<i>Pedomicrobium</i>	0	1
			<i>Rhizobium</i>	0	2
			<i>Rhodoplanes</i>	0	2
			<i>Xanthobacter</i>	0	1
		Rhodobacterales	<i>Falsirhodobacter</i>	0	1
			<i>Oceanicaulis</i>	0	1
			<i>Paracoccus</i>	0	1
			<i>Rhodobaca</i>	0	1
			<i>Rhodobacter</i>	0	1
			<i>Rubellimicrobium</i>	0	1
		Rhodospirillales	<i>Acetobacter</i>	13	7
			<i>Asaia</i>	0	1
			<i>Azospirillum</i>	0	2
			<i>Gluconacetobacter</i>	1	2
			<i>Gluconobacter</i>	8	8
			<i>Inquilinus</i>	0	1
			<i>Kozakia</i>	1	2
			<i>Rhodospirillum</i>	0	1
			<i>Roseococcus</i>	0	1
			<i>Roseomonas</i>	0	1
		Rickettsiales	<i>Wolbachia</i>	0	1
		Sphingomonadales	<i>Blastomonas</i>	0	1
			<i>Kaistobacter</i>	0	1
			<i>Novosphingobium</i>	0	3
			<i>Sphingobium</i>	0	2
			<i>Sphingomonas</i>	1	6
		Xanthomonadales	<i>Sphingopyxis</i>	0	1
			<i>Dokdonella</i>	0	1
			<i>Dyella</i>	1	2
			<i>Luteibacter</i>	0	1
			<i>Luteimonas</i>	0	2
			<i>Stenotrophomonas</i>	0	2
			<i>Xanthomonas</i>	1	1
		Verrucomicrobia	<i>Chthoniobacter</i>	0	1
			<i>Candidatus</i>	0	1
			<i>Xiphinematobacter</i>		
Fungi	Ascomycota	Botryosphaerales	<i>Microdiplodia</i>	1	1
		Capnodiales	<i>Antennariella</i>	1	1
			<i>Capnodium</i>	0	1
			<i>Strelitziana</i>	0	1
		Chaetothyriales	<i>Cladosporium</i>	11	29
		Dothideales	<i>Aureobasidium</i>	0	1
			<i>Aspergillus</i>	48	67
		Eurotiales	<i>Byssoschlamys</i>	1	2
			<i>Eurotium</i>	2	8

(Continued)

TABLE 5 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
Fungi	Ascomycota	Eurotiales	<i>Paecilomyces</i>	0	3
			<i>Penicillium</i>	39	45
			<i>Talaromyces</i>	1	5
		Glomerellales	<i>Colletotrichum</i>	3	3
			<i>Plectosphaerella</i>	1	1
		Helotiales	<i>Articulospora</i>	0	2
			<i>Botrytis</i>	1	2
			<i>Cadophora</i>	1	1
		Hypocreales	<i>Monilia</i>	0	1
			<i>Acremonium</i>	0	5
			<i>Beauveria</i>	2	2
			<i>Cylindrocarpon</i>	1	2
			<i>Fusariella</i>	0	1
			<i>Fusarium</i>	18	37
			<i>Gibberella</i>	2	4
			<i>Myrothecium</i>	1	1
			<i>Sarocladium</i>	1	1
			<i>Stachybotrys</i>	0	1
		Microascales	<i>Trichoderma</i>	1	4
			<i>Microascus</i>	0	1
		Mycosphaerellales	<i>Scopulariopsis</i>	1	2
			<i>Cercospora</i>	0	3
			<i>Neodevriesia</i>	0	1
		Onygenales	<i>Zymoseptoria</i>	0	1
			<i>Chrysosporium</i>	0	1
		Orbiliates	<i>Arthrobotrys</i>	0	1
		Pleosporales	<i>Alternaria</i>	1	4
			<i>Drechslera</i>	0	1
			<i>Epicoccum</i>	0	1
			<i>Leptosphaerulina</i>	1	1
			<i>Phaeosphaeria</i>	0	1
			<i>Phoma</i>	0	2
			<i>Pyrenochaeta</i>	0	1
			<i>Pyrenochaetopsis</i>	0	2
			<i>Setophoma</i>	0	1
			<i>Stemphylium</i>	0	1
			<i>Ulocladium</i>	0	1
		Saccharomycetales	<i>Eremothecium</i>	0	1
		Sordariales	<i>Neurospora</i>	1	1
		Trichosphaeriales	<i>Nigrospora</i>	1	2
		Xylariales	<i>Pestalotia</i>	0	1
			<i>Pestalotiopsis</i>	0	1
	Basidiomycota	Agaricostilbales	<i>Bensingtonia</i>	0	1
		Cantharellales	<i>Rhizoctonia</i>	0	1
		Leucosporidiales	<i>Leucosporidiella</i>	0	1
		Malasseziales	<i>Malassezia</i>	0	1
		Tremellales	<i>Fellomyces</i>	1	1
			<i>Hannaella</i>	1	4
			<i>Rhynchogastrema</i>	0	1
			<i>Vishniacozyma</i>	2	3
		Ustilaginales	<i>Pseudozyma</i>	0	1
		Walleniales	<i>Wallenia</i>	1	3
	Mucoromycota	Mucorales	<i>Absidia</i>	1	4
			<i>Circinella</i>	0	1
			<i>Lichtheimia</i>	1	1
			<i>Mucor</i>	2	16
			<i>Rhizopus</i>	2	16
	Zoopagomycota	Zoopagales	<i>Syncephalastrum</i>	1	3
			<i>Syncephalis</i>	0	1

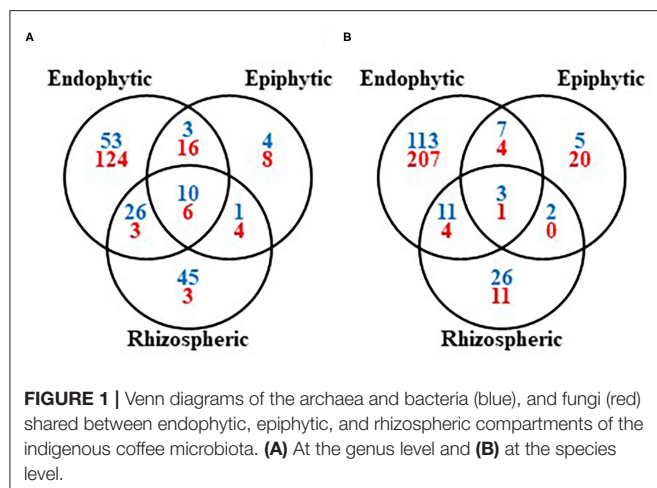
(Continued)

TABLE 5 | Continued

Kingdom	Phylum	Order	Genus	No. of species	No. of citations
(Yeasts)	Ascomycota	Saccharomycetales	<i>Arxula</i>	1	2
			<i>Barnettozyma</i>	1	1
			<i>Blastobotrys</i>	2	2
			<i>Brettanomyces</i>	0	1
			<i>Candida</i>	32	26
			<i>Citeromyces</i>	1	1
			<i>Clavispora</i>	1	1
			<i>Cyberlindnera</i>	1	2
			<i>Debaryomyces</i>	1	10
			<i>Dekkera</i>	1	1
			<i>Dipodascus</i>	1	2
			<i>Geotrichum</i>	3	8
			<i>Hanseniaspora</i>	4	16
			<i>Hyphopichia</i>	1	3
			<i>Issatchenkia</i>	0	1
			<i>Kazachstania</i>	2	3
			<i>Kloeckera</i>	1	3
			<i>Kluyveromyces</i>	2	5
			<i>Kodamaea</i>	1	2
			<i>Lachancea</i>	2	2
			<i>Lodderomyces</i>	1	1
			<i>Meyerozyma</i>	3	12
			<i>Ogataea</i>	1	1
			<i>Pichia</i>	13	28
			<i>Saccharomyces</i>	3	21
			<i>Saccharomycopsis</i>	3	3
			<i>Saturnispora</i>	1	1
			<i>Schwannomyces</i>	1	3
			<i>Sporopachydermia</i>	1	1
			<i>Starmerella</i>	1	3
			<i>Torulaspora</i>	1	15
			<i>Wickerhamomyces</i>	4	13
			<i>Williopsis</i>	1	1
			<i>Yarrowia</i>	1	1
			<i>Zygotorulaspora</i>	0	1
	Basidiomycota	Schizosaccharomycetales	<i>Schizosaccharomyces</i>	1	3
		Cystofilobasidiales	<i>Cystofilobasidium</i>	2	6
			<i>Naganishia</i>	0	1
			<i>Holtermannia</i>	1	2
		Heteromycetales	<i>Trichosporonoides</i>	1	2
			<i>Leucosporidium</i>	0	1
		Sporidiobolales	<i>Rhodosporeidium</i>	1	1
			<i>Rhodotorula</i>	7	9
			<i>Sporidiobolus</i>	1	3
		Tremellales	<i>Sporobolomyces</i>	2	3
			<i>Bullera</i>	1	1
			<i>Cryptococcus</i>	4	7
		Trichosporonales	<i>Papillotrema</i>	2	4
			<i>Sirobasidium</i>	0	1
			<i>Apiotrichum</i>	1	1
			<i>Cutaneotrichosporon</i>	0	1
Bacteria	14	59	227	176	51
Fungi (yeasts)	4	34	119	270	105
	(2)	(10)	(51)	(117)	(47)
Total	18	93	346	446	127

belong to the Ascomycota phylum, the Eurotiales order, and the genera *Aspergillus*, *Byssoschlamys*, and *Penicillium* with 23 toxigenic species identified to date (Supplementary Table 3).



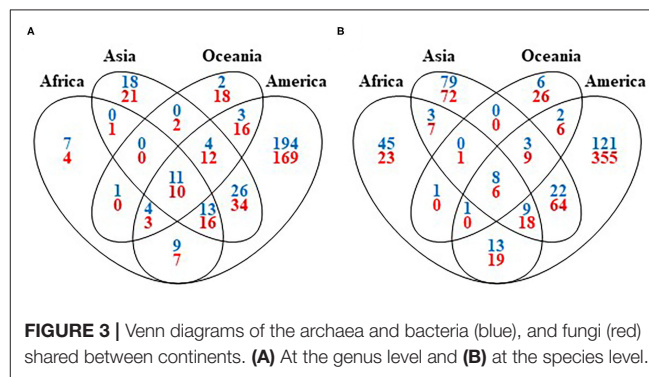
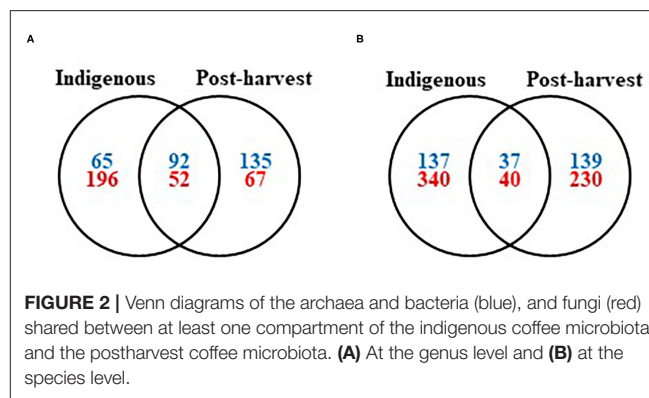


Regarding yeasts, the most cited genera are *Pichia* (28), *Candida* (26), *Saccharomyces* (21), *Hanseniaspora* (16), *Torulaspora* (15), *Wickerhamomyces* (13), *Meyerozyma* (12), and *Debaryomyces* (10). Furthermore, the yeast genera *Pichia* and *Candida* are also reported to be the dominant genera in term of relative abundance during the coffee fermentation across all the metabarcoding studies (De Bruyn et al., 2017; De Oliveira Junqueira et al., 2019; Zhang et al., 2019b; Elhalis et al., 2020a,b).

Regarding the bacterial kingdom, it contains 14 phyla, 59 orders, 227 genera, and 176 species described across 51 publications. In matter of recurrence in the literature, the most cited are the Firmicutes (38), Proteobacteria (33), Actinobacteria (14), and the Bacteroidetes (7) at the phylum level and the *Bacillus* (21), *Leuconostoc* (20), *Lactobacillus* (18), *Enterobacter* (18), *Pseudomonas* (17), *Klebsiella* (16), *Pantoea* (12), *Erwinia* (11), *Serratia* (11), *Lactococcus* (10), and *Acinetobacter* (10) at the genus level. These findings are in accordance with the relative abundances registered using metabarcoding/metagenomic approach. Indeed, in such studies the lactic acid bacteria (LAB), especially from the genus *Leuconostoc*, and acetic acid bacteria (AAB) are often reported to be the dominant bacteria during the fermentation along with other bacterial genera (e.g., *Bacillus*, *Erwinia*, *Pseudomonas*) belonging to the Firmicutes and Proteobacteria phyla (De Bruyn et al., 2017; De Carvalho Neto et al., 2018; De Oliveira Junqueira et al., 2019; Zhang et al., 2019a,b; Elhalis et al., 2020a,b). Thus, a total of 22 species of AAB belonging to the genera *Acetobacter*, *Gluconacetobacter*, *Gluconobacter*, and *Kozakia*, as well as 23 species of LAB from the *Bifidobacterium*, *Bacillus*, *Clostridium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, and *Weissella* genera have been described during the coffee postharvest steps (see also **Supplementary Table 4** for the complete list of species).

## The Core Coffee Microbiota Related to Specific Functional Roles

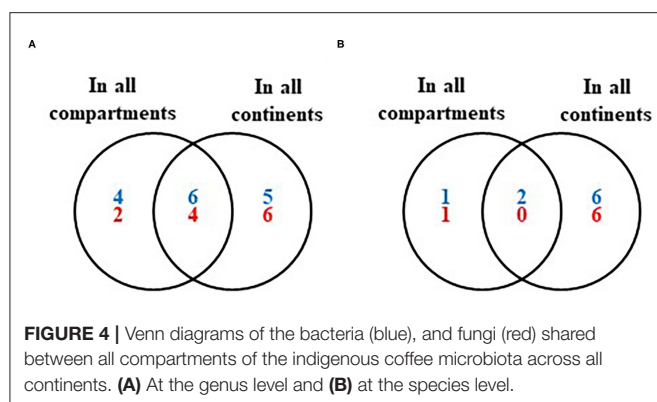
The plant “core microbiota” can be described at different taxonomic, spatial, and temporal levels and is likely to



contain highly competitive plant colonizers representing choice candidates for functional studies (Vandenkoornhuyse et al., 2015; Müller et al., 2016). Furthermore, it is hypothesized that the “core microbiota” is the result of a coevolution process in which the microbes have been selected to achieve essential functions for their hosts (Lemanceau et al., 2017; Compant et al., 2019).

In the framework of this review, we defined the “core coffee microbiota” corresponding to the microbial taxa (at genus and species levels) shared between (i) indigenous coffee microbiota plant compartments, namely, the rhizosphere, episphere, and endosphere (**Figure 1**; see also **Supplementary Table 5** for the complete list of genera and species in each partitions), (ii) at least one indigenous coffee microbiota compartment and postharvest coffee microbiota (**Figure 2**; see also **Supplementary Table 6** for the complete list of genera and species in each partitions), (iii) the continents (**Figure 3**; see also **Supplementary Table 7** for the complete list of genera and species in each partitions), and (iv) all indigenous coffee microbiota plant compartments across all continents (**Figure 4**; see also **Supplementary Table 8** for the complete list of genera and species in each partitions).

Considering the taxa shared between the rhizosphere, endosphere, and episphere, a total of 10 bacterial genera (*Bacillus*, *Burkholderia*, *Citrobacter*, *Enterobacter*, *Gluconacetobacter*, *Kocuria*, *Pseudomonas*, *Serratia*, *Stenotrophomonas*, and *Streptomyces*) and three species (*B. subtilis*, *P. putida*, *S. maltophilia*), as well as six fungal genera (*Aspergillus*, *Cladosporium*, *Cylindrocarpum*, *Fusarium*, *Penicillium*, and *Trichoderma*), and one species (*A. niger*), are able to



colonize all the coffee plant compartments (Figure 1; see also Supplementary Table 5). These microorganisms can therefore be considered as those presenting the highest competitiveness to colonize and survive in the coffee rhizosphere but also at the surface and inside coffee plant tissues/organs.

Regarding the microorganisms associated both to coffee plants in the field and during coffee processing, we highlighted 92 genera (32% of the total) and 37 species (12% of the total) for bacteria, as well as 52 genera (17% of the total) and 40 species (7% of the total) for the fungi, shared between the postharvest microbiota and at least one indigenous coffee microbiota compartment (Figure 2; see also Supplementary Table 6). Furthermore, 135 bacterial and 67 fungal genera, as well as 139 bacterial and 230 fungal species, were found only in the postharvest microbiota and are therefore introduced during the processing steps. These observations are particularly relevant regarding toxigenic fungi because among the 23 species found during postharvest processing, only 10 are member of indigenous microbiota. Indeed, the species *A. flavus* and *A. ochraceus* are also present as epiphytes while *A. sclerotiorum*, *A. versicolor*, *A. westerdijkiae*, *P. crustosum*, *P. olsonii*, and *P. oxalicum* as endophytes (Supplementary Table 3). Finally, *A. niger* and *P. brevicompactum* are present in all coffee plant compartments. Therefore, the presence of the other 13 toxigenic species could be avoided by taking stricter hygiene measures in the processing facilities and thus reducing the losses associated with fungal toxins contaminations.

Concerning the diversity between the coffee growing continents, 11 bacterial genera (*Bacillus*, *Citrobacter*, *Enterobacter*, *Enterococcus*, *Erwinia*, *Klebsiella*, *Leuconostoc*, *Pantoea*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*) and eighth species (*B. megaterium*, *B. subtilis*, *E. cloacae*, *K. pneumoniae*, *L. mesenteroides*, *P. agglomerans*, *P. fluorescens*, and *S. maltophilia*), together with 10 fungal genera (*Aspergillus*, *Candida*, *Cladosporium*, *Fusarium*, *Hanseniaspora*, *Penicillium*, *Pichia*, *Rhodotorula*, *Torulaspora*, and *Wickerhamomyces*) and six species (*A. westerdijkiae*, *H. uvarum*, *P. brevicompactum*, *P. citrinum*, *R. mucilaginosa*, and *W. anomalus*), are shared all across the coffee world (Figure 3; see also Supplementary Table 7).

Thus, these microbial taxa, which are ubiquitous across continents, are able to adapt themselves to various environmental conditions.

Finally, a total of six bacterial genera (*Bacillus*, *Citrobacter*, *Enterobacter*, *Pseudomonas*, *Serratia*, and *Stenotrophomonas*) and two species (*B. subtilis* and *S. maltophilia*), as well as four fungal genera (*Aspergillus*, *Cladosporium*, *Fusarium*, and *Penicillium*), are present in all coffee plant compartments across all continents (Figure 4; see also Supplementary Table 8).

To summarize, the coffee microbiota contains some microorganisms that are either some competitive coffee plant colonizers or able to adapt themselves to various environmental conditions, and sometimes both. Therefore, these microorganisms should be further studied in order to exploit their capacities for the development of new biotechnological applications.

## Biotechnological Applications of the Coffee Microbiota

### Potential Uses as Plant Growth Promoting Agents

Coffee-associated microorganisms have been extensively studied for their potential use as plant-growth promoting agents including rhizospheric bacteria and fungi (Jimenez-Salgado et al., 1997; Posada et al., 2013; Kejela et al., 2016; Perea Rojas et al., 2019), epiphytic bacteria (Estrada-De Los Santos et al., 2001; Teshome et al., 2017), endophytic bacteria (Jimenez-Salgado et al., 1997; Silva et al., 2012) and AMF (Caldeira et al., 1983; Perea Rojas et al., 2019).

The microorganisms' capacity to promote the plant growth is often linked with the improvement of the plant mineral nutrition or the regulation of the plant hormonal balance (Egamberdieva et al., 2017; Kudoyarova et al., 2019; Aeron et al., 2020). Since nitrogen, phosphorus, and iron are among the most limiting nutrients for plants, the use of N-fixing, phosphorus-solubilizing (P-solubilizing), and siderophore-producing microorganisms could represent a sustainable strategy to reduce the reliance on chemical fertilizers (Vitousek et al., 2002; Scavino and Pedraza, 2013; Alori et al., 2017; Pahari et al., 2017; Prabhu et al., 2019; Smercina et al., 2019).

Thus, *in vitro* screenings were often used to highlight the microbiota potential capacity to improve the coffee plant nutrition. For example, the ability to fix the atmospheric nitrogen was demonstrated for some bacterial species associated with *C. arabica* roots belonging to the genera *Gluconacetobacter* (Jimenez-Salgado et al., 1997; Fuentes-Ramírez et al., 2001), *Burkholderia* (Estrada-De Los Santos et al., 2001), *Azotobacter*, *Leifsonia*, and *Stenotrophomonas* (Wedhastris et al., 2012). Another valuable microbial process is the improvement of nutrient availability. To this end, the capacity to solubilize the phosphorus has been demonstrated not only for numerous bacterial species associated with *C. arabica*, *C. canephora*, and *C. liberica* roots and seeds, belonging to the genera *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brachy bacterium*, *Burkholderia*, *Caballeronia*, *Cellulomonas*, *Chromobacterium*, *Chryseobacterium*, *Chryseomonas*, *Citrobacter*, *Curtobacterium*,

*Enterobacter*, *Gordonia*, *Kocuria*, *Luteibacter*, *Mycolicibacterium*, *Nocardia*, *Paenibacillus*, *Paraburkholderia*, *Pasteurella*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, *Staphylococcus*, *Stenotrophomonas*, and *Vibrio* (Baon et al., 2012; Muleta et al., 2013; Teshome et al., 2017; Duong et al., 2021) and also for some fungi from the genera *Aspergillus*, *Chaetomium*, *Cladosporium*, *Cylindrocarpon*, *Fusarium*, *Humicola*, *Paecilomyces*, and *Penicillium* (Posada et al., 2013; Perea Rojas et al., 2019). Another capacity is the iron mobilization through the production of siderophores as displayed by the bacterial genera *Acinetobacter*, *Bacillus*, *Burkholderia*, *Caballeronia*, *Cellulomonas*, *Enterobacter*, *Escherichia*, *Luteibacter*, *Mycolicibacterium*, *Paraburkholderia*, *Lechevalieria*, *Mycobacterium*, *Pseudomonas*, *Nocardia*, *Paenibacillus*, and *Rhizobium* associated with roots, leaves and seeds of *C. arabica*, *C. canephora*, and *C. liberica* (Silva et al., 2012; Kejela et al., 2016; Duong et al., 2021). Finally, the production of phytohormones (e.g., auxins) or regulators (e.g., the ACC deaminase enzyme able to lower ethylene level) was established for some members of the genera *Bacillus*, *Brachyбактерium*, *Burkholderia*, *Erwinia*, *Escherichia*, *Kocuria*, *Luteibacter*, *Methylobacterium*, *Mycobacterium*, *Mycolicibacterium*, *Nocardia*, *Ochrobactrum*, *Paenibacillus*, *Paracoccus*, *Pseudomonas*, *Rhizobium*, *Serratia*, *Sinomonas*, and *Sphingobium* (Muleta et al., 2009; Baon et al., 2012; Silva et al., 2012; Kejela et al., 2016; Duong et al., 2021).

Even though highlighting some plant growth-promoting capacities *in vitro* represents a tool to select some potential beneficial microorganisms, the most important step to support the use of microorganisms in coffee production is the validation of the effects on the plants. Therefore, several *in planta* experiments under various controlled conditions (nursery, greenhouse, and phytotron) were performed to confirm the growth-promoting effect of the microorganisms. For example, Chattopadhyay et al. (2006) and Wedhastris et al. (2012) demonstrated the capacity of some N-fixing bacteria to promote the growth and the nutrient acquisition of *C. canephora* seedlings. Baon et al. (2012) and Cisneros-Rojas et al. (2017) also highlighted the increase in biomass of *C. arabica* and *C. canephora* seedlings after the inoculation of P-solubilizing bacteria. Furthermore, Medina et al. (2003) carried out some *C. arabica* seed inoculations with an N-fixing bacterial strain alone or in combination with some P-solubilizing bacteria and they were able to demonstrate a plant height increase of 33% with the co-inoculation. By testing numerous endophytic isolates (bacteria and fungi), Silva et al. (2012) showed that only 6 bacterial strains out of 234 isolates tested significantly promoted the growth of *C. arabica* seedlings while some isolates even had a deleterious effect. Trying to understand the mechanisms involved, the authors further characterized the most efficient strains and demonstrated their ability to solubilize phosphorus, as well as to produce auxins and siderophores. This result underlines the interest to perform some preliminary beneficial capacity screenings before undertaking further larger experiments with the coffee plants.

The majority of other *in planta* experiments in controlled condition were focused on AMF. Several authors were able to demonstrate the positive effect of these symbiotic fungi

on the growth of *C. arabica* seedlings and their nutrient acquisition including nitrogen, phosphorus, potassium, calcium, magnesium, and manganese (Caldeira et al., 1983; Vaast and Zasoski, 1992; Siqueira et al., 1995; Vaast et al., 1997b; Osorio et al., 2002). Other authors also emphasized the potential of the AMF co-inoculation with other plant growth-promoting microorganisms. Indeed, Pérez et al. (2002) observed an increased AMF colonization of *C. canephora* seedlings when combined with N-fixing bacteria. Moreover, González et al. (2004) underlined a consistent increase of the AMF effects on height and foliar area of *C. arabica* seedlings when N-fixing bacteria were applied simultaneously with the mycorrhizal fungi. Recently, Perea Rojas et al. (2019) confirmed the positive effect of AMF on *C. arabica* seedling growth and showed an improved efficiency by the addition of P-solubilizing fungi with a significant increase of the phosphorus content in the leaves.

The last step toward the development of sustainable alternatives to chemical fertilizers is the confirmation of the results obtained in controlled conditions *in situ*. Field experiments were exclusively conducted with AMF so far. Siqueira et al. (1993) were among the first authors to study the effect of mycorrhiza during 3 years in the field by inoculating different AMF strains (alone or in combination) on *C. arabica* seedlings under greenhouse conditions before to transfer the plants in the field. At the transplantation, the authors reported that most of the treatments were able to increase the plant biomasses and nutrient content (P and Cu) and after 6 months all the treatments increased the survival rate, stem diameter, and height of the plants. Finally, when superphosphate was applied some treatments also increased the yield with a mean increase of 74% with the most efficient one.

These beneficial effects of AMF were confirmed by several other studies. Indeed Colozzi-Filho et al. (1994) and Trejo et al. (2011) also observed an increase in *C. arabica* vegetative growth and nutrition (P, K, and Cu) under controlled conditions after the inoculation of different AMF isolates. These effects were maintained after the field transplantation along with an improvement of survival and first yield (up to 100% for some treatments). Siqueira et al. (1998) performed a 6-year field study during which they also confirmed the beneficial effect of AMF inoculation during the early development of coffee seedlings. However, after 26 months in the field the differences between the inoculated and control plants started to decrease and became insignificant during the following years. This finding was explained by the fact that the non-inoculated plants started to be colonized by indigenous mycorrhizal fungi after the transplantation. Therefore, the authors suggested that the AMF inoculation is really relevant only to increase the initial development of coffee seedlings and the first productions.

These findings also highlighted the need to further characterize the coffee-associated microorganisms especially under field conditions in order to develop some efficient microbiota-based alternatives to conventional fertilizers.

## Potential Uses as Biocontrol Agents

Microorganisms colonizing several coffee plant compartments have been examined for their potential use as biocontrol agents



including bacteria and fungi isolated from the rhizosphere (Mulaw et al., 2010; Kejela et al., 2016), the episphere (Haddad et al., 2014; Leong et al., 2014), and the endosphere (Silva et al., 2012; Hoang et al., 2020; Duong et al., 2021).

As for the plant growth-promoting agents, several *in vitro* screenings of potential biocontrol capacities were employed. For example, it was successfully demonstrated that numerous bacterial isolates (from the genera: *Acinetobacter*, *Aeromonas*, *Alcaligenes*, *Bacillus*, *Brachybacterium*, *Brevibacillus*, *Burkholderia*, *Cedecea*, *Caballeronia*, *Cellulomonas*, *Chromobacterium*, *Chryseobacterium*, *Chryseomonas*, *Curtobacterium*, *Enterobacter*, *Erwinia*, *Escherichia*, *Flavobacterium*, *Herbaspirillum*, *Kitasatospora*, *Lechevalieria*, *Leifsonia*, *Luteibacter*, *Microbacterium*, *Micrococcus*, *Mycobacterium*, *Mycolicibacterium*, *Nocardia*, *Ochrobactrum*, *Paenibacillus*, *Paraburkholderia*, *Pasteurella*, *Pectobacterium*, *Pseudomonas*, *Rhizobium*, *Salmonella*, *Serratia*, *Sinomonas*, *Streptomyces*, and *Vibrio*) were able to produce some enzymes including chitinases, gelatinases, lipases, and proteases (Muleta et al., 2009; Tiru et al., 2013; Kejela et al., 2016; Asyiah et al., 2018; Hoang et al., 2020; Duong et al., 2021) as well as some other active compounds like HCN and siderophores (Muleta et al., 2007; Silva et al., 2012; Tiru et al., 2013; Duong et al., 2021), already known to be involved in the biocontrol mechanisms (Compant et al., 2005; Saraf et al., 2014; Köhl et al., 2019).

Another approach that was extensively employed to highlight the biocontrol capacity of bacterial and fungal isolates involved the confrontation of the pathogen either directly with the antagonist (dual culture method) or with the compounds secreted in the culture medium (agar diffusion method). This strategy was successfully employed to demonstrate the ability of some biocontrol agents to inhibit the development of some of the major coffee diseases such as the CLR caused by the fungal pathogen *H. vastatrix* (Shiomi et al., 2006; Bettiol et al., 2007; Silva et al., 2008, 2012; Daivasikamani and Rajanaika, 2009; Haddad et al., 2013) or the coffee wilt disease (CWD) also known as tracheomycosis caused by the fungal pathogen *Gibberella xylarioides* (Muleta et al., 2007; Mulaw et al., 2010, 2013; Tiru et al., 2013). This methodology was also used to reveal the microorganisms biocontrol potential toward numerous other phytopathogens including *Alternaria alternata*, *A. solani*, *Ambrosiella macrospora*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *C. coffeicola*, *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, *Glomerella* sp., *Macrophomina phaseolina*, *Myrothecium roridum*, *Pestalotia longisetula*, *Phoma* sp., *Phytophthora capsici*, *P. meadii*, *Pythium aphanidermatum*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* (Nair et al., 2002; Mulaw et al., 2013; Bongiorno et al., 2016; Kejela et al., 2016; Monteiro et al., 2017; Ranjini and Raja, 2019; Hoang et al., 2020; Duong et al., 2021) but also some pests such as the coffee berry borer *Hypothenemus hampei* (Vega et al., 2008), the root knot nematode *Meloidogyne incognita* (Mekete et al., 2009; Hoang et al., 2020), the burrowing nematode *Radopholus duriophilus*, and the root lesion nematode *Pratylenchus coffeae* (Duong et al., 2021), as well as some toxigenic fungi including *Aspergillus carbonarius*, *A. flavus*, *A. niger*, *A. ochraceus*, and *A. westerdijkiae* (Masoud and

Kaltoft, 2006; Ramos et al., 2010; Djossou et al., 2011; Leong et al., 2014; De Melo Pereira et al., 2015a; De Almeida et al., 2019).

Several *in planta* experiments on *C. arabica* grown under controlled conditions (growth chamber and greenhouse) confirmed the capacity of several bacterial antagonists to decrease the spore germination, the disease severity, and the sporulation of *H. vastatrix* (Shiomi et al., 2006; Bettiol et al., 2007; Silva et al., 2008, 2012; Haddad et al., 2013, 2014; Culliao and Barcelo, 2015). Some authors underlined the fact that the treatment was more efficient when applied before the pathogen inoculation (Shiomi et al., 2006; Silva et al., 2012; Haddad et al., 2014), and other studies also demonstrated that the simultaneous applications of some bacterial isolates were as efficient as copper hydroxide in reducing CLR severity (Haddad et al., 2013).

In another greenhouse studies, Tiru et al. (2013) corroborated the *in vitro* results obtained with some *G. xylarioides* (CWD) antagonists. They showed that depending on the isolate and timing of application (before, after, or simultaneously), the inoculation of the biocontrol agents was able to significantly control the pathogen with a reduction of the disease severity comprised between 40 and 82.4%.

Nematode control capacity was also confirmed *in planta*. Indeed, Asyiah et al. (2018) demonstrated that a bacterial endophyte isolate was able to inhibit the penetration of the migratory endoparasitic nematode, *P. coffeae*, in the roots of *C. arabica* seedlings. Moreover, Vaast et al. (1997a) demonstrated that the inoculation of *C. arabica* seedlings with AMF 4 months before introducing *P. coffeae* significantly improved the tolerance to nematodes compared to the control without AMF. Finally, Mekete et al. (2009) confirmed the results obtained *in vitro* on tomato seedlings and showed that the inoculation of several bacterial endophytes significantly reduced the number of egg masses and galls caused by the root knot nematode *M. incognita*.

The only *in situ* experiments were conducted in order to test some microbial antagonists against *H. vastatrix* (CLR) under the field conditions. Vélez and Rosillo (1995) evaluated the efficiency of an isolate of the fungus *Verticillium lecanii* by spraying the antagonist 48 h before the inoculation of CLR spores. They noticed a delayed latent period (5 days), but the biocontrol agent failed to display a significant protecting effect although the number of lesions was reduced compared to the controls. More recently, Daivasikamani and Rajanaika (2009) tested some bacterial isolates as prevention treatment over a 2-year period and compared the results to those obtained with a copper-based (Bordeaux mixture) and a systemic fungicide (Triadimefon). By taking the average of the 2 years, the authors highlighted that the two best biocontrol agents were able to decrease the disease incidence by 36% with *B. subtilis* and 28% with *P. fluorescens*. However, the chemical fungicides were still more efficient with a mean disease incidence reduction of 44% with the Bordeaux mixture and 64% with Triadimefon. In another study, Haddad et al. (2009) assessed the efficiency of two bacterial isolates (*Bacillus* sp. and *Pseudomonas* sp.) compared to copper hydroxide in controlling CLR. Depending on the rate and time of application, the *Bacillus* isolate was as efficient as



the copper-based fungicide in reducing the disease incidence and severity.

Another interesting way to control CLR was explored with the use fungal hyper-parasites. Indeed, the pathogenicity of fungal mycoparasites toward *H. vastatrix* has already been demonstrated on infected leaves (Carrion and Ruiz-Belin, 1988; Gómez-De La Cruz et al., 2017). The identification of CLR fungal hyper-parasites is therefore of prime interest and has already been done with a standard microbiological procedure (Carrion and Rico-Gray, 2002) as well as by a comparative metabarcoding analysis of the fungal communities associated with healthy and diseased coffee leaves (James et al., 2016).

Finally, the AMF abundance, diversity, and root colonization in *C. arabica* infested or not with *H. vastatrix* in the field were compared. Some authors reported a higher mycorrhizal colonization and spore density as well as the prevalence of some AMF genera in healthy plants, therefore highlighting the potential implication of mycorrhizal fungi in CLR tolerance (Monroy et al., 2019).

Together, these results emphasized the potential use of microbes as biocontrol agents that might be as efficient as the chemical pesticides and fungicides in controlling some of the major coffee pests and diseases.

### Potential to Improve the Quality and the By-product Management

The quality of the coffee is not only influenced by several parameters during the production such as the plant genotype, environmental conditions, cultivation techniques and the associated microbiota (Toledo et al., 2016; Martins et al., 2020), but also by the type of postharvest processing (Gonzalez-Rios et al., 2007b; Lee et al., 2015; Poltronieri and Rossi, 2016), the storage conditions (Bucheli et al., 1998; Urbano et al., 2001; Geremew et al., 2016), and the roasting and brewing methods (Gonzalez-Rios et al., 2007a; Frost et al., 2020; Hu et al., 2020).

The involvement and potential use of microorganisms to improve the coffee quality is being increasingly studied. For example, the coffee undergoing the wet processing is often associated with a higher cup quality and it is now established that several groups of microorganisms especially the acetic acid bacteria (AAB), the lactic acid bacteria (LAB), and the yeasts are involved in the improvement of the quality by producing several metabolites, organic acids, and volatile compounds (De Bruyn et al., 2017; De Oliveira Junqueira et al., 2019; Zhang et al., 2019a,b; Elhalis et al., 2020b). In a recent study, Elhalis et al. (2020a) inhibited the yeast growth during the fermentation and compared the bean composition as well as the quality and sensory characteristics of coffee fermented with or without yeasts. Using this strategy, they were able to clearly demonstrate the implication of yeasts in the quality improvement of the wet-processed coffee. Finally, several authors also demonstrated the improvement of the quality after the inoculation of selected yeasts and lactic acid bacteria strains on coffee undergoing not only the wet (De Melo Pereira et al., 2015b, 2016; Da Silva Vale et al., 2019; Bressani et al., 2020) but also the semidry processing (Martinez et al., 2017).

Another explored aspect where microorganisms could have an impact on coffee quality is the potential control of toxigenic

fungi. Indeed, it has been demonstrated that some yeasts and lactic acid bacteria are able to control the development and toxin production by toxigenic fungi during artificially contaminated coffee fermentation (Massawe and Lifa, 2010; De Melo Pereira et al., 2015a).

It is also important to mention that coffee processing generates several by-products including the fruit skin, pulp, parchment, silverskin, and used coffee grounds (Iriondo-DeHond et al., 2020). Several strategies have been explored to valorize these wastes by using them among others as animal feed, fertilizer, substrate for biogas/biodiesel production, compost for plants, and edible fungi and for earthworm production (Perraud-Gaime et al., 2000; Kondamudi et al., 2008; Ronga et al., 2016). However, these strategies are difficult to implement because of caffeine, tannin, and polyphenol contents that make the wastes toxic. For these reasons, microorganisms have also been studied for their potential utilization in detoxifying the coffee wastes and were therefore screened for their capacity to degrade the caffeine and tannins (Aquiahuatl et al., 1988; Roussos et al., 1995; Brand et al., 2000; Mazzafera, 2002; Nayak et al., 2012). Finally, microorganisms have also been explored for some industrial applications such as the production of enzymes with by-products such as amylases (Murthy et al., 2009), pectinases (Antier et al., 1993; Boccas et al., 1994; Sakiyama et al., 2001; Serrat et al., 2002; Masoud and Jespersen, 2006), proteases (Rodarte et al., 2011), and xylanases (Murthy and Naidu, 2012).

Consequently, the application of microbes in the coffee industry is of prime interest, not only to improve the quality of the final product but also to achieve a better management of the wastes and potentially add some value to by-products.

## CONCLUSION AND FUTURE TRENDS

The coffee microbiota has been extensively studied and the potential applications of the microorganisms to improve the coffee plant fitness, either by directly promoting its growth or by acting as pathogen antagonists are now well-documented. Despite that a substantial work has been done *in vitro* and *in planta* under a controlled environment, there is a lack of assessment of the real potential of these microorganisms *in situ* under field conditions. This should be included as a research priority despite the difficulty of implementing this kind of experimental approach for a perennial crop such as coffee, which requires the monitoring of the plants for several years. Consumers, producers, industries, and governments are more and more concerned about the environmental and health issues associated with intensive agriculture and chemical inputs. Thus, microorganisms represent a promising alternative to improve the sustainability not only of the coffee production but also of the wastes' management as well as the quality of the final product. Despite the fact that metabarcoding studies now provide a more global understanding of the microbial communities associated with coffee, there are still some limitations regarding the taxonomic resolution. However, this issue should be resolved in the coming years with the improvement of both the sequencing technologies and the bioinformatics treatments. Nevertheless, this strategy remains complementary of the culture-dependent applied research that could be improved by some culturomics

approaches, for example. The present review provides an extensive description of the diversity of microorganisms at both farming and processing levels and an overview of their potential uses. The present paper also highlights the need of further researches in this area.

## AUTHOR CONTRIBUTIONS

BD: writing—original draft. PM, J-LM, PV, ML, and RD: writing—review & editing. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.607935/full#supplementary-material>

**Supplementary Table 1** | Coffee microbiota full description.

**Supplementary Table 2** | Arbuscular mycorrhizal fungi in association with coffee.

**Supplementary Table 3** | Toxigenic fungi producing Ochratoxin A (OTA), Ochratoxin B (OTB), and Aflatoxin B (AFB) in association with coffee.

**Supplementary Table 4** | Acetic acid bacteria (AAB) and lactic acid bacteria (LAB) in association with coffee.

**Supplementary Table 5** | Venn diagrams and partitions of the archaea, bacteria, and fungi shared between endophytic, epiphytic, and rhizospheric compartments of the indigenous coffee microbiota.

**Supplementary Table 6** | Venn diagrams and partitions of the archaea, bacteria, and fungi shared between at least one compartment of the indigenous coffee microbiota and the postharvest coffee microbiota.

**Supplementary Table 7** | Venn diagrams and partitions of the archaea, bacteria, and fungi shared between continents.

**Supplementary Table 8** | Venn diagrams and partitions of the bacteria and fungi shared between all compartments of the indigenous coffee microbiota across all continents.

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# Production of Microsclerotia From Entomopathogenic Fungi and Use in Maize Seed Coating as Delivery for Biocontrol Against *Fusarium graminearum*

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The commercial use of the entomopathogenic fungi *Metarhizium* spp. in biopesticides has gained more interest since the discovery that several species of this genus are able to colonize roots. In general, commercial products with *Metarhizium* are formulated based on conidia for insect pest control. The process of mass production, harvesting, and formulation of infective conidia can be detrimental to conidial viability. Entomopathogenic fungi such as *Metarhizium* spp. are able to produce high concentrations of resistant structures, known as microsclerotia, when grown in liquid media. Microsclerotia are desiccation tolerant, with excellent storage stability, and are capable of producing high quantities of infective conidia after rehydration. The aim of this study was to evaluate microsclerotia production by different isolates of *Metarhizium* spp. and determine the effect of microsclerotia coated onto maize seeds on plant growth in the presence of soil-borne pathogen *Fusarium graminearum*. On average,  $\sim 1 \times 10^5$  microsclerotia/mL were produced by selected isolates of *M. anisopliae* (A1080 and F672) and *Metarhizium robertsii* (F447). Microsclerotia were formulated as granules with diatomaceous earth and used for seed coating, after which propagules produced around  $5 \times 10^6$  CFU/g of seeds. In the presence of the plant pathogen, maize plants grown from untreated seeds had the lowest growth, while plants treated with the *Metarhizium* microsclerotia had significantly greater growth than the control plants. Hyphae were observed growing on and in root tissues in all the *Metarhizium* spp. treatments but not in samples from control plants. *Metarhizium* hyphal penetration points on roots were observed 1 month after sowing, indicating the fungi were colonizing roots as endophytes. The results obtained indicate that microsclerotia can be coated onto seeds, providing plant protection against soil plant pathogens and a method to establish *Metarhizium* in the ecto- and endo-rhizosphere of maize roots, allowing the persistence of this biocontrol agent.

**Keywords:** plant growth promotion, microsclerotia, plant protection, *Metarhizium* spp, *Fusarium graminearum*, seed coating, endophytic entomopathogenic fungi, biocontrol

## INTRODUCTION

The commercial use of entomopathogenic fungi such as *Metarhizium* (Metschnikoff) Sorokin, 1883 (*Hypocreales: Clavicipitaceae*) or *Beauveria* (Balsamo) Vuillemin, 1912 (*Hypocreales: Clavicipitaceae*) as “mycoinsecticides” or “biopesticides” is generally practiced using the inundative biocontrol approach, where the environment harboring the insect pest is treated with high concentrations of infective fungal propagules (Eilenberg et al., 2001; Jackson et al., 2010). Generally, fungal antagonists of plant pathogens or fungal biopesticides for biocontrol purposes are produced using solid substrate fermentations on grains which are subsequently formulated as powders or liquids for topical applications or as granules for soil applications (Jackson et al., 2010; Tripathi et al., 2010; Jaronski, 2014). However, the different steps involved in mass production of infective conidia as well as during harvesting and formulation can be detrimental for conidia viability and survival. Additionally, proper application with standard farming equipment and homogenous distribution through the soil to target phytopathogens or soil dwelling insect pests, can be difficult to achieve (Jaronski and Jackson, 2008). Granular formulations are a practical alternative to agrochemicals for the application of fungal antagonists for plant protection targeting soil plant pathogens and soil-dwelling insect pests (Burgess, 1998; Jaronski and Jackson, 2008).

The development of mycoinsecticides as conidiogenic granules is preferred since fresh conidia are produced *in situ* where the target plant pathogens or insect pests dwell (Jackson and Jaronski, 2012). Using this propagule alternative, conidia avoid all the necessary steps from mass-production up to application, which can damage these fungal reproductive structures. When incorporated into soil, biocontrol agents formulated as conidiogenic granules have some additional advantages such as protection from the harmful effects of UV radiation or from unfavorable conditions such as high temperatures and low humidity to which microorganisms can be exposed after foliar applications. Another benefit is that in soil, moisture content and temperatures are generally within the optimal range for fungal survival and growth (Jackson and Jaronski, 2009).

Over the past decade, it has been discovered that entomopathogenic fungi belonging to *M. anisopliae*, *M. brunneum*, *Beauveria bassiana*, *B. brongniartii*, and *B. pseudobassiana* are able to produce high concentrations of microsclerotia (MS) when grown in liquid media (Jackson and Jaronski, 2009, 2012; Vega et al., 2009; Wang et al., 2011; Villamizar et al., 2018). These environmentally resistant structures are desiccation tolerant, have excellent storage stability, and are capable of producing high quantities of infective conidia suitable for management insect pests or as antagonists of phytopathogens (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Vega et al., 2009; Song, 2018). Microsclerotia from *M. anisopliae* can be produced by culturing in liquid substrate fermentation for ~3 to 4 days in continuous agitation, while further fermentation up to 8 days allows the complete MS melanization. Subsequently, obtained MS are

harvested and formulated in diatomaceous earth (DE) and air-dried to produce MS-DE granules (Jackson and Jaronski, 2009). MS-DE granules can survive a moderate drying process without significant viability losses and can be safely stored at 4° for up to 12 months. In appropriate conditions, MS germinate (hyphal development) and produce high quantities of conidia, in general above  $1 \times 10^8$  conidia/g dried MS-DE (Jackson and Jaronski, 2009, 2012). As for conidiogenic granules, the soil environment is suitable for reactivation of MS, since humid conditions are favorable for the rehydration and germination process of these fungal resting structures. Consequently, MS obtained from entomopathogenic fungi have been recognized as a viable alternative for use in biocontrol programmes (Jaronski and Jackson, 2008).

Another interesting feature of some entomopathogenic fungi like *Metarhizium* and *Beauveria* is their natural ability to associate endophytically with plant roots and to colonize the rhizosphere (Hu and St. Leger, 2002; Vega et al., 2009; Rivas-Franco et al., 2019). This property not only allows entomopathogenic fungi to persist in the root system, but also fulfill additional ecological roles such as antagonism of plant pathogens, plant growth promotion, biofertilization, and promotion of the plant induced resistance (Goettel et al., 2008; Vega et al., 2009; Sasan and Bidochka, 2012; Rivas-Franco et al., 2020). In order to make use of these advantages, an alternative to MS formulated as granules (0.6–1.2 mm) and incorporated into soils at planting is to formulate the MS directly as a seed coating. Therefore, after sowing, MS coated onto seeds reactivated by soil humidity produce hyphae in a process known as MS germination. When nutrients in microsclerotia are scarce, hyphae develop phialides which, in turn, produce fresh conidia. These conidia are released in proximity to the nutrient-rich environment of the rhizosphere, where conidia soon germinate, and produce hyphae. Finally, either hyphae from microsclerotia or from conidia will interact and associate with the growing roots. Entomopathogenic fungal colonization of the rhizosphere allows fungal persistence, exerting biocontrol activity against the pests or plant pathogens threatening plant roots (Rivas-Franco et al., 2019).

Previous studies with seed treatments with different species of *Metarhizium*, resulted in 50% of larvae of *Anomala cincta* (Coleoptera: Rutelidae) or up to 60% of *Costelytra giveni* larvae (Coleoptera: Scarabaeidae) infected with the entomopathogenic fungi (Peña-Peña et al., 2015; Rivas-Franco et al., 2019). However, the biocontrol ability of *Metarhizium* is not limited only to insects but also against plant pathogens like *Fusarium*. Several studies have shown that *Metarhizium* spp. can reduce *Fusarium* root rot symptoms after seeds were coated with conidia obtained from this biocontrol agents (Sasan and Bidochka, 2013; Rivas-Franco et al., 2019). Additionally, maize roots provide support for *Metarhizium* rhizosphere colonization as well as increasing fungal persistence in the root system through endophytic growth (Keyser et al., 2014; Peña-Peña et al., 2015; Rivas-Franco et al., 2020). The ability of *Metarhizium* to colonize the rhizosphere and plant roots endophytically supports the bodyguard hypothesis (Elliot et al., 2000) where the host plant plays an important role driving this interaction in the presence of insect pests or phytopathogens threatening roots (Rivas-Franco et al., 2020).

Previously, we found that several species of *Metarhizium* coated on maize seeds showed a significant decrease in their ability to colonize the rhizosphere in the presence of larvae of *C. givens*, while endophytic colonization by *Metarhizium* increased in the presence of the pathogen *Fusarium graminearum* (Rivas-Franco et al., 2020). In those studies, root colonization by *Metarhizium* spp. altered maize physiology modifying the content of the phytohormones salicylic acid and jasmonic acid, possibly leading to induction of the systemic response in maize (Rivas-Franco et al., 2020).

Numerous species of the genus *Fusarium* are well-known plant pathogens affecting yield, nutritive value and hygienic quality of agricultural crops in most countries from Africa, Asia, Europe, North America, Oceania, and South America (van der Lee et al., 2015; Oldenburg et al., 2017; Kazan and Gardiner, 2018). In particular, the *Fusarium graminearum* Schwabe species complex cause *Fusarium* head blight in several plant cereals leading to yield losses above 50% in some cases and compromising grain quality (van der Lee et al., 2015; Garmendia et al., 2018). Infections of maize with *Fusarium* spp. can cause other serious plant diseases such as seedling blight, seed rot, root and stem rot, ear and kernel rot, and rudimentary ear rot (Oldenburg et al., 2017; Zhou et al., 2018). *Fusarium graminearum* also produces a class of mycotoxins known as trichothecenes (deoxynivalenol, nivalenol, and their derivatives) and zearalenone, with acute toxicity for animals and humans (van der Lee et al., 2015; Kazan and Gardiner, 2018; Zhou et al., 2018). Maize plants are susceptible to *Fusarium* spp. infections throughout the cultivation period and mycotoxins can be accumulated in the affected tissues and grains, which represents a risk for human and animal health (Oldenburg et al., 2017; Kazan and Gardiner, 2018). Management of *F. graminearum* can be difficult because standard seed fungicide treatments are not always sufficient, while aerial applications are costly. Additional difficulties are associated with correct application timing, environmental risk and the development of *Fusarium* resistance to fungicides (Kazan and Gardiner, 2018; Zhou et al., 2018).

Seed coating with conidia from entomopathogenic fungi has proved to be a strong tool for the delivery of these biocontrol agents, promoting fungal persistence in the system after entomopathogenic fungal colonization of the rhizosphere or even roots endophytically (Rivas-Franco et al., 2019). The aim of this study was to evaluate MS production by different isolates of entomopathogenic fungi and determine the effects of MS coated onto maize seeds on plant growth performance in the presence of *F. graminearum*. The ability of the entomopathogenic fungal isolates, coated on seeds as MS, to associate with roots was determined through fluorescent and laser confocal microscopy.

## MATERIALS AND METHODS

### Plants

Maize seeds (*Zea mays*) of the hybrid 34H31 (US patent 6,897,360 B1) were used. This hybrid is characterized by a stable yield performance in different environments, early flowering and good drought tolerance.

## Fungal Isolates

The isolates selected for this study (Table 1) are all held in the fungal collections of the Bio-Protection Research Center and AgResearch (both at Lincoln, New Zealand) and the Agricultural Research Service Collection of Entomopathogenic Fungal Cultures (ARSEF-USDA). The plant pathogen *F. graminearum* 13083 was obtained from a maize root rot sample and held in the Manaaki Whenua Landcare Research culture collection (Auckland, New Zealand). For liquid culture fermentation studies, monosporic cultures of each fungal isolate were used. Monosporic cultures were obtained by transferring a sole conidium from each fungal isolate to a Petri plate containing potato dextrose agar (PDA, Difco) and incubating at  $25 \pm 2^\circ\text{C}$  in light:dark conditions (12:12). After 3 weeks, a conidial suspension was prepared for each fungal isolate from the monosporic fungal colony grown on PDA. The resulting monosporic conidial suspension was aliquoted in tubes with 10% glycerol and stored at  $-80^\circ\text{C}$  (stock cultures) until use. Conidial inoculants for liquid culture experiments were obtained by inoculating PDA Petri plates with 100  $\mu\text{L}$  from fungal stock cultures. Petri plates were then incubated at  $25 \pm 2^\circ\text{C}$  in light:dark conditions (12:12) for 2 weeks until complete sporulation of colonies. Subsequently, a 250 mL flask containing 90 mL of liquid medium was inoculated with 10 mL of a conidial suspension of  $5 \times 10^6$  conidia/mL. All conidial suspensions were obtained by rinsing the fungal colony grown on a PDA plate with 3–5 mL of 0.01% Triton X-100 and transferring the resulting conidial suspension to a 15 mL Falcon tube. Conidial suspensions were quantified using a haemocytometer (Neubauer Improved).

## Fungal Growth and Microsclerotia Production in Liquid Substrate Fermentation

### Media Composition and Fermentation Conditions

Media composition and fermentation conditions were as described by Jackson and Jaronski (2009, 2012) with slight modifications in carbon and nitrogen content. The liquid media used to produce hyphal inoculum and MS from the fungal isolates were composed of a basal salts solution with trace metals and vitamins (Jackson and Jaronski, 2009). The pre-culture medium [C:N ratio 33:1; (C) = 40 g/L] used for producing the hyphal inoculum of fungal isolates contained the basal medium supplemented with 80 g/L glucose and 15 g/L acid hydrolysed casein (Casamino acids, Difco). Production medium for MS contained the same composition but with 25 g/L of hydrolysed casein [C:N ratio 23:1, (C) = 45 g/L]. Carbon concentration and C:N ratio calculations were based on 40% carbon in glucose and 53% carbon and 8% nitrogen in acid hydrolysed casein (Jackson and Jaronski, 2009). Glucose solutions (20% w/v) were autoclaved separately and added to the basal salts solution with trace metals and vitamins prior to inoculation. Pre-cultures were obtained by inoculating 90 mL pre-culture medium in baffled Erlenmeyer flasks (250 mL) with 10 mL conidial suspension of  $5 \times 10^6$  conidia/mL. Production cultures were obtained by inoculating 90 mL of production medium in baffled Erlenmeyer flasks (250 mL) with 10 mL of 4 day-old pre-culture broth.



**TABLE 1** | Voucher information for the specimens used in this study.

Isolate	Species	Origin	Source	Location	Collection <sup>a</sup>
A1080	<i>Metarhizium anisopliae</i>	Insect larvae	<i>Trichoplusia ni</i> (Lep: Noctuidae)	Florida, USA	ARSEF
Bb21	<i>Beauveria bassiana</i>	Endophyte from leaf husk	<i>Zea mays</i> (Poa: Poaceae)	Ashburton, Canterbury, NZ	BPRC
F16	<i>Metarhizium guizhouense</i>	Insect cadaver	<i>Costelytra giveni</i> (Col: Scarabaeidae)	Nelson, Marlborough, NZ	AgR
F327	<i>Trichoderma harzianum</i>	Endophyte from roots	<i>Mentha</i> spp. (Lam: Nepetoideae)	Waitakaruru, Waikato, NZ	BPRC
F447	<i>Metarhizium robertsii</i>	Endophyte from roots	<i>Actinidia deliciosa</i> (Eri: Actinidiaceae)	Avondale, Auckland, NZ	BPRC
F672	<i>Metarhizium anisopliae</i>	Endophyte from root	<i>Pinus radiata</i> (Pin: Pinaceae)	Taupo, Waikato, NZ	AgR
F99	<i>Metarhizium novozealandicum</i>	Insect cadaver from farm soil	<i>Costelytra giveni</i> (Col: Scarabaeidae)	Methven, Canterbury, NZ	AgR
BK41	<i>Metarhizium guizhouense</i>	Endophyte from leaves	<i>Actinidia deliciosa</i> (Eri: Actinidiaceae)	Nelson, Marlborough, NZ	BPRC
13083	<i>Fusarium graminearum</i>	Root rot	<i>Zea mays</i> (Poa: Poaceae)	Pukekohe, Auckland, NZ	ICMP

<sup>a</sup>ARSEF, USDA-ARS Collection of Entomopathogenic Fungal Cultures, Ithaca, NY; BPRC, Bio-Protection Research Center Fungal Collection, Lincoln, Canterbury; AgR, AgResearch Fungal Collection, Lincoln, Canterbury; ICMP, International Collection of Microorganisms from Plants, Manaaki Whenua Landcare Research, Lincoln Canterbury.

Production cultures were grown for 8 days at 28°C in an orbital shaker incubator (Cocono TU 4540) at 300 RPM. Total fermentation time for pre-cultures plus production cultures was 12 days. All pre-cultures and production cultures were inoculated in duplicate. All experiments were carried out at least three times.

### Quantification Methods and Sample Processing During Liquid Fermentations

Samples for the determination of biomass, blastospore and MS were taken after inoculation for pre-cultures (days 3 and 4) and for production cultures (days 3, 4, 6, 7, and 8). At each sampling day, 5 mL replicates of culture broth were taken from each isolate and processed for quantification of biomass, blastospore, and MS content determination. Biomass was determined as dry weight after incubating 1 mL of culture broth in a 1.5 mL Eppendorf tube for 96 h at 65°C. Before drying biomass, the medium culture was removed by two consecutive washes with 1 mL of 0.01% Triton X-100 solution. Blastospore concentration was determined by transferring 1 mL of culture broth to a tube containing 9 mL of distilled water and quantifying microscopically using a haemocytometer (Neubauer Improved). MS production was determined using 70 µL of sample placed onto a glass slide and covered with a large (50 × 24 mm) coverslip. All MS on the slide were counted. Only discrete hyphal aggregates larger than 50 µm in diameter and melanized were counted as MS (Jaronski and Jackson, 2012). All quantifications were done in duplicate. Culture broth was diluted as appropriate for quantifications and all broth suspensions were vortexed to ensure homogeneity before quantification. Microscopic analysis was done using a Leica DM 2500 microscope and images were taken with the Celsens Standard software (Olympus). Quantifications (biomass, blastospores, and MS) from each sampling day and fungal isolate were averaged. Data were analyzed by a two-way analysis of variance (ANOVA).

### Microsclerotia Production for Characterization and Seed Coating

Microsclerotia for maize seed coating were produced following the procedure indicated above using the isolates of *M. anisopliae* A1080 and F672 and *M. robertsii* F447. These isolates exhibited

highest MS production among remaining *Metarhizium* isolates and were selected for the following studies. The pre-cultures for biomass and blastospore production were evaluated only on day 4 after inoculation, and production cultures were evaluated for biomass and MS production on harvest at day 6 after inoculation. MS production cultures were completed using two replicates per isolate. Experiments were repeated three times. Biomass and MS quantification from each fungal isolate at sampling day were averaged for data analysis.

### Microsclerotia Harvest and Drying Process

After six fermentation days, total fungal biomass volume including MS was determined and 5% (W/V) of diatomaceous earth (DE, Hyflo Sigma-Aldrich) was added. The combined MS-DE were mixed, and vacuum filtered with a Büchner funnel using Whatman N°54 filter paper. The resulting filtered MS-DE solid block was broken up with a coffee blender by applying short pulses until small crumbs were obtained. The resulting crumbs were layered on glass Petri plates of 15 cm diameter and air-dried overnight in the air flow within a laminar-flow. The moisture content of the MS-DE granules was determined with an activity meter (Aqualab Lite V4). The granules were dried until the water content was between 3–5 % and then sealed in plastic bags and stored at 4°C until further processing. Moisture content ( $a_w$ ) determination from the MS of each fungal isolate was completed in duplicates. Each experiment was repeated three times. Data were analyzed by a two-way ANOVA.

### Viability and Conidiogenesis of MS-DE Granules

Microsclerotial viability and conidial production from MS were determined by sprinkling 25 mg of the air-dried MS-DE granules onto the surface of a water agar plate (Jackson and Jaronski, 2009). Two water agar plates were used for each of the MS-DE granules obtained from the corresponding *Metarhizium* isolate. After 24 hrs incubation at 28°C in light:dark conditions (12:12 h), a total of 100 MS-DE granules on each plate were examined with a stereo microscope (Olympus SZX 12) to determinate hyphal emergence (MS germination) as an indicator of viability. Conidial production was determined after further incubation of MS-DE granules on water agar plates in the same conditions for 8 days. Then, MS-DE water agar plates were

flooded with 7 mL of a sterile 0.01% Triton X-100 and agitated at 60 RPM in a rotary shaker platform for 30 min at room temperature. After agitation, granules were dislodged from agar with the help of a hockey stick cell spreader and the resulting suspension containing granules and conidia was transferred to a 15 mL Falcon tube, and the recovered volume was recorded. The concentration of conidia was determined microscopically using a haemocytometer (Neubauer Improved) and total conidia per gram of MS was calculated from duplicate samples. Experiments were repeated three times. Data were analyzed by a two-way ANOVA.

### Maize Seed Coating With Microsclerotia

After MS characterization, isolates *M. anisopliae* F672 and *M. robertsii* F447 were selected for maize seed coating based on their high MS production. Seed coating was prepared as described in Rivas-Franco et al. (2019) with slight modifications as follows. The conidial suspension in the polymer gel was substituted by an amount of MS-DE estimated to provide, after MS germination and conidiation, a final conidial concentration of  $\sim 1 \times 10^8$  conidia/mL per gram of seeds. The corresponding quantity of MS-DE was added to the polymer gel, mixed and then used for coating maize seeds with the same proportions of polymer with MS-DE, bentonite and talc as previously described (Rivas-Franco et al., 2019). Control seeds (CS) were coated with the polymer and the other corresponding ingredients without the microsclerotia. Germination of maize seeds with a MS-DE coating was determined using the between-paper method as described in Rivas-Franco et al. (2019). In this evaluation test four replicates, each consisting of 50 maize seeds with MS-DE coating or CS, were used. Experiments were repeated twice. Statistical design was a randomized block design with two blocks of the MS fungal seed coatings treatments. Data were analyzed with a one-way ANOVA.

### MS-DE Coated Maize Seeds Propagule Viability

After maize seeds were coated with *Metarhizium* MS-DE granules, 10 g of coated seeds were placed in a Falcon centrifuge tube (50 mL) containing 30 mL of a solution of Triton x-100 (0.01%). The resulting mix composed of seeds and solution was agitated vigorously and incubated at 4°C overnight (mother suspension). After incubation, the mix was agitated vigorously and serial dilutions from mother suspension were prepared. From each dilution 0.1 mL was spread onto Petri plates containing half strength acidified PDA. To prepare half strength acidified PDA, two drops of Lactic acid were added to the sterilized medium before pouring in the Petri plates. This simple strategy of medium acidification reduces bacterial contamination with no effects on fungal development. Inoculated plates were incubated at 20°C with 12:12 h light:dark conditions. After 7 days, fungal colonies originated from MS were quantified as colony forming units (CFU). Data were analyzed by a one-way ANOVA.

## Evaluation of Maize Plant Performance in the Presence of *Fusarium graminearum* After Seeds Were Coated With Microsclerotia of *Metarhizium* spp

Maize seeds coated with MS from *M. anisopliae* F672 and *M. robertsii* F447 were sown in 1 L pots containing 920 g of potting-mix with 0.5% (w/w) of *F. graminearum* 13083. Control seeds without MS treatment were grown in the absence (CS) or the presence of *F. graminearum* (CSfg). Plants were allowed to grow in the Biotron (controlled environment facility, Lincoln University), at  $24.3 \pm 2.5^\circ\text{C}$ , (referred as  $25^\circ\text{C}$ ) in light:dark conditions (16:8 h),  $65 \pm 10$  RH% and watered every fourth day with 300 mL of tap water. Three weeks after sowing, plants were harvested and dry weight of shoots and roots determined (Rivas-Franco et al., 2019). Each maize plant trial consisted of two randomized blocks with five pots in each block with the corresponding MS seed coating treatments (*M. anisopliae* F672 and *M. robertsii* F447) and CS. All pots contained the fungal pathogen *F. graminearum* 13083 incorporated in the soil potting mix. Additionally, there were two randomized blocks, also with five pots in each block, with the corresponding CS without the fungal pathogen in the potting mix. Experiments were repeated four times. Data were analyzed by a two-way ANOVA.

## Fusarium Graminearum 13083 Biomass Production for Maize Trials With MS-DE Coated Seeds

*Fusarium graminearum* isolate 13083 was maintained in slant tubes on PDA at 4°C and grown on PDA plates at 25°C with 12:12 h light:dark conditions for experiments. For maize trials, *F. graminearum* 13083 biomass was obtained as follows. From the edge of a *Fusarium* colony grew in PDA for 14 days, 10 plugs of  $\sim 0.5$  cm diameter were taken and transferred to 1 L Erlenmeyer flasks containing 500 mL of malt extract broth (MEB). Flask were incubated in an orbital shaker at 180 rpm and  $25 \pm 2^\circ\text{C}$ . After 7 days, biomass was harvested by centrifugation at 400 rpm for 5 min at 4°C. Following this procedure, up to  $69.7 \pm 3.4$  g/L of *F. graminearum* 13083 biomass containing  $\sim 1.3 \pm 0.5$  ( $\times 10^8$ ) blastospores/mL was obtained. Then, the *F. graminearum* 13083 biomass was incorporated with the potting mix at 0.5% w/w and mixed by hand to homogenize. Immediately, the mix was used to fill pots and then CS and MS-DE coated maize seeds were sown. Filled pots were watered and maize plants let to grow for 3 weeks at 25°C before plant performance evaluation.

## Determination of Fungal Endophytism by Confocal Fluorescent Microscopy After Maize Seed Coating With *Metarhizium* spp MS-DE Granules

Maize seeds were coated with MS from *M. anisopliae* F672 and A1080, and *M. robertsii* F447 as described above, while for CS the coating consisted of polymer gel without MS. After coating, maize seeds were sown in 1 L pots containing 600 g of vermiculite (fine grade 2). Pots were watered with 400 mL of tap water, and transferred to a growth chamber at 25°C with light:dark

conditions (12:12 h). Plants were watered every fifth day with 400 mL of distilled water. After 1 month, maize plants were gently pulled out, and maize roots, stems and leaf sheath were cleared and fluorescent dyed as described by Rivas-Franco et al. (2019). Maize samples of roots, stems and leaves from seeds coated with MS, and control plants were analyzed for the presence of fungal structures on the surface or internally as endophytes using confocal microscopy (LSM 510 META–Zeiss, Germany). Confocal fluorescence images were recorded on a multichannel confocal microscope (LSM 510 META–Zeiss, Germany) using the program ZEN 2009.

## Statistical Analysis

For each individual trial data were analyzed by the ANOVA test that was appropriate for the experimental design. For treatment factors with several levels (e.g., isolates, trial, day), an unrestricted LSD procedure was used to compare means (Saville, 2015). To combine data over several identical trials, an ANOVA test was used, as above, followed by an unrestricted LSD procedure, with data being treatment means from individual trials, and with “trial” being specified as a blocking factor.

## RESULTS

### Fungal Growth and Microsclerotia Production in Liquid Substrate Fermentation

#### Biomass Production

All the fungal isolates had produced their highest biomass by day 3 ( $89.7 \pm 16.5$  mg/mL) and 4 ( $87.1 \pm 15.1$  mg/mL) after inoculation followed by a subsequent steady weight decrease ( $F_{28/78} = 5.1$ ;  $p < 0.01$ ; **Figure 1A**). By day 7, *Trichoderma harzianum* F327 and *B. bassiana* Bb21 had the lowest biomass among the isolates, 38.4 and 42.2 mg/mL, respectively, while *M. anisopliae* F672 and *M. novozelandicum* F99 had the highest 68.4 and 71.7 mg/mL also, respectively ( $LSD_{5\%} = 0.075$ ; **Figure 1A**). At the end of the fermentation process on day 8, only *T. harzianum* F327 and *B. bassiana* Bb21 showed a significant increase in biomass compared to their biomass on day 7 ( $LSD_{5\%} = 0.075$ ; **Figure 1A**).

#### Blastospore Production

Contrary to the development of fungal biomass, blastospore production of each isolate increased over the fermentation period but with almost no significant variation during consecutive sampling days ( $F_{28/78} = 1.2$ ;  $p = 0.288$ ; **Figure 1B**). Only *T. harzianum* F327 and *M. robertsii* F447 had a significant increase in the number of blastospores per mL by day 6 of the experiment,  $1.4 \times 10^7$  and  $1.9 \times 10^6$  blastospores/mL, respectively ( $LSD_{5\%} = 0.557$ ). In contrast, the remaining isolates had the highest number of blastospores per mL by day 7. The highest production of blastospores was by *B. bassiana* Bb21 and *M. guizhouense* Bk41, both with  $9.1 \times 10^8$  blastospores/mL, while the lowest was found in *T. harzianum* F327 with  $9.6 \times 10^6$  blastospores/mL ( $LSD_{5\%} = 0.557$ ; **Figure 1B**).

## Microsclerotia Production

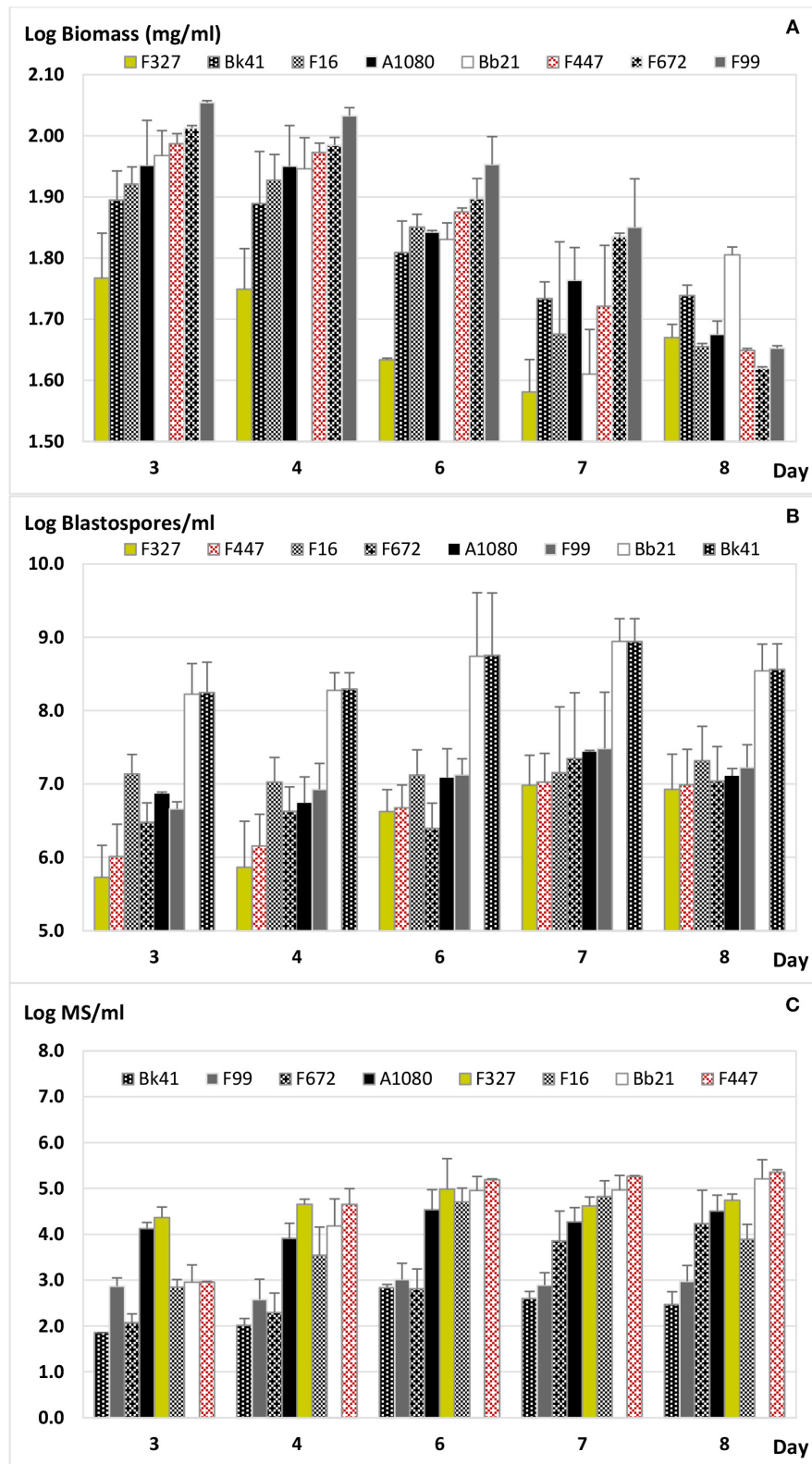
As well as the trend observed with blastospore production, the amount of MS increased with fermentation time for most of the isolates. The highest concentration,  $7.3 \pm 7.9 (\times 10^4)$  MS/mL, was found around the sixth day after inoculation and the level remained stable until the last sampling day ( $F_{28/78} = 7.0$ ;  $p < 0.01$ ). The exception was *M. novozelandicum* F99 which showed little variation in MS content over the 8 days of fermentation ( $LSD_{5\%} = 0.498$ ; **Figure 1C**). This isolate, together with *M. guizhouense* Bk41, produced, on average, the lowest number of MS,  $9.9 \pm 4.3 (\times 10^2)$  MS/mL (**Figure 1C**). At the end of the fermentation process, most MS were melanised heterogenous round structures of differing sizes (**Figure 2**). The highest MS production was determined in *M. robertsii* F447 at day 8 with  $2.3 \times 10^5$  MS/mL followed closely by *B. bassiana* Bb21 with  $1.6 \times 10^5$  MS/mL ( $LSD_{5\%} = 0.498$ ). The lowest production of MS was found in *M. guizhouense* Bk41 with 684 MS/mL at day 6, while the plant growth promotor *T. harzianum* showed its highest production with  $9.7 \times 10^4$  MS/mL, also at 6 days after inoculation (**Figure 1C**). Microsclerotia formation during fermentation were first recorded on day 3, undergoing on the following days different developmental stages until complete melanization was achieved (**Figure 2**). At days 3 and 4 after inoculation, MS were still immature as aggregated hyphae were not completely melanized. By day 6, all isolates had slightly melanized compact hyphal structures, conforming to previous descriptions of MS structures. The stages in MS development differed among the isolates in rate of development, size and melanization. For example, MS from *T. harzianum* F327 were relatively small when compared to those from *Metarhizium* isolates. The MS in *M. guizhouense* melanized faster than those in *M. novozelandicum* F99 (data not shown). The longer the fermentation time, the greater melanization of the MS. Complete melanization of MS occurred after 7 days of fermentation culture (**Figure 2**).

## Microsclerotia Production for Seed Coating and Characterization

The isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447 were selected for MS production and incorporation into a coating for maize seeds. These isolates produced the highest concentration of MS among the *Metarhizium* isolates. Interestingly, they were obtained from different sources, *M. anisopliae* A1080 from an insect and the other two isolates from plant samples as endophytes (**Table 1**). Following the procedure for MS production described above, cultures yielded on average around 80 mg/mL of biomass and  $\sim 1 \times 10^5$  MS/mL (**Figure 3**).

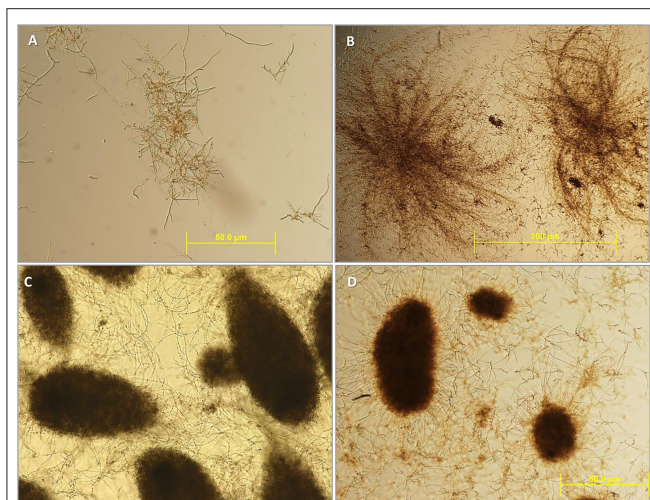
All MS-DE granules obtained from the three isolates had germinations rates above 90% with no significant differences among isolates ( $F_{2/13} = 1.59$ ;  $p = 0.242$ ; **Table 2**). However, isolate-specific differences were found in the water content of MS-DE after drying. The lowest water content was found in *M. anisopliae* F672 granules (**Table 2**). Conidia production by the different MS-DE granules was always above  $1 \times 10^9$  conidia/g,



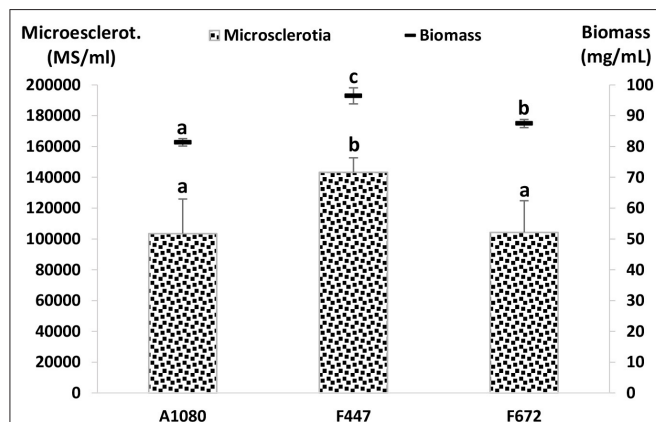


**FIGURE 1 |** Fungal growth in liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). **(A)** Fungal biomass (dry weight Log<sub>10</sub> mg/mL); LSD<sub>5%</sub> = 0.075,  $p < 0.01$ . **(B)** Blastospore production (Log<sub>10</sub> number/mL); LSD<sub>5%</sub> = 0.557,  $p = 0.288$ . **(C)** Microsclerotia production (Log<sub>10</sub> number/mL); LSD<sub>5%</sub> = 0.498,  $p < 0.01$ . Error bars = standard error.





**FIGURE 2 |** Microsclerotia development during liquid fermentation in basal salts solution with trace metals and vitamins (C:N ratio 23:1). The initial process for the formation of microsclerotia (MS) started early in the fermentation under liquid conditions with the formation of hyphal aggregates [(A,B), 4 days after inoculation]. After 7 days of inoculation, the MS formed as round structures with compact pseudoparenchymatous cells layers highly melanised (C,D). Images from: *M. anisopliae* A1080 (A); *M. robertsii* F447 (B); *M. anisopliae* F672 (C); and from *M. novozelandicum* F99 (D).



**FIGURE 3 |** Biomass and MS production by entomopathogenic fungi in basal salts solution with trace metals and vitamins (C:N ratio 23:1). Biomass (mg/mL);  $LSD_{5\%} = 4.9$ ,  $p < 0.01$ . Microsclerotia (MS/mL);  $LSD_{5\%} = 32,542.3$ ,  $p < 0.05$ . Error bars = standard error.

with the highest production achieved by *M. anisopliae* F672 ( $p < 0.01$ ; Table 2).

Maize seeds were coated with an amount of MS-DE granules which provided an estimated final concentration of  $1 \times 10^8$  conidia per gram of seeds. Control seeds (CS) were coated with the same amount of DE and the rest of the coating ingredients, but without fungal MS or conidia. Seeds coated with MS-DE from *M. anisopliae* F672 had a viable conidia production of  $7.8 \times 10^6$  CFU per gram of maize seeds. This did not differ from *M. anisopliae* A1080 but was greater than for *M. robertsii*, which

produced only  $2.0 \times 10^6$  CFU per gram of maize seeds (Figure 4). These CFU values were expected since previous results showed that CFU decreased an estimated two orders in relation to the initial conidial concentration coated onto maize seeds. From CS no fungal colonies were obtained.

### Maize Plant Performance in the Presence of *Fusarium graminearum* After Seeds Were Coated With Microsclerotia of *Metarhizium* spp

#### Maize Plant Dry Weight

Maize growth, determined as total dry weight of shoots and roots, differed significantly among treatments ( $F_{3/153} = 145.6$ ;  $p < 0.01$ ). Maize dry weight was highest in CS in the absence of *F. graminearum*, while CS in the presence of this phytopathogen (CSfg) had a significantly lower dry weight (Figure 5;  $LSD_{5\%} = 0.029$ ). In the presence of *F. graminearum*, maize plants with the *Metarhizium* spp. MS-DE coated seed treatments had a significantly greater dry weight than CSfg ( $LSD_{5\%} = 0.029$ ). In the presence of *F. graminearum*, plants coated with the *M. anisopliae* F672 MS-DE treatment had, on average, a dry weight gain of 32% compared to the maize plants without the coating, while the dry weight of plants was almost 26% lower when compared to CS in the absence of *F. graminearum* (Figure 5;  $F_{3/153} = 145.6$ ;  $p < 0.01$ ).

The negative effect of *F. graminearum* on total plant dry weight was due to the phytopathogen's impact on both shoot and root dry weight, which was significantly lower in the CSfg compared to the CS treatment (Figure 5;  $p < 0.01$ ). The *Metarhizium* spp. seed coating with MS-DE partially compensated this reduction in shoot dry weight caused by the plant pathogen. Compared to CS grew in the absence of *F. graminearum*, the dry weight of shoots from plants treated with *M. anisopliae* F672 was reduced by 27% ( $F_{3/153} = 73.5$ ;  $p < 0.01$ ). In plants treated with *M. robertsii* F447 a 37% shoot dry weight reduction occurred when plants were grown in the presence of *F. graminearum* (Figure 5;  $LSD_{5\%} = 0.054$ ). In the presence of the phytopathogen maize plants with MS-DE treatments had a greater root dry weight when compared to plants without a fungal treatment (Figure 5;  $LSD_{5\%} = 0.034$ ). However, in the presence of *F. graminearum*, dry weight of roots from plants with the MS-DE treatments with *M. anisopliae* F672 and *M. robertsii* F447 were 14 and 28%, respectively, lower than root dry weight in CS in the absence of *F. graminearum* (Figure 5;  $F_{3/153} = 80.5$ ;  $p < 0.01$ ).

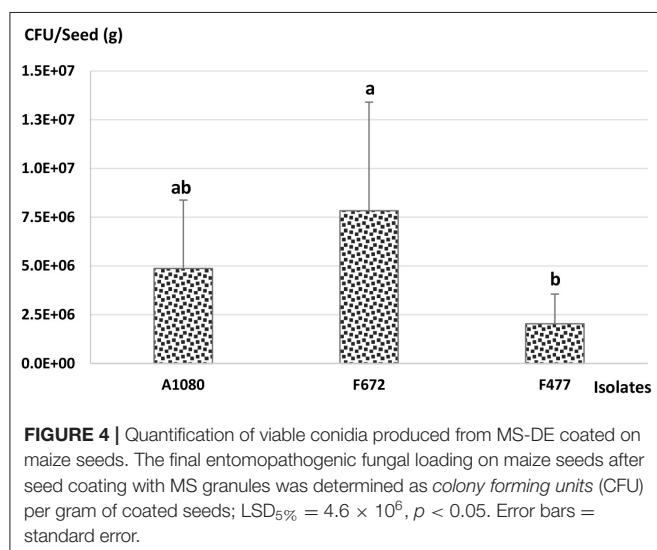
### Determination of Fungal Endophytism by Confocal Fluorescent Microscopy After Maize Seed Coating With *Metarhizium* spp. MS-DE Granules

The presence of hyphae on and in root tissues was observed in all the *Metarhizium* MS-DE treatments but not in samples from CS without fungal treatment. Fungal hyphae growing on the surface of roots were observed growing in proximity to the root crown, in seeds remains and all along the length of the root, but not in root apices. The protrusion zone, where secondary

**TABLE 2** | Characterization of *Metarhizium* spp. microsclerotia–diatomaceous earth granules.

Isolate	Species	Germination $\pm$ SE (%)	$a_w \pm$ SE	Conidia production $\pm$ SE (conidia/g MS-DE)
A1080	<i>M. anisopliae</i>	95.2 $\pm$ 1.8 <sup>a</sup>	0.75 $\pm$ 0.03 <sup>a</sup>	2.1 $\pm$ 0.1 ( $\times 10^9$ ) <sup>a</sup>
F447	<i>M. robertsii</i>	91.0 $\pm$ 3.4 <sup>a</sup>	0.70 $\pm$ 0.12 <sup>a</sup>	4.4 $\pm$ 0.3 ( $\times 10^9$ ) <sup>b</sup>
F672	<i>M. anisopliae</i>	95.2 $\pm$ 1.9 <sup>a</sup>	0.58 $\pm$ 0.01 <sup>b</sup>	8.8 $\pm$ 0.3 ( $\times 10^9$ ) <sup>c</sup>
LSD <sub>5%</sub>		5.7	0.09	1.8 $\times 10^9$
<i>p</i> -value		= 0.236	< 0.01	< 0.01

Microsclerotia were obtained from production cultures and filtered with diatomaceous earth to obtain granules (MS-DE). The MS-DE granules after drying were evaluated for water content determined as water activity ( $a_w$ ), hyphae production (germination) and conidiogenesis (conidia production). Average values with no superscript letters in common represent statistically significant differences at  $p < 0.05$  within the same column (germination,  $a_w$  or conidia production).

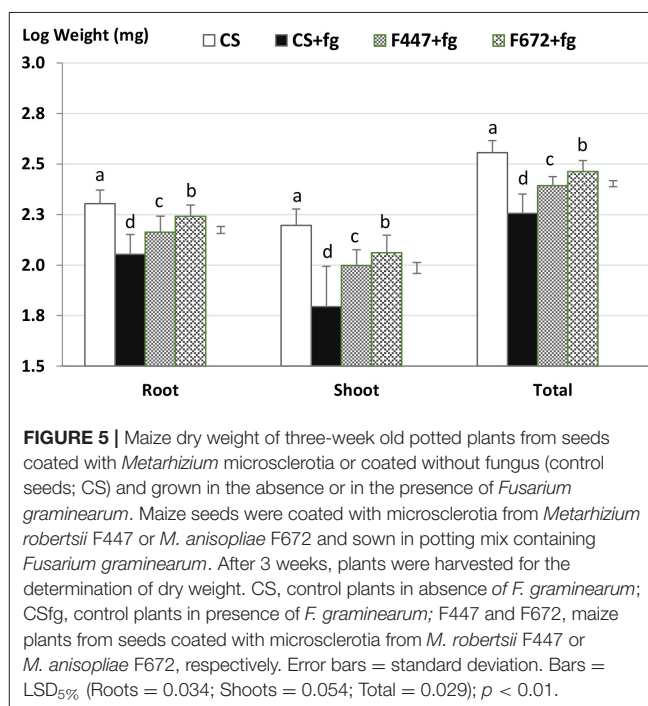


roots emerge, seemed to be one of the access points into the roots. In this area, extensive colonization by *M. anisopliae* A1080 was observed (Figures 6A–D). The presence of hyphae growing inside the cortical root cells was observed 1-month after sowing the MS-DE coated seeds (Figure 7). The lectin, CoA-AF688, binds to glycoproteins,  $\alpha$ -mannopyranosyl, and/or  $\alpha$ -glucopyranosyl residues allowing the identification of hypha penetration sites through the root cell wall which are visualized as red dots (Figure 7A, arrow heads). The constriction points in the hypha also confirmed these penetration zones (Figure 7B, arrow heads). At this plant stage, the roots showed extensive fungal colonization on the surface of the roots but also endophytic colonization (Figure 7D).

Samples of stems and leaves were also analyzed in all the MS-DE treatments by fluorescent and confocal microscopy, but no endophytic colonization was observed. At least 1 month after sowing *Metarhizium* MS-DE coated seeds, fungal colonization seemed to be limited only to roots.

## DISCUSSION

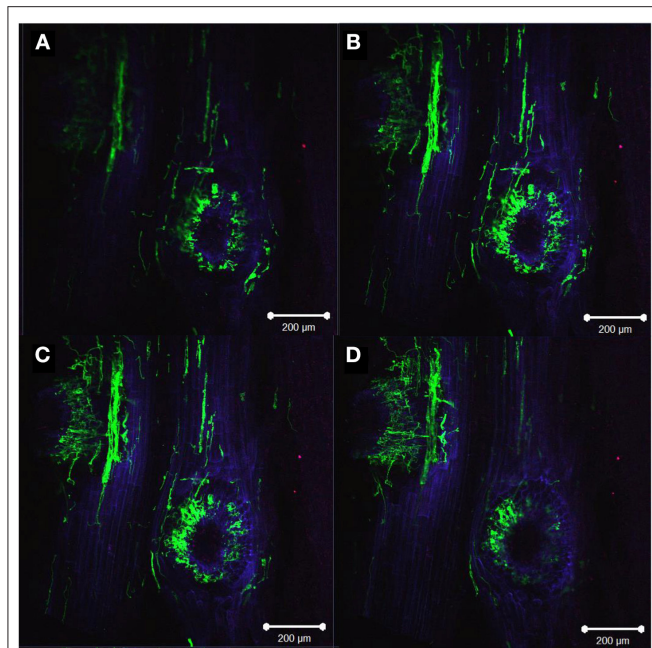
In this study the production and formulation of microsclerotia (MS) by isolates of *M. anisopliae*, *M. guizhouense*,



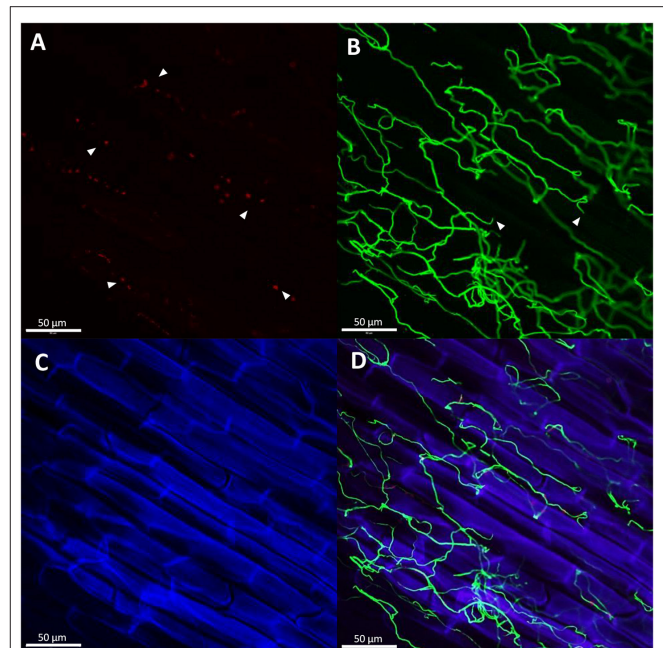
*M. novozealandicum*, *M. robertsii*, *B. bassiana*, and *T. harzianum* was determined. All isolates produced MS, compact hyphal aggregates that become pigmented with culture age, in addition to more typical blastospores and mycelia. Previous reports have reported the production of these resistant structures in *M. anisopliae*, *M. brunneum*, *M. humeri*, *M. rileyi*, *M. robertsii*, *B. bassiana*, *B. brogniartii*, *B. pseudobassiana*, and *T. harzianum* (Jaronski and Jackson, 2008; Jackson and Jaronski, 2009; Behle et al., 2013; Kobori et al., 2015; Villamizar et al., 2018; Lira et al., 2020). However, this study also reports on the production of MS in several *Metarhizium* species, *T. harzianum*, and *B. bassiana* simultaneously, and it is the first report of MS obtained from isolates of *M. guizhouense* and *M. novozealandicum*.

The biomass obtained by the New Zealand *Metarhizium* spp. isolates (42–55 mg/mL) was higher than values previously reported which vary between 7.0 and 33.0 mg/mL (Jackson and Jaronski, 2009; Mascarin et al., 2014). In a previous study with *M. rileyi*, with a biomass production of 40.7 mg/mL (Song et al., 2016), similar values were obtained as in the present





**FIGURE 6 |** *Metarhizium anisopliae* A1080 colonization of roots from two weeks old maize plants, showing the protrusion zone of secondary root emergence with fungi growing on the surface and inside the tissues. Overlay of images: plant membranes were stained with propidium iodide (blue) and Congo red (red), while fungal structures were stained with WGA-AF488 (green). Z-Stack image (A–D): upper left image (A) distal focus, bottom right panel (D) closest focus (total distance 51.7  $\mu$ m). Images were taken with a confocal microscope (LSM 510 META–Zeiss) using the program ZEN 2009. The bar represents 200  $\mu$ m.



**FIGURE 7 |** *Metarhizium anisopliae* F672 hyphae growing on 1-month old maize roots. The presence of glycoproteins,  $\alpha$ -mannopyranosyl, and/or  $\alpha$ -glucopyranosyl residues, around hyphal adhesion sites were stained with ConA-AF633. These sites indicate the hyphal penetration points through the root cell wall visualized as red dots (A), arrowheads). The net of hyphae growing around and inside the root cell stained with WGA-AF488 are visualized in green (B). The constriction zones in the hypha also confirm the vegetal cell wall penetration points [(B), arrowheads]. The root cells were stained with propidium iodide visualized in blue (C). Overlay of the three previous images (D). Images were taken with a confocal microscope (LSM 510 META–Zeiss) using the program ZEN 2009. The bar represents 50  $\mu$ m.

study. The highest biomass production was in *B. bassiana* Bb21 with 64 mg/mL. This yield was higher than in previous work under similar conditions where biomass weight obtained was between 15.3 and 20.5 mg/mL (Bidochka et al., 1987; Lohse et al., 2014). The variances in biomass yield might be due to differences in growth conditions and media [C:N ratio 23:1, (C) = 45 g/L], or to the different isolates used. The reasons behind the biomass increase observed in *Trichoderma* and *Beauveria* isolates compared to *Metarhizium* could be to their well-known fast growth and the ability to produce different types of fungal propagules such as submerged conidia, blastospores, blastoconidia, and budding hyphal cells from the developing hyphae (microcycle conidiation).

The production of blastospores by the *M. anisopliae* isolates varied between  $6.5 \times 10^5$  and  $1.3 \times 10^7$  blastospores/mL after six fermentation days. Blastospore concentration obtained was slightly lower than that reported by Jackson and Jaronski (2009) for *Metarhizium* spp., which was around  $2.0$ – $1.6 \times 10^8$  blastospores/mL after eight fermentation days in similar conditions. The differences in blastospore production might be based on the slight differences in C:N ratios but is more likely to be due to the intrinsic properties of the isolates used. *Beauveria bassiana* Bb21 blastospore production after 3 days of fermentation at  $2.7 \times 10^8$  blastospore/mL was close to the values reported by Mascarin et al. (2015) of between 0.95 and

$7.9 \times 10^8$  blastospores/mL in a similar study. However, in the present work, *B. bassiana* Bb21 maximum production,  $4.3 \times 10^9$  blastospore/mL, was obtained after six fermentation days.

The quantity of MS obtained from both *M. anisopliae* isolates ( $3.5$ – $4.0 \times 10^4$  MS/mL) was close to those reported previously by Jackson and Jaronski (2009) for *M. anisopliae* ( $1.8 \times 10^4$ – $6.4 \times 10^4$  MS/mL) using identical fermentation conditions and similar C:N ratios. Although in their study the highest production was by *M. brunneum* F52 ( $1.2 \times 10^5$  MS/mL), in this study the highest MS production was found in *M. robertsii* F447 ( $2.3 \times 10^5$  MS/mL). Mascarin et al. (2015) reported yields of around  $6.1$ – $7.3 \times 10^6$  MS/L for *M. anisopliae*, *M. acridum*, and *M. robertsii* isolates after growth in liquid fermentation for 3 days, with a maximum yield of  $0.7$ – $1.1 \times 10^4$  MS/mL after 5 days in liquid fermentation. In a different study with *M. rileyi* MS production of up to  $9.7 \times 10^4$  MS/mL was reported (Behle et al., 2013). Lira et al. (2020) screened 48 isolates of different species of *Metarhizium* using only 3-day liquid fermentation obtaining a range from  $1.3 \times 10^2$  to  $3.6 \times 10^3$  MS/mL.

Evidently, all *Metarhizium* species tested are capable of producing MS although, as expected, the production of the resting structures varied between species and isolates, as reported previously (Lira et al., 2020). In this study, the lowest MS

production was by *M. guizhouense* Bk41 with  $3.3 \times 10^2$  MS/mL, while for *M. guizhouense* F16,  $9.5 \times 10^3$  MS/mL was achieved. However, *M. guizhouense* Bk41 produced higher amounts of blastospores ( $2.6 \times 10^7$  blastospores/mL) than *M. guizhouense* F16 ( $6.1 \times 10^5$  blastospores/mL). This fact highlights the importance of isolate characterization to determine potential use and applicability. The results obtained in the current study confirm that growth conditions required for formation of MS can vary among fungal species and even among isolates of a particular species as stated also by Wang et al. (2013).

Another factor to consider during MS production is maturation. Although the production of these resistant structures can be obtained at an early stage of fermentation, a complete melanization of MS did not occur until after at least 6 days of liquid fermentation. The longer the fermentation process, the higher the melanization of the MS, not only in entomopathogenic fungi but also in *T. harzianum* (Jackson and Jaronski, 2009; Mascarin et al., 2014; Kobori et al., 2015). On average, MS can be observed after 4 days of fermentation, but this period might not be sufficient for complete melanization. Further studies are needed to determine the relationship between melanization and subsequent performance of MS. If these resistant structures are not fully mature, an early harvest, and following downstream processing (drying and formulation) could result in MS viability losses. Melanization has been associated with prolonged persistence in soil and resistance to desiccation (stress tolerance) in various filamentous fungi (Jackson and Jaronski, 2009; Kobori et al., 2015). Melanin has also been reported as a natural barrier against the harmful effects of UV-B radiation as well as resistance to toxic substances (Eisenman and Casadevall, 2012).

The rehydration and incubation of air-dried MS-DE granules from the isolates *M. anisopliae* A1080 and F672, and *M. robertsii* F447, on water agar plates resulted in hyphal formation after just 24 h. The development of sporogenic structures able to produce high numbers of conidia was obtained in less than a week. This was also reported in previous studies for *M. brunneum* and *M. anisopliae* (Jackson and Jaronski, 2009; Mascarin et al., 2014), *Lecanicillium lecanii* (Wang et al., 2013), *T. harzianum* (Kobori et al., 2015), *M. robertsii*, and *M. acridum* (Mascarin et al., 2014). In the current study, the concentration of conidia/g of dried MS-DE reached were one log higher than those reported by Jackson and Jaronski (2009), but similar to those values reported by Mascarin et al. (2014).

Lira et al. (2020), showed that coating maize seeds with MS is a feasible method for delivery of entomopathogenic fungi for biocontrol and plant growth promotion purposes. In our study, we demonstrated that after coating, MS were viable, and provided protection to maize plants by counteracting the negative effects of *F. graminearum*. As stated by Partida-Martínez and Heil (2011) the plant gain from any beneficial microorganism is mainly noticed when challenged by plant pathogens. Thus, the potential benefits of MS coated to maize seeds on maize growth was observed in the presence of the plant pathogen *F. graminearum*. Control plants grown in the absence of *F. graminearum* (CS) had significantly greater dry weight than plants treated with *M. robertsii* F447 or *M. anisopliae* F672. Conversely, in the presence of this phytopathogen, shoot development in plants

grown from seeds coated with MS from *M. robertsii* and *M. anisopliae* was significantly better, when compared to maize plants from seeds without the MS coating.

Previous work found that bean or maize plants colonized by *Metarhizium* spp., and then exposed to *F. solani* or *F. graminearum*, had greater plant growth and lower disease indices compared with uncolonized plants (Sasan and Bidochka, 2013; Rivas-Franco et al., 2019). The mechanisms used by *Metarhizium* to counteract the negative effects of the presence of the plant pathogen could be due to multiple factors including competence for rhizosphere colonization, promotion of the plant induced response or production of *Metarhizium* secondary metabolites that inhibit *Fusarium* growth (Sasan and Bidochka, 2013; Ravindran et al., 2014). Thus, the overall results suggest that species of *Metarhizium* may be used as a control for plant pathogens as well as insect pests. The mechanisms behind the entomopathogenic fungi *Metarhizium* spp. ability to reduce the impact of this plant root pathogen represents an area that warrants further research.

Using laser confocal microscopy, we showed that the hyphae were closely associated with the ecto and endorhizosphere of maize roots. In the present work, two isolates, *M. anisopliae* F672, and *M. robertsii* F447, were obtained from plant material, *Pinus radiata* and *Actinidia deliciosa*, respectively, while *M. anisopliae* A1080 was isolated from a lepidopteran larva, *Trichoplusia ni*. The ability to produce MS as well as the capability to associate with maize roots seemed to be conserved in *Metarhizium* independent of origin. This reinforces the fact that *Metarhizium*, as soil dwelling fungi, have the capability to form resting structures to survive unsuitable conditions, and the ability to associate with roots which guarantees long term persistence.

Vega et al. (2009) stated “the ability of *Metarhizium anisopliae* to form sclerotia may be important for rhizosphere competence following a pattern seen in phytopathogenic fungi.” In this study, *M. anisopliae* and *M. robertsii* strongly colonized the differentiation and the root hair zones, inter- and intracellularly, and were infrequently detected in the elongation and meristematic zones. This colonization pattern was also observed in the plant pathogen *Piriformospora indica* (Zuccaro et al., 2011). However, the endophytic ability of *Metarhizium* and the ability to colonize cortical cell roots, places this genus closer to ecto- and arbuscular mycorrhizal fungi, which either grow intercellularly or predominantly colonize the deeper cortex layers of younger parts of the root (Zuccaro et al., 2011). This suggests that entomopathogenic fungi might also follow a pattern similar to that observed in *P. indica* and mycorrhiza. In *P. indica*, the failure of WGA-AF488 to stain the hyphae inside living cells strongly suggested that the fungus remained enveloped in an intact plant-derived membrane throughout intracellular growth (Zuccaro et al., 2011). *Metarhizium anisopliae* and *M. robertsii* were observed inside the cells stained with the WGA-AF488 which may indicate that entomopathogenic fungi growing inside the plant cell were not enveloped by an endomembrane as in *P. indica*. Whether the association pattern between hyphae of entomopathogenic fungi and roots resembles a similar process to that of phytopathogenic fungi or mycorrhizae, or even represents a novel process, requires further studies.



In summary, the results obtained provide insights into liquid culture production of MS from different species of *Metarhizium* and demonstrates the potential use of these structures when coated onto seeds for the biocontrol of plant pathogens and plant growth promotion. Additionally, this MS coating strategy places the fungus close to the roots which can be colonized both superficially and endophytically. This fungus-root association allows persistence of the biocontrol agent, and places it close to harmful plant challengers such as plant pathogens and soil-dwelling insect pests. Innovation in the development of *Metarhizium* as a biocontrol agent needs to consider all its capabilities including insect pathogenicity, antagonism of fungal phytopathogens, root colonization, and stimulation of the plant induced response, making *Metarhizium* a multifunctional bioinput for plant health.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

FR-F: conceptualization, methodology, investigation, formal analysis, and writing—original draft. JH, MR, TJ, MJ, and

TG: conceptualization, resources, writing—review & editing, and supervision. NA: resources, writing—review & editing, and supervision. JS: conceptualization and methodology. PW: conceptualization, resources, and supervision. DS: validation, formal analysis, and writing—review & editing. All authors contributed to the article and approved the submitted version.

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# Long Term Comparison of Talc- and Peat-Based Phytobeneficial *Pseudomonas fluorescens* and *Pseudomonas synxantha* Bioformulations for Promoting Plant Growth

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Use of plant growth promoting rhizobacteria (PGPR) is an important strategy in sustainable agriculture. Among PGPR, many *Pseudomonas* strains are of great interest due to their abilities to colonize and thrive in the rhizosphere, in addition to displaying mechanisms in plant growth promotion and biocontrol activities. However, as *Pseudomonas* strains are non-spore forming micro-organisms, their development into stable bioformulations for commercial applications can be difficult. This study examined over a long term period the effect of two different carriers, peat and talc, to prepare bioformulations using phytobeneficial *Pseudomonas* strains belonging to two taxonomical groups of interest: *P. fluorescens* and *P. synxantha*. Each strain has previously demonstrated plant growth promotion activity when inoculated in the rhizosphere. Each bioformulation was stored at ambient temperature and their viability was measured up to 180 days. In parallel, every 30 days (up to 180 days) each bioformulation was also applied in the rhizosphere of plantlets to validate their plant growth promotion activity, and their establishment in the rhizosphere was quantified by using strain-specific quantitative polymerase chain reaction assays. The viability of both *Pseudomonas* strains in the bioformulations was found to decrease after the first 15 days and remained relatively stable for up to 180 days. When applying the bioformulations to *Buglossoides arvensis* plantlets, the expected plant growth promotion was observed when using up to 180 day-old formulations of *P. fluorescens* and up to 120 day-old formulations of *P. synxantha*, with similar results for both carriers. Establishment of both *Pseudomonas* strains in the rhizosphere inoculated with the peat-based carrier bioformulations stored for up to 180 days was found to be stable. While a lower establishment of *P. fluorescens* in the rhizosphere was observed when talc-based bioformulations were stored for 90 days or more, rhizosphere colonization by *P. synxantha* talc-based bioformulations remained stable for up to 180 days. In conclusion, both peat and talc appear to be suitable carriers for *Pseudomonas* bioformulations,

however strain-specific variability exists and therefore the viability of each *Pseudomonas* strain and its capacity to maintain its plant growth promotion activity should be validated in different substrates before determining which formulation to use.

**Keywords:** bioformulation, *Pseudomonas*, peat, talc, carrier, plant-growth-promotion

## INTRODUCTION

The world population is increasing at an alarming rate and is set to reach 9 billion by the year 2050, while the land available for agriculture has been decreasing due to industrialization (Aamir et al., 2020). Food production has increased through advances in agriculture such as the use of chemical pesticides and fertilizers, as well as breeding efforts to develop plant cultivars that are more productive and more stress tolerant (Aamir et al., 2020). However, plant productivity is still impacted by different environmental stresses such as drought, salinity, and limited nutrient availability (Basu et al., 2017; Aamir et al., 2020). Additionally, global climate change will also increasingly affect agriculture worldwide through increased surface temperature and reduced soil moisture (Basu et al., 2017). Conventional agriculture relies heavily on chemical pesticides and fertilizers to control pests and improve plant growth. The use of chemical pesticides varies among crops but data has shown that up to 93% of row crops such as corn, soybean, and cotton are treated with some kind of pesticide (Pimentel, 1993). Chemical pesticides have proven successful at preventing some crop diseases and enhancing crop productivity, however their role in damaging agro-ecosystems is now better understood (Mishra and Arora, 2016). Studies show that most chemical pesticides used in conventional agriculture can have detrimental effects on human health (Moses et al., 1993; Reigart and Roberts, 2013). In addition to their negative effects on human health, chemical pesticides can also negatively affect aquatic systems, the wildlife and the richness of soil microorganisms (Dorigo et al., 2009). The use of chemical fertilizers has also been beneficial to achieve greater crop productivity, however long-term experiments on the effects of fertilizers on soil fertility have shown that continual use can decrease soil quality (Kumar and Yadav, 2001; Yang, 2006).

Sustainable agriculture has been increasing in interest as a method to produce food to feed the growing population using environmentally friendly approaches. One attractive and economical approach is the use of plant growth promoting rhizobacteria (PGPR) to improve crop productivity and achieve higher yields (Antoun and Kloepper, 2001). PGPR are a functional group of bacteria that include different genera such *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Azotobacter*, *Enterobacter*, *Xanthomonas*, *Bacillus*, and *Pseudomonas* (Bashan, 1998; Bishnoi, 2015; Zayed, 2016; Berninger et al., 2018; Aamir et al., 2020). PGPR make up from 2 to 5% of all bacteria found in the rhizosphere (the soil area surrounding plant roots) (Antoun and Kloepper, 2001) and specific PGPR strains can be used alone or in combination to achieve different ecological functions leading to improved plant yields. Their use coupled with other crop management practices can lead to a

reduction in the amount of chemicals used in the field without reducing crop yields. Among PGPR of interest, *Bacillus* and *Pseudomonas* are the most widely reported genera (Podile and Kishore, 2007). PGPR, and especially *Bacillus* and *Pseudomonas*, are of interest due to their ease of growth in the laboratory and their abilities to increase plant yields and/or improve plant health through various direct and indirect mechanisms of action. Direct methods include phosphate solubilization, nitrogen fixation, phytohormone production, iron acquisition, and the stimulation of plant defenses. Indirect methods include antibiosis, production of protective enzymes, induced systemic resistance, and the production of ACC-deaminase, which reduces the amounts of ethylene in the plant (Podile and Kishore, 2007; Martínez-Viveros et al., 2010; Zayed, 2016; Berninger et al., 2018; Bhattacharyya et al., 2020). Other beneficial traits of PGPR include their ability to efficiently colonize the root surface or the soil surrounding the roots, survive, compete and multiply in the soil (Bhattacharyya et al., 2017). PGPR that facilitate nutrient acquisition are known as biofertilizers, while PGPR that produce plant hormones are known as phytostimulators, and those that suppress diseases are known as biocontrol agents (Martínez-Viveros et al., 2010; Bishnoi, 2015). Some PGPR strains belong to more than one category. Biofertilizers, phytostimulators and biocontrol agents are considered key components of sustainable agriculture and are known to improve crop productivity in an ecofriendly manner. The biofertilizer market (comprising biofertilizers, phytostimulators, and biocontrol agents) is estimated to be worth 1.66 billion USD in 2022 and displayed an annual growth rate of 13.2% between 2015 and 2022, representing ~5% of the overall chemical-based fertilizers market (Macik et al., 2020).

A promising microbial inoculant must first be developed into a bioformulation to be used as a commercial product. A bioformulation is developed by combining a biologically active ingredient (living microbe or spore) with inert substances, known as carriers, to obtain a formulated product (Aamir et al., 2020). Common carriers include peat, talc, vermiculite, and diatomaceous earth (Bashan, 1998; Zayed, 2016). The carrier provides a temporary protective surface to the microorganism and must demonstrate the ability to support the growth of the target organism and maintain desired populations over an acceptable period of time (Macik et al., 2020). Carriers must have high water holding capacity, be well-buffered, environmentally safe, easy to use, and cost-effective (Bashan, 1998; Zayed, 2016; Bhattacharyya et al., 2020). Bioformulations are often divided into categories based on the composition of the carrier. These can include peat, liquid, granules, and freeze-dried powders (Abadias et al., 2005; Guijarro et al., 2007; Bhattacharyya et al., 2020). Among these, peat is one of the most common



carriers used worldwide (Bhattacharyya et al., 2020). Peat has a high water-holding capacity, high organic matter content, and is easily available (Bhattacharyya et al., 2020). Sticking agents are often added to peat-based biofertilizers to evenly distribute and stabilize the microorganism and can include carboxymethylcellulose, arabic gum, and polyalcohol derivatives (Bhattacharyya et al., 2020). Liquid formulations are aqueous suspensions made in oil, water, or a combination of both (Schisler et al., 2004). They are also a popular type of bioformulation; however, they can have reduced survival and propagation rates when compared to solid carriers (Berninger et al., 2018).

Developing a successful bioformulation can be a difficult process, as extensive trials must be conducted before the product can be marketed. Many bacteria are tested in pot assays under strict controlled settings, and their subsequent testing under field conditions can be inconsistent with pot studies or even unsuccessful (Lugtenberg and Kamilova, 2009; Backer et al., 2018). Many uncontrollable factors are found in the field such as variations between different field sites, year to year environmental variation, and even from growing different crops in the same field (Raaijmakers et al., 2009; Compant et al., 2010; Bishnoi, 2015). Soil is notoriously heterogeneous and introduced microorganisms can be unable to find an empty niche (Bishnoi, 2015). It is of utmost importance that the inoculated microorganism is able to compete with indigenous microorganisms and establish itself and survive in the rhizosphere. Many studies have shown that following a PGPR application, a sharp decline in its population is observed after a few weeks (Verma et al., 2013; Bishnoi, 2015; Corrêa et al., 2015; Novinscak and Filion, 2019). Additionally, the microorganism used to develop the bioformulation should be compatible with mass multiplication and not display any toxicological effects to the plants or other beneficial microorganisms (Bishnoi, 2015). Another difficulty encountered when developing bacterial bioformulations is the use of Gram-negative strains because they do not produce spores (Berninger et al., 2018), which are often used for Gram-positive bacterial formulations. Gram-negative bacteria have however been found to be important PGPR, but due to their inability to produce spores, have a shorter shelf-life and are easily killed during desiccation of the formulation (Ramakrishna et al., 2019). One method used to counter the short shelf-life of Gram-negative bacterial formulations is to increase the number of microbes in the inoculant (Bashan et al., 2014; Tabassum et al., 2017). Even with a declining population over time, sufficient cells often remain alive to achieve a positive effect on the plants when applying the bioformulation in the field (Bashan et al., 2014).

*Pseudomonas* strains are some of the most recognized strains of PGPR found in the rhizosphere (Podile and Kishore, 2007), however, they have not been widely commercialized in large part due to the rapid loss of viability of the available bioformulations (Corrêa et al., 2015). *Pseudomonas* strains represent only 6% of the commercial biocontrol and biofertilizer products available for plants as compared to *Bacillus* strains which represent 15% and *Trichoderma* fungal species, which lead the way with 43% (Bettiol et al., 2012). *Pseudomonas* strains are also found to be highly susceptible to environmental stress factors, such as

drought and high temperatures (Paulitz and Bélanger, 2001). Greater commercial use of *Pseudomonas* strains in crops will depend on the formulation of products with greater shelf life and the demonstration that the products are viable and effective when applied to plants. High moisture levels, favorable pH values and low temperature favors the survival of *Pseudomonas* strains in carrier materials (Corrêa et al., 2015). Our research group has thoroughly examined the plant growth promoting effects of two strains of *Pseudomonas* species, *Pseudomonas fluorescens* LBUM677 and *Pseudomonas synxantha* LBUM223. Previous studies conducted with LBUM677 have shown that in addition to plant growth promotion properties, the bacterium is able to increase seed oil content in various oilseed plants, including in corn gromwell (*Buglossoides arvensis*), a plant of high nutraceutical interest, under various field settings (Novinscak and Filion, 2018, 2019; Jiménez et al., 2020). LBUM223 has demonstrated plant growth promotion properties and has been successful in reducing the symptoms of the bacterial pathogen *Streptomyces scabies* on potato plants in controlled and field settings (St-Onge et al., 2010, 2011; Arseneault et al., 2015). To reach the goal of eventually commercializing these strains, we wished to evaluate the survival and viability of these strains over a long period of time when produced in bioformulations. The present study examined their viability in two solid carriers, peat and talc, their capacity to colonize the rhizosphere when formulated in these carriers and stored for up to 180 days and finally their ability to conserve their plant growth promotion activity. Interestingly, many *Pseudomonas* strains isolated to date which display strong PGPR abilities belong to the *P. fluorescens* and *P. synxantha* species. We therefore anticipate that the findings obtained in this study using LBUM677 and LBUM223 will, at least in part, be applicable to other *P. fluorescens* and *P. synxantha* strains of interest.

## MATERIALS AND METHODS

### Bacterial Preparation

*P. fluorescens* LBUM677 and *P. synxantha* LBUM223 were originally isolated from the rhizosphere of strawberry plants cultivated in Bouctouche, NB, Canada. Three replicates of pre-cultures of each bacterium were prepared by inoculating each bacterium in King's B broth (King et al., 1954) and incubating at 25°C with agitation at 150 rpm for 24 h. Each pre-culture was used to inoculate larger volumes of King's B broth (used for inoculum preparation) and incubated at 25°C with agitation at 150 rpm for 48 h. The bacterial concentrations were determined to range from  $2.4 \times 10^{11}$  to  $3.7 \times 10^{11}$  cfu mL<sup>-1</sup> for LBUM677 and  $8.8 \times 10^{11}$  to  $1.0 \times 10^{12}$  cfu mL<sup>-1</sup> for LBUM223 based on OD<sub>600nm</sub> measurements and previously determined growth curves (Novinscak and Filion, 2018).

### Inoculum Preparation

Talc (Fisher Scientific, Ottawa, Canada) and peat-based (Green Island peat moss, Isle-Verte, Canada) formulations of each *Pseudomonas* strain were prepared by following the method of Vidhyasekaran and Muthamilan (1995). One large batch of each carrier was prepared by mixing eight kilograms of each carrier

with 80 g of carboxymethylcellulose (Sigma Aldrich, Oakville, Canada) and the pH was adjusted to 7 using calcium carbonate (Sigma Aldrich, Oakville, Canada). After adjusting the pH, eight aliquots of one kg of each substrate were prepared and were sterilized by autoclaving the carriers for 60 min at 121°C twice, on separate days.

Triplicates of each bacterium were inoculated in each carrier by aseptically adding 400 ml of bacterial suspension (see bacterial preparation) to 1 kg of carrier and mixing well. Duplicate negative controls were also prepared by aseptically adding 400 ml of uninoculated King's B broth to 1 kg of carrier and mixing well. Each bioformulation (eight for each carrier type; three inoculated with LBUM223, three inoculated with LBUM677 and two negative controls, inoculated with King's B broth) were air dried overnight and were then stored in the dark in polythene bags at room temperature (~25°C).

## Evaluation of the Shelf Life of the Talc and Peat Formulations

The viability of the bioformulations was tested after their storage for specific intervals of time (stored for 15 days, stored for 30 days, stored for 45 days, stored for 60 days, stored for 90 days, stored for 120 days, stored for 150 days, and stored for 180 days). After each storage time, one gram of each of the bioformulations was added to nine ml of sterile water, mixed, and six serial dilutions (from  $10^{-2}$  to  $10^{-7}$ ) were prepared. One hundred  $\mu$ l of each dilution was plated in triplicate on Petri containing King's B medium. The Petri were incubated at 25°C for 48 h before determining the number of colony-forming units on each plate.

## Growth Promotion of Corn Gromwell Using Talc and Peat Formulations

After the bioformulations were stored for 30 days, 60 days, 90 days, 120 days, and 180 days, the plant growth promotion effect of the bioformulations was also measured by mixing one gram of each bioformulation to 50 g of non-sterile soil and adding three *Buglossoides arvensis* seeds to each pot (to account for low germination rates). After germination, only one plant per pot was kept. The non-sterile soil used was obtained from a field at the Senator Hervé J. Michaud Agriculture and Agri-Food Canada Research Farm (Bouctouche, NB, Canada) and was characterized as a Gleyed Podzolic Gray Luvisol (GLPZ.GL), per the Canadian Soil Classification System with 62% sand, 25% silt, 13% of clay, 2.6% organic matter and a pH of 5.2 (National Research Council of Canada, 1987). A total of 320 experimental units was used (eight bioformulations for each carrier type; two carrier types; five time-points; four replicates). The *B. arvensis* plantlets were grown for 60 days at 20°C and a 12 h photoperiod. At 60 days, the rhizosphere soil was collected from each plant (destructive sampling), flash frozen in liquid nitrogen and stored at -80°C and the plant's total fresh weight (root and shoot weight) was measured.

## Rhizosphere Competence of *P. synxantha* LBUM223 and *P. fluorescens* LBUM677

As mentioned previously, on each sampling date, rhizosphere soil was harvested from each plant, flash frozen in liquid nitrogen

and stored at -80°C. A total of 320 rhizosphere samples were collected by the end of the experiment. The rhizosphere soil was subsequently lyophilized using a lyophilizer (Thermo Fisher Scientific, Mississauga, Canada). DNA was extracted from 0.5 g of rhizosphere soil using the method described in Griffiths et al. (2008). Following DNA extraction, all samples were diluted 1:10 before being used in qPCR. qPCR standards for LBUM223 and LBUM677 were created as previously described (deCoste et al., 2011) using a diluted cloned PCR fragment obtained from each bacterium using specific PCR primers. PCR primers and a TaqMan probe targeting a specific DNA sequence in LBUM223 were developed previously in Arseneault et al. (2016) (223phzCfor: 5'- ATA GAT GGA ATG CCG GTC ATG-3'; 223phzCrev: 5'- GCC CTC CTC CCT CTT TTG TTT-3' and 223phzCprobe: 5'- FAM-CGA CAA ACT CCA GTC AA-MGBNFQ-3') while PCR primers and a TaqMan probe targeting a specific DNA sequence in LBUM677 were developed previously in Novinscak and Filion (2019) (BOX18F 5'- GCC TGC ATC GCG GTC TT-3'; BOX18R 5'- CCC AAT CGG GTG ATC ATT G-3' and BOX18probe 5'- FAM-ATC ATA GAC TCG GAA TTG T-MGBNFQ-3'). The qPCR reactions were performed in 20  $\mu$ l and consisted of: 10  $\mu$ l of 10X iTaq Mix (Bio-Rad Laboratories, Mississauga, Canada), 0.8  $\mu$ l of 5  $\mu$ M of each probe, 0.8  $\mu$ l of 5  $\mu$ M of each primer, 3.0  $\mu$ l of DNA and 4.6  $\mu$ l of DEPC-treated H<sub>2</sub>O. The amplification protocol was performed in a 7500 Real Time PCR machine (Applied Biosystems, Mississauga, Canada) and consisted of 95°C for 3 min followed by 50 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 60 s. Data collection was performed at the end of the annealing/extension step.

## Statistical Analyses

The data from the bioformulation viability assay was rank transformed before statistical analysis. Data was separated by bacterium and a two factor ANOVA (sampling date and carrier type as factors) was used to examine the effects on the bacterial populations. For the plant growth promotion assays, each time-point was examined separately. Data were rank transformed when necessary. A two-factor ANOVA was used to measure the effect of the carrier type and the bacterial inoculation (LBUM677, LBUM223 or control) on plant weight. Finally, for the rhizosphere competency assay, the results for each bacterium were analyzed separately. A two-factor ANOVA examining the effect of the sampling date and the carrier type was performed. *P*-values below 0.05 were considered significant. SAS software version 9.2 (SAS Institute Inc, NC, USA) was used for all statistical analyses.

## RESULTS

### Evaluation of the Shelf-LIFE of Talc and Peat Bioformulations of *P. fluorescens* LBUM677 and *P. synxantha* LBUM223

LBUM677's survival rate was higher in the peat carrier than the talc carrier (Table 1), while no difference was observed between the two carriers for LBUM223 (Table 1). For both bacteria, the population levels decreased almost two log values (cfu g<sup>-1</sup>) from

**TABLE 1** | Shelf-life of talc and peat bioformulations of *P. fluorescens* LBUM677 and *P. synxantha* LBUM223 determined by plating dilutions of inoculum at each time point.

Carrier formulation and treatment	Population (10 <sup>7</sup> cfu per g) at various days of storage							
	15	30	45	60	90	120	150	180
Peat + LBUM677	634.0 ab <sup>#</sup>	306.0 ab	293.0 ab	205.0 ab	392.0 abc	11.0 cdef	3.7 ef	3.0 f
Talc + LBUM677	455.0 ab	45.1 bcd	12.5 cdef	25.2 bcde	8.4 def	6.0 ef	8.2 def	23.0 bcdef
Peat + LBUM223	1190.0 ab	268.0 ab	294.0 ab	197.0 ab	48.2 bcd	7.3 cdef	4.9 ef	5.2 def
Talc + LBUM223	476.0 ab	105.0 bc	5.1 cdef	27.1 bcde	3.2 f	6.1 cdef	3.4 ef	4.2 ef

<sup>#</sup> Mean of three replications. \*Means followed by a common letter in a row are not significantly different ( $P = 0.05$ ) according to Tukey's a posteriori test. The initial bacterial concentrations inoculated in the substrates were  $2.81 \times 10^{11}$  cfu per g for LBUM677 and  $9.60 \times 10^{11}$  cfu per g for LBUM223.

the initial inoculation of the carriers to the first sampling date, 15 days later (Table 1). A difference between the population levels of LBUM677 between peat and talc was first observed at 45 days and again at 90 days, however this difference between carriers was not maintained over time. Similarly, a difference between the LBUM223 population levels of both carriers was also observed at 45 and 90 days, however, as with LBUM677, the difference was not maintained. Over the course of the experiment, the population levels decreased by four log values from  $\sim 1 \times 10^{11}$  to  $1 \times 10^7$  cfu g<sup>-1</sup> for both bacteria in both substrates.

### Growth Promotion of Corn Gromwell by *P. fluorescens* LBUM677 and *P. synxantha* LBUM223

The effect of the bacterial treatment was found to significantly influence the plant weight at all time-points measured when compared to the controls having received the carriers only without the bacteria (Figure 1). However, a significant effect of the carrier was only observed sporadically when the carriers reached 60, 90, and 180 days of storage (Figure 1). The plant growth promotion effect was observed for both bacteria at most time points, however, a difference between the effects of the two bacteria was initially observed when the bioformulations were stored for 90 days and this trend remained to the end of the experiment. The LBUM677 talc bioformulation was found to increase the plant weight from 12 to 380% (as compared to controls) at all time-point analyzed. The LBUM677 peat bioformulation was found to increase the plant weight from 37 to 364% at all time-points except for the 30 days bioformulation, which actually decreased the plant weight by 8% as compared to the control. The LBUM223 talc bioformulation was found to increase the plant weight by 93 to 130%, but only when the bioformulation was between 60 and 120 days of age. This was also observed in the LBUM223 peat bioformulation, where the plant weight was increased by 67 to 139% during the same time-points. Both LBUM223 bioformulations had a negative effect on the plant weight at 30 days and 180 days (Figure 1).

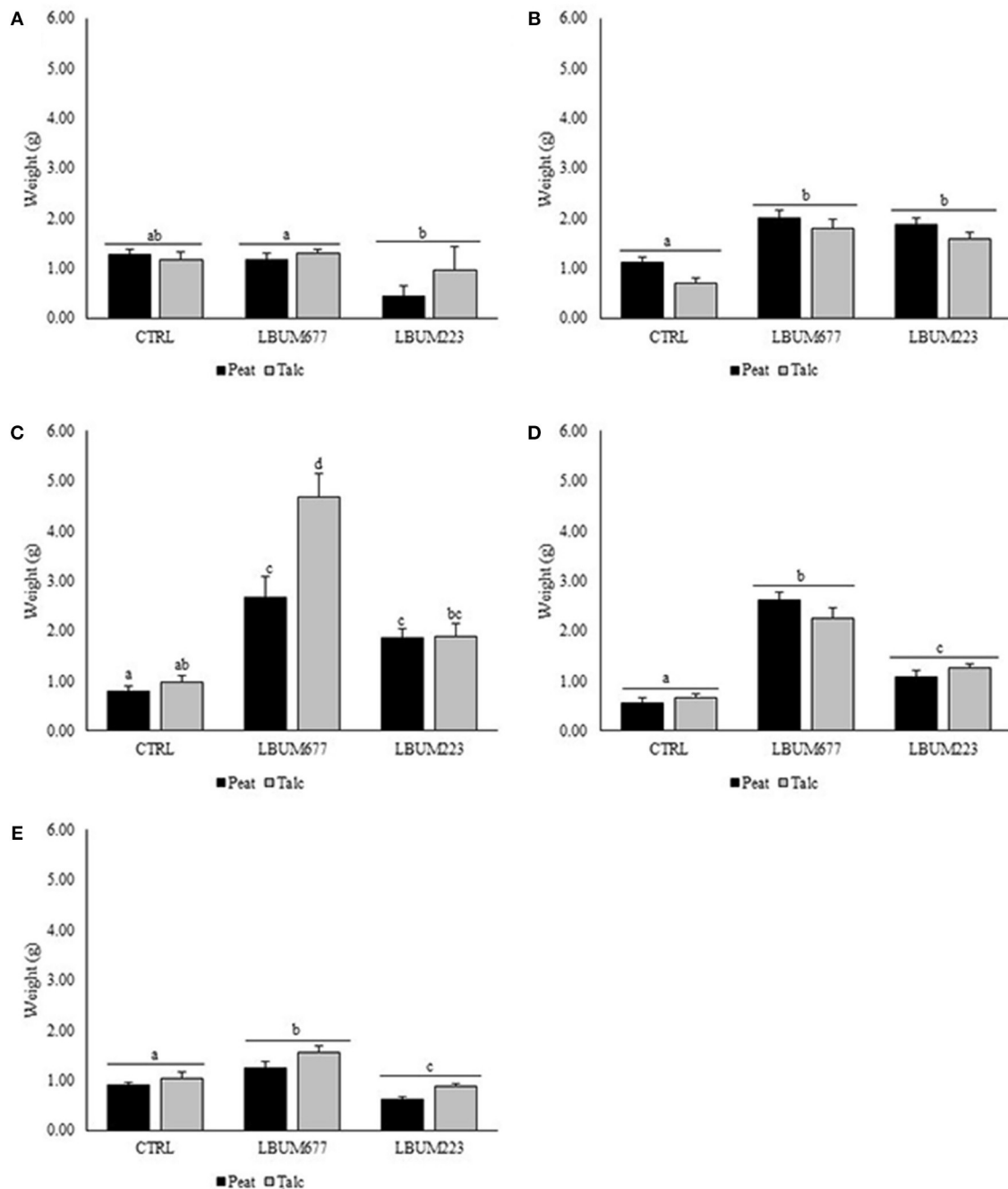
### Rhizosphere Competence of *P. fluorescens* LBUM677 and *P. synxantha* LBUM223

When examining the rhizosphere competency of both bacteria evaluated using specific qPCR assays, LBUM677 was found to establish itself with higher population levels in the

rhizosphere of corn gromwell than LBUM223 (Table 2). Additionally, LBUM677 populations were found to be higher when peat was used as a carrier than talc. When using the peat carrier, the LBUM677 populations were found to be stable throughout the experiment, however there was a significant decrease in the LBUM677 populations when talc was instead used. For LBUM223, no difference was observed in the rhizosphere competence between talc or peat carriers. The LBUM223 populations were found to be relatively stable throughout the entire experiment with significantly lower population levels observed at 120 days, however, this difference was no longer observed at 180 days.

## DISCUSSION

The overall results of this study show that bioformulations stable up to 180 days at room temperature were successfully developed for two distinct *Pseudomonas* species. Both *P. fluorescens* and *P. synxantha* remained viable, could colonize the rhizosphere, and achieve plant growth promotion. When trying to determine if peat or talc would be a better carrier in general for *Pseudomonas* strains, our results indicated that both carriers yielded similar results at most time-points for both bacteria and demonstrated a shelf-life up to 180 days. Cell viability at 180 days in peat was similar for LBUM677 and LBUM223 ( $3 \times 10^7$  and  $5 \times 10^7$  cfu g<sup>-1</sup>, respectively), while results for talc were better for LBUM677 than LBUM223 ( $2 \times 10^8$  and  $4 \times 10^7$  cfu g<sup>-1</sup>, respectively). These results differ from those obtained comparing talc and peat as carriers for *Pseudomonas fluorescens* strains Pf1 and Pf2 showing that talc formulations were viable after 180 days of storage while peat formulations were only effective for up to 120 days of storage (Vidhyasekaran et al., 1997). The survivability of *P. fluorescens* strains Pf1 and Pf2 in six different carriers was tested and talc was also found to be the best carrier and maintained the highest populations of *P. fluorescens* at the end of 6 months of storage at  $1.8 \times 10^9$  cfu g<sup>-1</sup> carrier (Gade et al., 2014). However, other results obtained using peat-based carriers, similar to the ones obtained in our study, showed that *P. fluorescens* Pf1, Pf2, and Pf27 remained viable for up to 8 months at a population of  $7 \times 10^6$  cfu g<sup>-1</sup> (Vidhyasekaran and Muthamilan, 1995), and *P. chlororaphis* PA23 successfully maintained a shelf life of more than 6 months in a peat carrier (Nakkeeran et al., 2004). The survival of *P. fluorescens* PfALR2



**FIGURE 1 |** Total plant fresh weight (in grams) of corn growwell inoculated with peat or talc bioformulations of LBUM677, LBUM223, or control that were stored for (A) 30 days, (B) 60 days, (C) 90 days, (D) 120 days, and (E) 180 days. All plants were harvested at the same age and destructively sampled at the same development stage (after 60 days of growth). Black bars represent bioformulations prepared with peat while gray bars represent bioformulations prepared with talc. Different letters indicate statistically significant differences between treatments.

in peat, talc, and lignite was measured up to 60 days and peat was found to be a better carrier for this bacterium than talc and lignite (populations of  $4.4 \times 10^7$  cfu g<sup>-1</sup> for peat as compared to  $2.9 \times 10^7$  cfu g<sup>-1</sup> for talc) (Rabindran and Vidhyasekaran, 1996). Another study also tested three different carrier materials

(cocopeat, talc, and farmyard manure) for their ability to support the growth of two *Pseudomonas* sp. isolates (An-2-nali and Pn-2-kho) (Verma et al., 2013). Cocopeat had the longest shelf-life of up to 7 months with a population of  $1 \times 10^4$  cfu g<sup>-1</sup> (An-2-nali strain), while the shortest shelf-life was observed in the



**TABLE 2 |** Rhizosphere competence of LBUM677 and LBUM223 bioformulations as determined by measuring the populations of each bacterium in rhizosphere soil using real-time PCR when the bioformulations reached different ages.

Population (10 <sup>7</sup> cfu per g) of bacteria in the rhizosphere soil detected using real-time PCR at various bioformulation ages (days)					
Carrier formulation and treatment	30	60	90	120	180
Peat + LBUM677	29.40 a <sup>#</sup>	7.94 ab	16.00 a	3.40 ab	12.00 ab
Talc + LBUM677	1.53 abc	7.04 bc	0.71 c	0.23 c	2.23 c
Peat + LBUM223	0.04 a	0.12 a	0.23 ab	0.01 b	0.11 a
Talc + LBUM223	0.16 a	0.34 a	0.08 ab	0.01 b	0.31 a

<sup>#</sup>Mean of twelve replications (3 substrates × 4 plants). \*Means followed by the same letter in a row are not significantly different ( $P = 0.05$ ) according to Tukey's a posteriori test.

farmyard manure. Their results indicated that a smaller particle size of the carrier lead to an increased shelf-life (Verma et al., 2013).

The initial quantity of bacteria used to prepare the inoculum in this study was higher than what has been previously reported in the literature, where concentrations ranged from  $1 \times 10^8$  cfu ml<sup>-1</sup> up to  $2.5 \times 10^9$  cfu ml<sup>-1</sup> (Rabindran and Vidhyasekaran, 1996; Shaharoon et al., 2007; Jorjani et al., 2011; Jambhulkar and Sharma, 2013; Verma et al., 2013; Gade et al., 2014; Dubey and Kumar, 2018). The higher quantity determined in our study was achieved using a starter culture of each bacterium to inoculate the larger volumes required for the inoculations of the carriers. Studies have expressed that one of the ways to circumvent the decline in bacterial populations in a carrier is to use higher initial levels of the bacteria, however this approach might not always be economically efficient (Berninger et al., 2018). Even using higher initial amounts of cells, the population levels of both bacteria in our study decreased by four log-values from  $\sim 1 \times 10^{11}$  to  $1 \times 10^7$  cfu g<sup>-1</sup> carrier. A previous study found a rapid decrease in population levels of two *P. chlororaphis* strains (63-28 and TX-1) from  $5 \times 10^9$  bacterial cfu ml<sup>-1</sup> to  $1 \times 10^8$  -  $1 \times 10^5$  bacterial cfu ml<sup>-1</sup> in peat and talc in as little as 15 h after inoculation (Corrêa et al., 2015). However, in that study, the pH of talc or peat was not adjusted to a neutral value of 7 (it was maintained at 9 for talc and 3 for peat), which could explain the rapid decrease in bacterial populations observed. As increasing initial bacterial populations in carriers does not always lead to longer survival, efforts should be made to optimize storage conditions for long-term bacterial survival, including determining optimal storage temperatures and water content (Berninger et al., 2018).

In addition to a long shelf-life, bioformulations must also maintain their effectiveness to colonize their target plants. Interestingly, when examining this factor, LBUM677 was able to maintain its positive effect of promoting plant growth at almost all time-points except the initial time-point of 30 days (Table 2). The effects of LBUM223 on plant growth promotion were not as significant as LBUM677 (as compared to control) and even displayed a negative effect on plant weight at 30 days and 180 days as compared to controls. Differences in the activity of the two strains under study is most likely due to differences in their biochemical and/or genetic characteristics. This has previously been observed in bentonite and talc formulations of *Pseudomonas* sp. and CKK-3 on cotton seedlings. Strain was

found to be more effective than strain CKK-3, likely due to differences in characteristics of this isolate (Sobhan et al., 2011). Other studies have shown the positive effects of *Pseudomonas* sp. bioformulations on various plant species. Specifically, four talc formulations of *Pseudomonas* sp. isolates CHAO, EP1, KKM, and VPT4 were successful in improving millable sugarcane height under field conditions (Viswanathan and Samiyappan, 2008). Wheat yield was improved by 15–25% over controls when using *Pseudomonas moraviensis* and *Bacillus cereus* bioformulations (Hassan and Bano, 2015). Seed treatment of wheat with *P. fluorescens* RRB-11 in talc led to significantly increased plant growth parameters and even increased seed germination by 94% over controls (Jambhulkar and Sharma, 2013). *Pseudomonas fluorescens* bioformulations showed the highest effectiveness of 8 different bioformulations on the fresh weight of sugar beets (Jorjani et al., 2011). *Pseudomonas putida* PT inoculated on maize seeds significantly enhanced the fresh and dry weights of the plants at harvest time (Khashei et al., 2020). Finally, chickpea seeds treated with talc or peat formulations of *Pseudomonas* spp. effectively controlled chickpea wilt disease in two field trials and increased the chickpea yield (Vidhyasekaran and Muthamilan, 1995).

In addition to the effect of the biofertilizers on the plant's weight, we also examined the persistence of each bacteria in the rhizosphere of corn growwell. The rhizosphere competency of a bacterial strain is important for its ability to be a successful PGPR (Compant et al., 2019; Rilling et al., 2019). Also, before a new PGPR strain is developed into a bioformulation and commercialized for field applications, it is essential for regulatory considerations to develop reliable bioassays capable of detecting and quantifying the strain of interest among the complex soil microbiome (Mathre et al., 1999). In our study, specific qPCR bioassays were used for both strains under study, which successfully allowed to detect and quantify both strains in rhizosphere soil. Surprisingly, only few studies to date examined the population levels of inoculated bacteria in the rhizosphere and instead relied on increases in plant biomass and other plant parameters to confirm successful PGPR activity (Rilling et al., 2019).

Another important consideration when testing a bioformulation's viability and establishment in the rhizosphere is the capacity to translate results obtained in controlled conditions, often in pots, to the field. Various *Pseudomonas* strains in peat

carrier have been tested in pot and field trials. Most strains displayed plant yield increases as compared to controls, however the results for a given strain were not consistent between pot and field trials (Shaharoon et al., 2007; Tabassum et al., 2017). Weather conditions, soils, plants, and the indigenous microorganisms in fields are all variable, leading to no specific rules for optimizing the inoculation of biofertilizers in soil (Khare and Arora, 2015). The success of the biofertilizer in field soil is dependent on its survival and ability to function in this heterogeneous environment. Abiotic factors (e.g., soil texture, pH, temperature and moisture levels) in the field exert their effects on inoculant population dynamics by imposing various stresses on the microbial cells (Evans et al., 1993; Khare and Arora, 2015). In our study, non-sterile field soil was used when performing pot studies, to help account for these abiotic factors found in this type of soil. This approach has previously allowed us to minimize the differences often observed between pot and field results with various PGPR and plant systems (St-Onge et al., 2011; Novinscak and Filion, 2018; Jiménez et al., 2020). The next step to validate the results obtained in this study will be to perform similar inoculation experiments under field conditions and track the PGPR strains in soil using the qPCR bioassays already available for LBUM223 and LBUM677. In parallel, the efficacy of the same carriers and inoculation approach will be validated for other *Pseudomonas* strains in development in our laboratory.

In conclusion, despite the apparent difficulty to develop stable bioformulations for non-sporulating plant beneficial *Pseudomonas* PGPR, significant progress is being made. Peat and talc-based carriers show great potential to assure viability of *Pseudomonas* strains at room temperature for many months

and retain their ability to colonize the rhizosphere and achieve plant beneficial activity. Despite strain-specific differences, which need to be validated for each *Pseudomonas* strain of interest, both carriers yield similar results with no clear superiority of one over the other. The development of stable bioformulations is required for the commercialization of these organisms to be successfully used for plant growth promotion and/or biocontrol of plant pathogens in sustainable farming practices.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

AN and MF contributed to the conception, design of the study, contributed to manuscript revision, read, and approved the submitted version. AN was responsible for conducting the experiments, the analyses, and writing the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. A patent has been granted on *P. fluorescens* LBUM677 and its use to enhance total lipid and SDA yields in an oilseed crop (US patent 10,165,743).

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# Effect of Plant Growth Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF) on Salt Stress Tolerance of *Casuarina obesa* (Miq.)

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Salinity is one of the main abiotic stresses limiting plant growth and development. However, the use of salt-tolerant plants combined with beneficial soil microorganisms could improve the effectiveness of biological methods for saline soil recovery. The aim of this study is to identify the *Casuarina obesa*/ Arbuscular Mycorrhizal fungi (AMF)/Plant Growth Promoting Rhizobacteria (PGPR) association that could be used in salt-land rehabilitation programs. Thus, the plants were grown under greenhouse on sandy soil, inoculated either with PGPR (*Pantoea agglomerans* and *Bacillus* sp.), or with AMF (*Rhizopogon fasciculatus* and *Rhizopogon aggregatum*) or co inoculated with PGPR and AMF and watered with a saline solution (0, 150, and 300 mM). After 4 months of cultivation, the plants were harvested and the results obtained showed that inoculation improves the survival rate, height and biomass of the plants compared to the control plants. The results also showed that inoculation increases the total amount of chlorophyll and the accumulation of plant proline at all levels of salt concentration. However, *P. agglomerans* and *Bacillus* sp. strains alone or in combination with *R. fasciculatus* increased plant growth. This study showed that these strains of PGPR, whether or not associated with AMF, could be biological tools to improve *C. obesa* performance under saline stress conditions.

**Keywords:** *Casuarina obesa*, beneficial microorganisms, land rehabilitation, salt stress, plant tolerance

## INTRODUCTION

Soil salinization is one of the major environmental stress for plant growth (Barnawal et al., 2014). About 10% of the total arable land as being affected by salinity and sodicity (Shahid et al., 2018). This phenomenon is constantly increasing and constitutes an obstacle to agricultural production (Munns and Tester, 2008). In Senegal, about 55% of arable land is affected by salinization (LADA, 2009), which considerably affects the potential for national agricultural production. Land salinization is most often the result of natural factors such as rainfall deficiency, seawater intrusion, capillary rise due to evaporation and rock solubilization, but also of anthropogenic factors such as poor agricultural practices (Legros, 2009). This salinization leads to a degradation of the biological, chemical and physical properties of soils, resulting in decreased of soil fertility and crop yields (Ndour, 2006). With increasing population growth, access to land is becoming more difficult. Therefore, there is an urgent to develop effective strategies to rehabilitate salt land despite the many economic and climatic constraints around the world. Thus, several strategies have been adopted by rural populations and development actors. These include: (i) mechanical control through the installation of anti-salt structures (dams, or bunds), (ii) chemical control through the application of chemical amendments such as gypsum, sulfur or sulphuric acid to neutralize alkalinity, and (iii) biological control through the introduction of salt-tolerant plant species or organic matter to rehabilitate saline soils (Fall et al., 2018). The use of salt-tolerant plants is an appropriate approach to salt land management (Singh, 2000). Indeed, the presence of trees favors the development of micro flora and micro fauna essential for biodegradation processes, which represent the basis for the soil fertility. However, the success of a revegetation strategy in these salt areas will require the use of local or introduced plant species that are best adapted to environmental conditions. To this end, trees of the *Casuarinaceae* family are widely used in these programs because of their ability to grow on very poor soils (Patil et al., 2005; Diagne et al., 2014). The growth of *Casuarina* species on these degraded soils may be related to their ability to associate with nitrogen-fixing bacteria (*Frankia*) or PGPR and AMF that improve the plant's nitrogen and phosphate nutrition. Indeed, these symbiotic associations constitute means of adapting plants to unfavorable environmental conditions such as saline stress (Beltrano et al., 2013). They also play an important role in plant growth under various conditions by modifying their root systems and increasing the mobilization and absorption of essential elements such as N and P (Hashem et al., 2016). The aim of this study were to select the most salt-tolerant *C. obesa*/PGPR/AMF combinations for the rehabilitation of land degraded by salinization.

## MATERIALS AND METHODS

### Plant Material

The plant species used in this experiment were derived from seeds of *C. obesa* (lot number: 17994) provided by CSIRO/ATSC (Australian Tree Seed Centre; <https://www.csiro.au/en/Research/Collections/ATSC/Purchasing-seed>). They were collected in the

localities of Mullewa (latitude 28°15' North, longitude 115°38' East, altitude 250) in Australia. About 2,800 viable seeds were counted in a 10 g lot. These seeds were not pre-treated and were stored at 4°C in the laboratory.

### Bacterial and Fungal Material

The strains *Pantoea agglomerans* lma2 (*P. agglomerans*) and *Bacillus* sp. were provided by the Laboratory of Applied Microbiology, Department of Microbiology, Faculty of Natural and Life Sciences, Ferhat Abbas Setif-Algeria University. These strains have been isolated in the Bou-saâda region of Algeria (Hasfa, 2014). They were isolated from the rhizosphere of wheat under arid soil affected by salinity. *P. agglomerans* (accession number GQ478022) is a Gram-negative bacterium and has been identified by sequencing RNA16S. These strains were initially cultured in tubes containing 5 ml of liquid Luria-Bertani (LB) Broth medium and placed under agitation in a bacterial culture chamber at 30°C for 48 h. After a second culture was made in tubes containing 500 ml of Broth LB medium and placed in a culture chamber for 24 h under the same conditions. The liquid bacterial cultures thus produced were used for inoculation of the plants.

The arbuscular mycorrhizal fungi used in this study were: *Rhizophagus fasciculatus* (Rf) (Thaxt.) C. Walker and A. Schüßler DAOM227130 isolated in Quebec (Schüßler and Walker, 2010) and *Rhizophagus aggregatum* (Ra) (N.C. Schenck and G.S. Sm.) C. Walker DAOM2277128 isolated in Burkina Faso. These AMF provide from the collection of "Laboratoire Commun de Microbiologie" (LCM). AMF inoculum was produced during 6 months under greenhouse using a mycotrophic plant (*Zea mays* L.) grown in the presence of AMF in pots containing 1.5 kg of sandy soil previously sterilized at 120°C for 2 h. The soil used for the study was collected at Sangalkam in Senegal (14° 46'52" N, 17° 13'40" O) with the following physico-chemical characteristics: pH (H<sub>2</sub>O) 6.5; clay 3.6%; fine silt 7.4%; fine sand 36.6%; coarse sand 21.55%; total carbon 0.54%; total nitrogen 0.06%; C/N 8.5; total phosphorus 39 mg kg<sup>-1</sup> and soluble phosphorus 4.8 mg kg<sup>-1</sup> (Diouf et al., 2005). The inoculum consisted of a mixture of spores and root fragments. The spore count was estimated using the Gerdemann and Nicolson (1963) method. The spore density of *R. fasciculatus* and *R. aggregatum* per 100 g of soil was 1,210 and 1,635 spores, respectively.

### Plant Growth, Experimental Design, and Application of Salt Stress

*C. obesa* plants were cultivated in nursery (30°C) on sterile soil and watering with tap water at a frequency of 2 times/day. After 3 months of cultivation the seedlings were carefully dug out and transferred into pots of dimensions (25 × 12 × 50 cm) filled with a sandy soil sieved to 2 mm and autoclaved at 121°C for 120 min. Each pot was received a seedling and kept in a nethouse at 30°C at ISRA/CNRA (Institute Senegalese of Agriculture Research/National Center for Agronomical Research, 14°71 North - 16°48 West Bambey, Senegal). The soil used in our experiment was taken at CNRA (14°71 North - 16°48 West Bambey, Senegal). The physicochemical characteristics of this soil were determined at Laboratory of soil, plant and water,

ISRA/CNRA, Bambey, Senegal and are as follows: pH (H<sub>2</sub>O) 7.78; pH (KCl) 7.46; EC 218.4  $\mu$ S/cm; assimilable phosphorus 8.915 ppm; total phosphorus 3.896 ppm; total nitrogen 0.057%; organic carbon 0.631%; organic matter 1.09% and C/N 11.15. Fungal inoculum was applied at the time of plant transplantation. Treatments with AMF received 10 g of inoculum *R. aggregatum* strain or 10.12 g of the *R. fasciculatus* strain placed at about 3–5 cm depth. Bacterial inoculum was applied 1 week after transplanting close to the seedling root system. Treatments with bacteria received 5 ml of bacterial inoculum from *P. agglomerans* or the *Bacillus* sp. strain with a final absorbance of 0.2 measured at  $\lambda = 595$  nm for each strain.

Salt stress was applied 2 months after inoculation to allow the establishment of mycorrhizal symbiosis. For each treatment, salt stress was gradually applied twice a week. Then, a weekly increase in NaCl concentration was performed to avoid osmotic shock. After 3 weeks of acclimatization, the control plants were watered with 0 mM and the stressed plants with 150 and 300 mM of NaCl. The choice of these concentrations was made on the basis of previous studies on *Casuarina* by Djighaly et al. (2018).

After 4 months the plants were harvested. Parameters such as height growth, total biomass, chlorophyll and proline contents and mycorrhizal frequency and intensity rate were evaluated.

The following treatments were applied: C: control plants not inoculated; P. agg: inoculated with *Pantoea agglomerans*; B25: inoculated with *Bacillus* sp.; Rf: inoculated with *Rhizophagus fasciculatus*; Ra: inoculated with *Rhizophagus aggregatum*; B25 + Rf: co-inoculated with *Bacillus* sp. and *Rhizophagus fasciculatus*; B25 + Ra: co-inoculated with *Bacillus* sp. and *Rhizophagus aggregatum*; P. agg + Ra: co-inoculated with *Pantoea agglomerans* and *Rhizophagus aggregatum*; P. agg + Rf: co-inoculated with *Pantoea agglomerans* and *Rhizophagus fasciculatus*. For each treatment seven repetitions were applied randomized device separated into blocks for each treatment to avoid contamination.

## Measurement of Height Growth, Above-Ground, Root, and Total Biomass

The height (cm) growth of the plants was measured each month using a graduated ruler. The survival rate was determined using the formula:

$$\text{Survival rate (\%)} = \frac{\text{number of survival plants}}{\text{number total of plants}} \times 100 \quad (1)$$

After 4 months of greenhouse cultivation, the plants were harvested and the fresh weight of the aerial (BA) and root (BR) biomass produced for each treatment was weighed. The aerial and root parts were dried in the oven at 65°C for 1 week. Once the samples were completely dry, their dry weight was determined using an electronic precision balance TE1245 (Sartorius AG, Germany).

## Determination of Chlorophyll and Proline Content

The chlorophyll content (a) and (b) of the aerial parts of the plants was determined using Arnon (1949) method, modified and

described by Tahri et al. (1998). The total chlorophyll content (a+b) was calculated according to Arnon (1949) formula:

$$\text{CHt (mg/l)} = [8, 02 \times \text{DO (663 nm)} + 20, 2 \times \text{DO (645 nm)}] \times V / M$$

where V = volume of acetone (10 ml); M = foliar mass (100 mg); DO = optical density in nm.

Proline levels in the leaves were determined using the method described by Monneveux and Nemmar (1986). A quantity of 100 mg of fresh leaves were used and the samples were measured with a spectrophotometer at a wavelength of 520 nm. Proline contents were calculated using the equation derived from the calibration curve constructed from a range of known and increasing proline concentrations from 0 to 800  $\mu$ moles.

## Mycorrhization

To determine the frequency and intensity of mycorrhization, the roots of the plants were cleaned and stained using the Phillips and Hayman (1970) method. For each plant, 100 fragments of 1 cm were used for the observations under the microscope. The frequency of mycorrhization was determined by the formula: F% = (number of mycorrhized fragments/total number of observed fragments)  $\times$  100. Mycorrhization intensity was analyzed using the method of Trouvelot et al. (1986) using a range of colonization intensity noted from zero (0) to five (5). It was estimated by the formula: I% = (95n<sub>5</sub> + 70n<sub>4</sub> + 30n<sub>3</sub> + 5n<sub>2</sub> + n<sub>1</sub>)/total number of observed fragments where n<sub>5</sub> = number of noted fragments 5; n<sub>4</sub> = number of noted fragments 4; n<sub>3</sub> = number of noted fragments 3; n<sub>2</sub> = number of noted fragments 2; n<sub>1</sub> = number of noted fragments 1.

## Statistical Analysis

The data were subjected to a two-factor analysis of variance (ANOVA) (inoculation and salinity level). The averages of the variables measured at the 5% probability threshold ( $p \leq 0.05$ ) were compared using the Student Newman-Keuls test. The tests and statistical analysis of the results were performed with GenStat Edition 17 software.

## RESULTS

Analysis of the variance reveals a significant effect of factor Inoculation and salt concentration (NaCl) on growth parameters such as plant height, shoot and root biomass (Supplementary Table 1). The interaction “Inoculation  $\times$  [NaCl]” had significant effects on chlorophyll and proline content.

## Effect of Inoculation With PGPR and/or AMF on the Survival Rate of *C. obesa* Plants Under Salt Stress Conditions

The results showed that all plants survived 1 month after salt stress application (Table 1). At harvest (2 months after salt application), plant mortality was noted only at 300 mM NaCl. The highest survival rate was observed in plants inoculated with *P. agglomerans* (100%) at 300 mM. However, this rate decreased in plants inoculated with B25 + Ra and Ra (80.95%), followed by control plants (C) and those inoculated with Rf + B25 and Rf

**TABLE 1** | Survival rate of *C. obesa* plants after 1 and 2 months of salt application.

Treatments	Survival rate of <i>C. obesa</i> plants (%)					
	After one (1) month of salt			After (2) months of salt		
NaCl	0 mM	150 mM	300 mM	0 mM	150 mM	300 mM
C	100 a	100 a	100 a	100 a	100 a	85,71 ab
P.agg	100 a	100 a	100 a	100 a	100 a	100 a
B25	100 a	100 a	100 a	100 a	100 a	90,47 a
Rf	100 a	100 a	100 a	100 a	100 a	85,71 ab
Ra	100 a	100 a	100 a	100 a	100 a	80,95 b
B25 + Rf	100 a	100 a	100 a	100 a	100 a	85,71 ab
B25 + Ra	100 a	100 a	100 a	100 a	100 a	80,95 b
P.agg + Rf	100 a	100 a	100 a	100 a	100 a	90,47 a
P.agg + Ra	100 a	100 a	100 a	100 a	100 a	90,47 a

C, control plants not inoculated; P. agg, inoculated with *Pantoea agglomerans*; B25, inoculated with *Bacillus* sp.; Rf, inoculated with *Rhizophagus fasciculatus*; Ra, inoculated with *Rhizophagus aggregatum*; B25 + Rf, co-inoculated with *Bacillus* sp. and *Rhizophagus fasciculatus*; B25 + Ra, co-inoculated with *Bacillus* sp. and *Rhizophagus aggregatum*; P. agg + Ra, co-inoculated with *Pantoea agglomerans* and *Rhizophagus fasciculatus*; P. agg + Rf, co-inoculated with *Pantoea agglomerans* and *Rhizophagus fasciculatus*. Each value represents the mean of plants used for each treatment ( $n = 7$ ); lower case letters (a, b) indicate significant differences to a two-factor analysis (inoculation and salinity level) according Student Newman-Keuls test ( $p < 0.05$ ).

with (85.71%) and in plants inoculated with P.agg + Rf, P.agg + Ra and B25 (90.47%) at 300 mM (Table 1).

### Effect of Inoculation With PGPR and/or AMF on Height, Total Dry Biomass of *C. obesa* Plants Under Saline Stress Conditions

Results obtained after 4 months of growing in greenhouse showed that the increasing of salt concentration lead to a decrease of plant height from 35.39% to 150 mM and 64.67% to 300 mM compared to controls at 0 mM (Table 2). In the presence of 150 mM, inoculation with P. agg + Rf improved the height of *C. obesa* plants by 13.27% compared to control plants (Table 3). At 300 mM, inoculation with PGPR and/or AMF have no significative effect on *C. obesa* height.

The Aerial biomass of *C. obesa* was improved by the inoculation with P. agg + Rf compared to control plants at all concentration of NaCl. At 300 mM, only inoculation with P.agg + Rf improved the root biomass of *C. obesa* plants.

The total biomass of *C. obesa* was improved by the inoculation with P. agg, P. agg + Rf and B25 + Rf compared to control plants in the absence of salt. At 150 and 300 mM, a significant increase was observed in plants co-inoculated with P. agg + Rf of 34.24 and 76.62%, respectively, compared to control plants (Table 2).

### Effect of Inoculation With PGPR and/or AMF on the Total Chlorophyll and Proline Contents of *C. obesa* Under Salt Stress Conditions

The total chlorophyll contents of *C. obesa* plants was evaluated after 4 months of salt stress and the results obtained

show a significant increase in chlorophyll contents in plants inoculated with *P. agglomerans* compared to control plants at all concentration. The inoculation with P. agg increase chlorophyll content by 25.56, 28.85, and 51.55% at 0, 150, and 300 mM, respectively, compared to controls plants (Table 3). At 150 mM, inoculation with P. agg + Rf had increased the total chlorophyll content of *C. obesa* compared to the control plants.

The results obtained show an increase in proline synthesis as a function of salt concentration. In the absence of salt, the proline content is not significantly different between inoculated and non-inoculated plants. In the presence of 150 mM, there is an increase in proline levels in plants inoculated with P. agg and B25 compared to control plants. At 300 mM, a significant difference was noted in plants inoculated with P. agg and B25 compared to plants (Table 3).

### Effect of Salt Stress on the Mycorrhization of *C. obesa* Plants Under Salt Stress Conditions

The highest frequency of mycorrhization was observed in co-inoculated plants inoculated with P.agg + Rf at all concentration. Concerning the intensity of mycorrhization, the higher intensity was observed with plants co-inoculated P. agg + Rf compared to controls plants at 0 and 150 mM. At 300 mM, no difference was noted between treatments compared to controls plants (Table 4).

## DISCUSSION

The effect of inoculation with AMF and PGPR strains was studied on *C. obesa* plants subjected to different concentrations of NaCl (0, 150, and 300 mM) for 4 months under greenhouse. The results obtained show that inoculation with *P. agglomerans* strain improves the survival rate of plants compared to control plants. After 2 months of salt stress application, the survival rate of plants inoculated with *P. agglomerans* strain was 100%. This result could be explained by the intrinsic ability of this strain to tolerate salt. The works of Hasfa (2014) was showed that *P. agglomerans* lma 2 tolerated high salt concentrations and that its growth increased to NaCl concentrations between 100 and 400 mM. Inoculation with *Bacillus* sp. and *P. agglomerans* strains combined with *R. fasciculatus* or *R. aggregatum* also increased the survival rate of *C. obesa* (90.47%) compared to control plants. *R. aggregatum* strain compared to controls did not improve plant survival.

The efficacy of co-inoculation with P.agg + Rf and P.agg + Ra could be related to the ability of PGPR to solubilize phosphates and the effective absorption of solubilized P from soil through AMF hyphae (Richardson et al., 2009; Saia et al., 2020). This association allows better colonization of the plant by AMF through these PGPR, which are well known as Mycorrhiza Helper Bacteria (Garbaye, 1994). This improved colonization results in better soil exploration by the mycorrhized roots of the plant and better absorption of mineral elements by the hyphae of fungi (Smith and Read, 2008). This statement is in agreement with our results which showed that the frequency of mycorrhization was significantly improved by co-inoculation P.agg + Rf compared to plants inoculated with AMF strains



**TABLE 2 |** Effect of inoculation with PGPR and/or AMF on height, shoot biomass, root biomass and total dry biomass of *C. obesa* plants under salt stress conditions.

	Control	P.agg	B25	Ra	Rf	P.agg + Rf	P.agg + Ra	B25 + Rf	B25 + Ra
<b>HEIGHT (cm)</b>									
0 mM	45.52 abc	52.50 a	52.21 ab	48.32 abc	44.41 abc	49.08 abc	43.78 c	45.11 abc	43.92 bc
150 mM	29.41 de	28.34 de	31.12 cd	26.22 de	29.81 de	33.91 c	25.54 de	27.98 de	23.21 e
300 mM	16.08 f	28.88 de	20.85 e	11.24 f	20.80 e	23.88 e	21.21 e	18.47 e	13.60 f
<b>AERIAL BIOMASS (g)</b>									
0 mM	1.26 bcd	1.65 ab	1.58 abc	1.22 cd	1.37 abcd	1.72 a	1.15 d	1.46 abcd	1.54 abcd
150 mM	0.38 fgh	0.40 fgh	0.44 ef	0.23 gh	0.42 efg	0.61 e	0.20 h	0.34 fgh	0.30 fgh
300 mM	0.12 i	0.42 efg	0.34 fgh	0.11 i	0.12 i	0.57 e	0.25 h	0.27 h	0.09 j
<b>ROOT BIOMASS (g)</b>									
0 mM	0.32 b	0.47 ab	0.44 b	0.32 b	0.39 b	0.48 ab	0.30 b	0.65 a	0.33 b
150 mM	0.12 cde	0.13 cd	0.11 cde	0.08 e	0.11 cde	0.14 c	0.08 e	0.11 cde	0.09 de
300 mM	0.05 e	0.12 cde	0.08 e	0.02 e	0.05 e	0.20 c	0.09 de	0.06 e	0.03 e
<b>TOTAL DRY BIOMASS (g)</b>									
0 mM	1.58 bc	2.12 a	2.03 ab	1.55 bc	1.76 abc	2.21 a	1.45 c	2.11 a	1.87 abc
150 mM	0.50 de	0.53 de	0.55 de	0.31 f	0.54 de	0.76 d	0.28 f	0.45 de	0.39 ef
300 mM	0.18 f	0.54 de	0.42 de	0.14 g	0.19 f	0.77 d	0.34 ef	0.33 f	0.13 g

Each value represents the mean of plants used for each treatment ( $n = 7$ ); lower case letters (a–j) indicate significant differences to a two-factor analysis (inoculation and salinity level) according Student Newman-Keuls test ( $p < 0.05$ ).

**TABLE 3 |** Total chlorophyll and proline content of *C. obesa* plants inoculated with PGPR and/or AMF subject to 0, 150, and 300 mM NaCl.

	Chlorophyll content (mg.g-1FM)			Proline content ( $\mu\text{mol/g MF}$ )		
	0 mM	150 mM	300 mM	0 mM	150 mM	300 mM
Control	2.33 b	2.12 b	0.96 f	57.14 e	51.82 e	25.03 fg
P.agg	3.09 a	2.98 a	1.98 bc	49.54 e	103.24 bc	90.19 c
B25	1.44 d	1.88 c	1.25 e	63.14 de	156.21 a	87.82 c
Ra	1.68 cd	1.82 c	0.58 g	53.97 e	136.52 ab	65.64 de
Rf	1.51 d	1.60 cd	0.98 f	47.18 e	61.4 de	77.71 d
P.agg + Rf	1.89 c	2.19 b	1.49 d	37.23 f	36.06 f	17.00 g
P.agg + Ra	1.92 bc	1.96 bc	1.25 e	31.85 f	70.21 d	33.45 f
B25 + Rf	1.93 bc	2.07 b	1.19 e	34.12 f	67.44 de	50.9 e
B25 + Ra	1.90 bc	1.57 d	0.92 f	52.13 e	46.61 e	38.92 f

Each value represents the mean of plants used for each treatment ( $n = 7$ ); lower case letters (a–g) indicate significant differences to a two-factor analysis (inoculation and salinity level) according Student Newman-Keuls test ( $p < 0.05$ ).

alone in the presence of salt. Several studies have shown that symbiotic associations between beneficial soil bacteria (PGPR or Rhizobia) and the PGPR can improve root colonization of plants by AMF in many species such as: *Acacia auriculiformis* and *Acacia mangium* (Diouf et al., 2005), *Acacia senegal* (Ndoye et al., 2012) and *Litchi chinensis* (Visen et al., 2017). These microorganisms are regulators of stress by adjusting nutritional and hormonal balance and inducing systemic stress tolerance (Ruiz-Lozano et al., 2012). This regulation can induce an increase in K<sup>+</sup> content accompanied by an effective decrease in Na<sup>+</sup> in plant tissues. Additional experiments aimed to evaluate the variations of K<sup>+</sup> and Na<sup>+</sup> and Cl<sup>-</sup> concentrations in response to the inoculation with P.agg + Rf and P.agg + Ra will help to determine whether a similar mechanism is involved. However, the result obtained with other combinations could be explained

by a less effective symbiosis between the two strains. Artursson et al. (2006) showed that the success of co-inoculation depends not only on the symbiotic efficacy of microorganisms but also on the compatibility between different symbiosis partners. Moreira et al. (2019) showed a synergistic effect between AMF and PGPR in increasing maize growth in saline conditions. This effect was related to an increase in K<sup>+</sup> content accompanied by an effective decrease in Na<sup>+</sup> in plant tissues.

The positive effect co-inoculation P. agg + Rf on growth parameters could be explained by the fact that *P. agglomerans*, is a halophilic, nitrogen-fixing and phosphate-solubilizing strain, capable of producing of indole acetic acid (AIA) up to 600 mM NaCl (Hasfa, 2014) and AMF (*R. fasciculatus*) in improving salt tolerance stress among *Casuarina* species (Djighaly et al., 2018). In the presence of salt, these microorganisms participate

**TABLE 4 |** Effects of the co-inoculation of PGPR and AMF on the frequency and intensity of mycorrhization of *C. obesa* plants under salt stress.

	Frequency of mycorrhization (%)			Intensity of mycorrhization (%)		
	0 mM	150 mM	300 mM	0 mM	150 mM	300 mM
Ra	26.25 b	23.75 b	16.00 b	1.37 c	3.14 b	1.57 c
Rf	34.50 a	31.25 b	15.00 b	6.34 b	3.26 b	0.82 c
P.agg + Rf	37.00 a	45.50 a	27.25 a	9.14 a	12.41 a	2.88 bc
P.agg + Ra	22.00 b	43.25 a	16.75 b	0.54 c	8.12 ab	0.45 c
B25 + Rf	15.00 c	35.00 ab	22.00 ab	1.12 c	4.91 b	0.90 c
B25 + Ra	23.00 b	29.55 b	25.75 a	1.74 c	2.35 bc	2.94 bc

Each value represents the mean of plants used for each treatment ( $n = 7$ ); lower case letters (a–c) indicate significant differences to a two-factor analysis (inoculation and salinity level) according Student Newman-Keuls test ( $p < 0.05$ ).

in the selective absorption of ions such as phosphorus, nitrogen and magnesium and in the reduction of  $\text{Na}^+$  ion absorption (Giri and Mukerji, 2004; Chen et al., 2014; Paul and Sinha, 2017). According to Ruiz-Lozano et al. (2012), improving plant tolerance to salinity by adding endomycorrhizae leads to increased photosynthetic activity and better water absorption efficiency.

The higher frequencies and intensities in co-inoculated P.agg + Rf plants would explain the salt tolerance of this strain. The work of Djighaly et al. (2018) showed significant metabolic activity of *R. fasciculatus* at 300 mM NaCl. But also to the additional effect of PGPR which improves the phosphate nutrition of the plant (Egamberdieva et al., 2019).

The high total chlorophyll content observed in plants inoculated with *P. agglomerans* in the presence of salt could explain the better growth rates observed in these plants. However, we noted that *P. agglomerans* strain associated with *R. fasciculatus* was more effective than *R. aggregatum* strain associated with the same strains. This result could be explained by a less effective symbiosis between the AMF (*R. aggregatum*) strain co-inoculated with the PGPR (*P. agglomerans*). A significant accumulation of proline was obtained with inoculated plants *P. agg* and B25 in the presence of salt (150 and 300 mM) compared to control plants. Proline accumulation is a mechanism of resistance to saline stress by adjusting intracellular osmotic pressure. These results are in agreement with works of Hasfa (2014) who showed that inoculation with *Bacillus* sp. and *Pantoea agglomerans* strain improve salt stress tolerance of wheat. However, our results showed that *P. agglomerans* + *R. fasciculatus* treatment had no significantly improve proline concentration in presence of NaCl. This result does not explain the good behavior of *C. obesa* plants co-inoculated with *P. agglomerans* + *R. fasciculatus*. However, it could also be supposed that this combination (*P. agglomerans* + *R. fasciculatus*) could establish other salt tolerance mechanisms such as the production of antioxidant enzymes, stress hormones and the overexpression of genes involved in salt stress tolerance. The works of Gond et al. (2015) showed that *P. agglomerans* improved the growth capacity of tropical maize compared to uninoculated controls. These results were explained by the up-regulation of the aquaporin gene family, especially plasma

membrane integral protein (ZmPIP) genes in *P. agglomerans*-treated plants.

## CONCLUSION

The results of the experiment show that inoculation with PGPR and/or AMF can improve resistance to salinity of *C. obesa* plants by increasing their growth parameters. In general, chlorophyll and proline content were also improved in plants inoculated under salt stress. This positive effect of inoculation was more pronounced with *P. agglomerans* and with *P. agglomerans* + *R. fasciculatus*. Thus, inoculation with salt tolerant PGPR and/or AMF could be a solution for the rehabilitation of land affected by the salt in Senegal. It would be interesting to carry out further research in field conditions to confirm the performance of these strains and then isolate indigenous strains of PGPR and *Frankia* from saline soils and determine their impact on salt tolerance in *Casuarina* inoculated with AMF.

As *Casuarina* species are associated with *Frankia* strains that play a critical role on their performance. It would be interesting the study the interaction of *Casuarina* species, salt tolerant AMF and PGPR strains.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

MNd, PID, MNg, GNd and ND did the experimental work and analysis thereof and wrote the manuscript. ND, DNg, SS, and HC-S contributed in designing, supervision, and interpretation of the results. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.601004/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Plant Growth-Promoting Bacteria Improve Growth and Modify Essential Oil in Rose (*Rosa hybrida* L.) cv. Black Prince

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Rose essential oil is rich in compounds widely used by the pharmaceutical and cosmetic industry, due to the biological activities it presents. However, obtaining oil is costly, as the yield per plant is low, which requires several techniques that aim to increase its production. The application of growth-promoting bacteria has been studied for this purpose. Thus, the objective of this work was to select efficient bacteria for production and evaluate their influence on the phytotechnical characteristics and composition of the essential oils of roses. Seven species of bacteria were evaluated for the potential to promote growth *in vitro*, being tested for nitrogen fixation, phosphate solubilization, protease production and auxin production. From bacteria tested, four were selected and inoculated on rose plants of cultivar Black Prince to evaluate the influence on phytotechnical variables of flower and stem and the oil production. The evaluation of the production of roses was performed through the characteristics of the flowers (size, weight, and diameter of the stem) and floral bud. The essential oils from the inoculated flowers were extracted and evaluated in terms of content, yield, and chemical composition. The application of *B. acidoceler*, *B. subtilis* and *B. pumilus* resulted in flowers with a diameter up to 29% larger. The floral stem was increased by up to 24.5% when *B. acidoceler* and *B. pumilus* were used. Meanwhile, the stem diameter was around 41% greater in the presence of *B. acidoceler*, *B. subtilis* and in the control. *Bacillus pumilus* also increased the weight of fresh petals (104%) and essential oil yield (26%), changing the chemical composition of the extracted essential oil. Thus, it is concluded that *B. acidoceler*, *B. pumilus*, and *B. subtilis* improved the phytotechnical characteristics of roses. Among bacteria, *B. pumilus* increased the essential oil content as well as positively changed the chemical composition of the extracted essential oil.

**Keywords:** *Bacillus* sp, biological activity, content, efficient microorganisms, *Rosa hybrida* L.

## INTRODUCTION

Rose (*Rosa hybrida* L. - Rosaceae) is one of the three main flowering plants in the world. Currently, there are approximately 35,000 cultivars bred throughout the world (Bendahmane et al., 2013; Kirov et al., 2014) and around 200 species cataloged (Fougère-Danezan et al., 2015). Roses are cultivated around the world for different purposes, from ornamentation to the manufacture of processed products such as perfumes, medicines, and food using bioactive molecules found in their flowers (Gil et al., 2019). Among these molecules, the essential oils (EO) are one of the most researched in roses. EO are secondary metabolites extracted from plants. They are commonly used in the food, cosmetic and pharmaceutical industries, due to their several biological activities, such as antimicrobial, analgesic, antiseptic, carminative, and diuretic; in many cases, EO also show pleasant diverse aromas (Calo et al., 2015; Basak and Guha, 2018; Sarkic and Stappen, 2018).

Brazil has a prominent place in the production of EO, alongside India, China and Indonesia, mainly due to the production of EO extracted from citrus fruits as by-products of the juice industry (Bizzo et al., 2009). EO of roses is rich in terpenic and sesquiterpenic compounds, which are widely explored by the pharmaceutical and cosmetic industries, mainly for their antioxidant capacity (Patrascu and Radoiu, 2016). Other compounds can also be found in other species of roses. In *Rosa roxburghii*, the EO presents significant amounts of linoleic acid, a powerful poly-unsaturated fatty acid possessing manifold health effects (Yang et al., 2020). The same oil also contains 2'-methylenebis (6-tert-butyl-4-methylphenol) considered a popular antioxidant and anti-aging agent. Phenols and flavonoids are compounds found in *Rosa damascena*, which presents antioxidant and free radical activity (Nikolova et al., 2019).

Worldwide, rose oil production is based on several species, such as *Rosa damascena*, *Rosa gallica*, *Rosa centifolia*, and *Rosa alba* L., the first two species being the most used in oil extraction (Kovatcheva et al., 2011). Among the several hybrid cultivars, the rose cv. Black Prince (also known as rose "Barcarolle") (Avdic et al., 2016) is very widespread throughout Brazil due to its rusticity and beauty. It has medium productivity, high architecture and elongated buttons (Barbosa, 2008). However, there are no reports of oil production of this cultivar, which makes this fact one of the main attractions of the present study.

In Brazil, the production of roses occupies the first position among the cut flowers, corresponding to 30% of the national market (Junqueira and Peetz, 2017). However, one of the problems faced for the acquisition of EO is the low yield per plant, being necessary the use of extraction techniques that increase the yield per flower, or the use of different managements during the cultivation that stimulate the plant production (Pirbalouti et al., 2013).

A new strategy that is being investigated is the use of efficient microorganisms aimed at increasing these molecules of interest. These microorganisms, also known as plant growth promoters (PGP), are used for the most diverse purposes and are capable of providing several benefits (Vejan et al., 2016). In agriculture,

**TABLE 1 |** Identification and origin of bacteria.

Code	Bacteria	Substrate
CCMA 0057	<i>Bacillus acidiceler</i>	Bush pepper fruit ( <i>Piper</i> sp.)
CCMA 0084	<i>Bacillus amyloliquefaciens</i>	Pineapple fruit
CCMA 0058	<i>Bacillus macauenses</i>	Bush pepper fruit
132	<i>Bacillus subtilis</i>	Strawberry fruit
CCMA 0098	<i>Bacillus pumilus</i>	Pequi fruit ( <i>C. brasiliense</i> )
121	<i>Pantoea ananatis</i>	Strawberry leaf
44	<i>Staphylococcus equorum</i>	Strawberry root

the use of PGP has influenced the growth and development of plants, stimulating increased production, aiding in the control of pests and diseases, and activating enzymes capable of producing compounds that improve the quality of products obtained by plants inoculated with them (Saharan and Nehra, 2011; Numan et al., 2018). PGPs can also influence the production of secondary compounds by plants, since many of them have the ability to produce hormones such as auxins, cytokinins and gibberellins or even improve the absorption of nutrients such as nitrogen and phosphorus, which directly affects production and quality of EO from plants (Glick, 2012; Younis et al., 2015).

Bacteria are the most commonly used efficient microorganisms. Studies indicate that bacteria are able to assist plants in their development as well as stimulate the production of compounds that assist in plant defense, such as the production of secondary metabolites responsible for the plant defense system to external factors, which can influence the chemical composition of its aromatic compounds, such as EO (Xie et al., 2019).

In view of the discussed, the objective of this study was to select growth-promoting bacteria for rose cultivation and evaluate their influence on the production of EO.

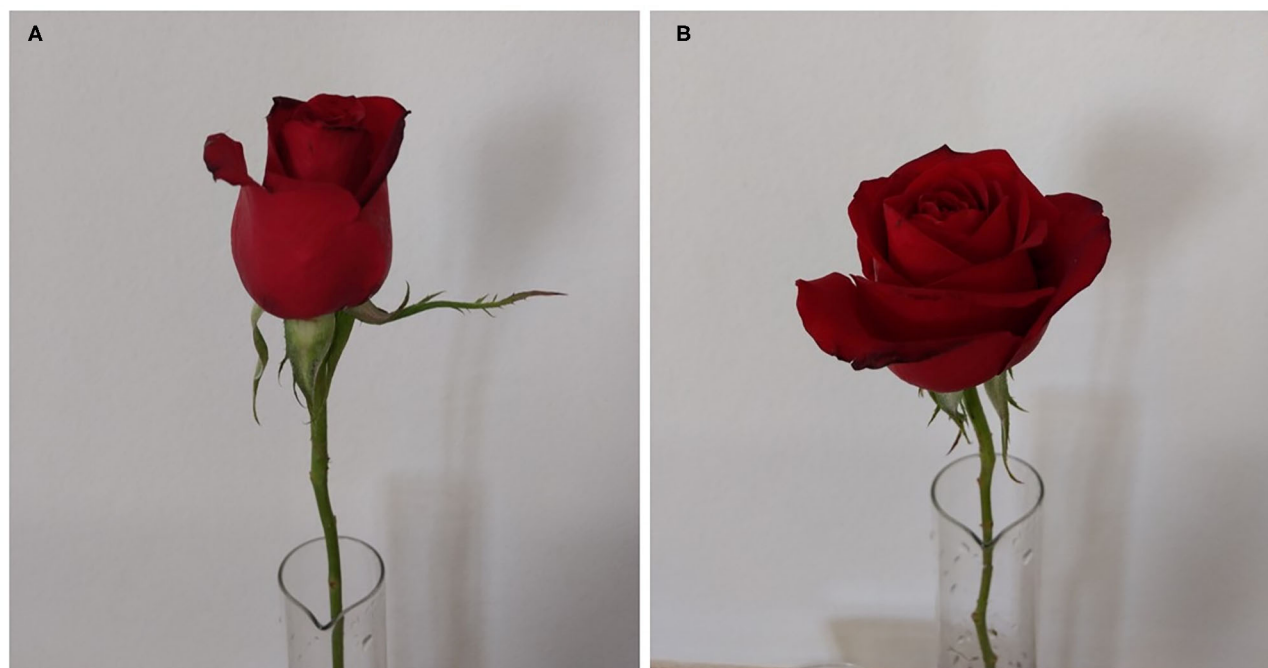
## MATERIALS AND METHODS

### Acquisition of Bacteria and Isolation of the Pathogen

Seven species of bacteria (*Bacillus acidiceler*, *Bacillus amyloliquefaciens*, *Bacillus macauenses*, *Bacillus subtilis*, *Bacillus pumilus*, *Pantoea ananatis*, *Staphylococcus equorum*) were isolated from strawberry plants and also from the Crop Collection of Agricultural Microbiology (CCMA) of the Federal University of Lavras, as shown in **Table 1**.

### Protease Production

Protease production was determined by the method of Sgroy et al. (2009). Petri dishes with Skim Milk Agar (SMA) culture medium were inoculated with 10 µL of bacterial isolates previously cultivated in Luria-Bertami medium (LB) and incubated at 28°C. Halo formation around the colonies was observed after 24 h after inoculation.



**FIGURE 1** | Flower bud opening stages. **(A)** Bud at the harvest point. **(B)** Completely open flower.

## Auxin Production

Cultures of bacteria activated in Petri dishes with medium 523 (10 g sucrose, 8 g casein hydrolysate, 4 g yeast extract, 2 g  $K_2HPO_4$ , 0.3 g  $MgSO_4 \cdot 7H_2O$ , 15 g agar, 1000 mL distilled water) for 24 h were used. Bacteria colonies were transferred to a micro tube containing 1 mL of nutrient broth added of  $0.1 \text{ g L}^{-1}$  of L-tryptophan; the material was maintained in agitation for 3 days in the dark. After this period, the micro tubes were centrifuged at 1500 rpm for 3 min and a 0.5 mL aliquot was removed, which was added of 0.5 mL of Salkowski's reagent. The reaction was incubated for 15 min in the dark, and then a pink or reddish color was observed which signifies the presence of auxins (Kado and Heskett, 1990).

## Phosphate Solubilization Test

For this test, the bacteria were activated 24 h in advance. 10  $\mu\text{L}$  of bacterial isolates grown in 523 liquid medium were inoculated in dishes containing the NBRIP medium (National Botanical Research Institute's Phosphate growth medium—India) (Nautiyal, 1999) which consists of glucose (10 g),  $Ca_3(PO_4)_2$  (5 g),  $MgCl_2 \cdot 6H_2O$  (5 g),  $MgSO_4 \cdot 7H_2O$  (0.25 g), KCl (0.2 g),  $(NH_4)_2SO_4$  (0.1 g), and agar (15 g). The material was incubated at  $28^\circ\text{C}$  with 12 h photoperiod. Halo formation was evaluated around the colonies after 24 h after inoculation.

## Biological Nitrogen Fixation Test

This test was performed using 10 mL flasks containing 5 mL of semi-solid NFB medium, consisting of malic acid (5 g),  $K_2HPO_4$  (0.5 g),  $MgSO_4 \cdot 7H_2O$  (0.2 g), NaCl (0.1 g),  $CaCl_2 \cdot 2H_2O$  (0.02 g), vitamin solution (1 mL), micronutrient solution (2 mL), FeEDTA

(4 mL), bromothymol blue (2 mL), KOH (4.5 g), agar (2 g) and pH 6.8 (Döbereiner et al., 1995). Bacteria with 24 h of growth, grown in medium 523 were inoculated in the medium. An inoculation loop was used to collect bacteria and puncture the medium. Then, they were incubated at  $28^\circ\text{C}$ , making it possible to observe the halo formation in the upper side of the culture medium, indicating the nitrogen fixation by bacteria.

## Effect of Bacteria on the Production of Roses

According to the growth-promoting tests, 4 (four) species of bacteria were selected and inoculated in roses of cultivar Black Prince to evaluate the bacterial effect on the productive characteristics of plants. To prepare the suspension, the bacteria were cultured for 48 h in nutrient broth and the concentration of each suspension was adjusted to  $10^6 \text{ cells mL}^{-1}$ . For inoculation, the plants were pruned at 30 cm from the grafting site, removed from the pots and their roots washed until complete exposure. The inoculation in the plants was done by immersing the bare roots in the suspension for 1 h. After that time, the plants were planted in pots with a capacity of 10 L, containing commercial substrate Topstrato®.

The experiment was carried out in a greenhouse, in a randomized block design with 5 treatments (each selected bacteria plus a control that corresponded to plants inoculated with distilled water only) and 5 replicates. The experimental unit consisted of 4 plants per block.

The evaluations started at the first cutting of stems with flowers at the point of harvest (**Figure 1**). At that point, the floral buds is closed and it changes from a pointed shape to a cup

shape. From the first to 5th day, the following phytotechnical characteristics were evaluated: diameter of the closed bud, diameter of the flower at the point of harvest, stem size, stem diameter, stem weight and total weight of petals per flower.

## Plant Material and EO Extraction

Rose EO was extracted from flowers at the point of harvest. The plants were acquired from a local producer (Roselanche Floriculture—Garden and Floriculture Brandão, Barbacena, Minas Gerais State, Brazil). The fresh petals were taken to an oven with air circulation for drying at 30°C. After drying, they were weighed and the EO was obtained using ~100 g of dry rose petals with distilled water in a round bottom flask. The plant material was subjected to the hydrodistillation process using the modified Clevenger apparatus for a period of 4 h (Brasil, 2010). Three extractions were performed. EO was separated from the hydrolate by a pentane partition process. The organic part was dried using anhydrous sodium sulfate, filtered and rotary evaporated to eliminate the solvent. EO without solvent residues was protected from light and refrigerated.

## Chemical Characterization of EO

The chemical constituents of EO were characterized by gas chromatography coupled to a mass spectrometer (GC-MS, Shimadzu Corporation, model QP2010 Plus, Kyoto, Japan) according to Adams (2007). The following conditions were performed: fused silica capillary column with phase connected to DB5 (30 m x 0.25 mm id, film thickness 0.25 µm); He 5.0 (White Martins, Rio de Janeiro, Brazil) as carrier gas and flow rate of 1 mL min<sup>-1</sup>. The injector temperature was 220°C and the detector temperature was 240°C. The injection volume of the sample was 0.5 µL diluted in hexane (1%) (Sigma-Aldrich®, St. Louis, MO, USA) with a division ratio of 1:100. Programmed temperature of 60°C increasing to 240°C at a rate of 3°C per min and from 240°C to 300°C at 10°C per min. The final temperature was maintained for 7 min. The operational parameters of the GC-MS followed the conditions: ionization potential (70 eV), ion source temperature (200°C), scan speed of 1000 Da sec<sup>-1</sup>, scan interval of 0.50 fragments sec<sup>-1</sup>. Mass analyzes were performed in full scan mode, ranging from 45 to 500 Da. The data regarding chemical constituents were acquired using LabSolutions LC/GC Workstation 2.72. The Van Den Dool and Kratz (1963) equation was used to calculate the retention index ( $IR = 100n + 100 [(tR(i) - tR(n)) / (tR(n+1) - tR(n))]$ , where  $tR(i)$ ,  $tR(n)$  and  $tR(n+1)$  correspond to the retention times of the tested compounds and standards, respectively. The standards used were the homologous series of n-alkanes (nC8–nC18). The identification of compounds was based on comparison with the retention indices with those of the literature (Adams, 2007) and the mass spectra of the EO constituents with up to 95% similarity were compared with those of the mass spectra libraries FFNSC 1.2, NIST 107, and NIST 21. The quantification of volatile compounds was determined by gas chromatography with a flame ionization detector (DIC) (Shimadzu GC - 2010, Kyoto, Japan). The experimental parameters were the same used by GC-MS, with the exception of the detector temperature, which was 300°C.

**TABLE 2 |** Production test of biochemical compounds by bacteria, evaluation of phosphate solubilization, protease production, biological nitrogen fixation, and auxin production.

Bacteria	Phosphate	Protease	Nitrogen	Auxin
<i>Bacillus acidicer</i>	+	+	+	-
<i>Bacillus amyloliquefaciens</i>	+	+	-	-
<i>Bacillus macauenses</i>	+	-	-	-
<i>Bacillus subtilis</i>	+	+	+	-
<i>Bacillus pumilus</i>	+	+	+	-
<i>Pantoea ananatis</i>	-	+	-	-
<i>Staphylococcus equorum</i>	+	+	+	-

(+) positive reaction and (-) negative reaction.

The percentages of compounds were calculated using the area normalization method.

## Yield and Content of Extracted Oils

Due the low amount of extracted oil, the amount was estimated using the difference in weight of the bottle before and after the extraction of the EO. For this purpose, the amber bottles were weighed before extraction and at the end of the extraction process, after the complete evaporation of hexane at room temperature. The weight difference determined the amount of oil extracted. With these values, the yield for each treatment was determined and the content of EO was calculated according to the following formula proposed by Santos et al. (2004): Oil content (%) = [Weight of oil (g) x Dry petal biomass (g)]/100. The yield of EO was expressed in weight of oil per unit weight of plant material in Moisture Free Base (% w/w MFB).

## Statistical Analysis of Data

The data were tested for normality and subsequently submitted to ANOVA. In case of significance, the means were compared by the Scott-Knott test ( $P \leq 0.05$ ), using the statistical software SISVAR version 5.2 (Ferreira, 2014).

## RESULTS AND DISCUSSION

The results of the growth promotion tests showed that the strains of *Bacillus acidicer*, *B. subtilis*, *B. pumilus* and *Staphylococcus equorum* were efficient in the phosphate solubilization, protease production and nitrogen fixation tests (Table 2). The *B. amyloliquefaciens* strain was efficient for phosphate solubilization and protease production and the *B. macauenses* and *Pantoea ananatis* strains were efficient only for phosphate solubilization and protease production, respectively. None of the strains of bacteria used showed the ability to produce auxins by the test evaluated.

Phosphate solubilization is a mechanism common to many microorganisms that are associated with plant roots. This solubilization capacity may be related to the decrease in the pH of the medium due to the production of organic acids (such as gluconic acid, oxalic acid, malonic acid, acetic acid, formic acid, citric acid, and succinic acid), which can dissolve



the phosphate mineral, as a result of anion exchange, or chelate ions associated with phosphate (Chagas Junior et al., 2010; Tenga et al., 2019). Acidification of the medium is correlated with phosphorus solubilization by acid phosphatase activity, indicating that some microorganisms have developed enzymes capable of releasing inorganic phosphate from organophosphate complexes, in addition to the activity of this enzyme proving to be a good indicator for the mineralization of organic phosphorus (Kumar, 2016; Tenga et al., 2019). Likewise, the capacity for nitrogen fixation is common to many groups of microorganisms and their association due to its beneficial potential has been studied for application in the most varied agricultural crops (Mondani et al., 2019; Schmidt et al., 2019).

Regarding the production of roses, the application of bacteria did not influence the size of the bud before the flower opened (Table 3). However, plants treated with *B. acidiceler*, *B. subtilis*, and *B. pumilus* had higher diameter of open flower. An increase in the weight of the petals of plants treated with *B. pumilus* was also observed, showing a positive effect of this bacterium in relation to the others evaluated.

Some growth-promoting bacteria can interfere with the production of hormones by plants, which can affect the structure of the roots, increasing their surface area, number and type (Ali et al., 2010; Saharan and Nehra, 2011). In addition to these factors, inoculation with endophytic bacteria can

promote increased photosynthesis inducing greater production of carbohydrates by plants (Olanrewaju et al., 2017). In roses, the accumulation of carbohydrates in the flower stems can favor the durability of the flowers in the post-harvest. Thus, the application of endophytic microorganisms that aid in the accumulation of carbohydrates in the flower stems can improve the final quality of the roses, leading to greater durability of the cut stems (Moubayidin et al., 2009; Wang et al., 2014, 2019; Castanheira et al., 2017).

Plants treated with *B. acidiceler* and *B. pumilus* had a larger stem size (Table 4). The diameter of the stems was smaller in plants treated with *S. equorum* and *B. pumilus*, but only plants treated with *S. equorum* had lower weight of flower stems.

The yield of oil extracted from roses treated with *B. pumilus* was superior to other bacteria. However, a negative effect was observed on the yield of plants treated with *B. acidiceler*, in which the oil content was also lower in plants treated with this bacterium, with no difference in oil content between the

**TABLE 3 |** Effect of growth-promoting bacteria on the bud diameter, flower diameter and weight of rose petals of the cultivar Black Prince.

Bacteria	Bud diameter (mm)	Flower diameter (mm)	Petals weight (g)
<i>Bacillus acidiceler</i>	30.68 ± 3.63 a	61.10 ± 11.62 a	8.56 ± 2.55 b
<i>Bacillus subtilis</i>	31.66 ± 3.36 a	58.48 ± 14.60 a	8.43 ± 2.52 b
<i>Bacillus pumilus</i>	27.16 ± 3.21 a	65.41 ± 2.91 a	15.89 ± 1.49 a
<i>Staphylococcus equorum</i>	28.93 ± 3.43 a	50.68 ± 7.59 b	7.76 ± 2.17 b
Control	29.05 ± 4.36 a	51.38 ± 10.20 b	9.00 ± 2.80 b
C.V. (%)	12.50	16.01	24.33

Means followed by the same letter in the columns do not differ by the Scott-Knott test ( $P \leq 0.05$ ).

**TABLE 4 |** Effect of growth-promoting bacteria on size, diameter and weight of stem of roses cv. Black Prince.

Bacteria	Size (cm)	Diameter (mm)	Weight (g)
<i>Bacillus acidiceler</i>	67.62 ± 5.99 a	4.15 ± 1.03 a	14.18 ± 4.35 a
<i>Bacillus subtilis</i>	60.86 ± 7.40 b	4.57 ± 0.58 a	16.14 ± 2.23 a
<i>Bacillus pumilus</i>	67.01 ± 6.09 a	3.88 ± 0.96 b	15.14 ± 2.54 a
<i>Staphylococcus equorum</i>	54.32 ± 6.17 b	3.35 ± 0.86 b	10.94 ± 3.72 b
Control	57.50 ± 8.76 b	4.74 ± 0.97 a	16.84 ± 3.70 a
C.V. (%)	10.06	23.28	22.14

Means followed by the same letter in the columns do not differ by the Scott-Knott test ( $P \leq 0.05$ ).

**TABLE 5 |** Effect of growth-promoting bacteria on yield and content of essential oil of roses cv. Black Prince.

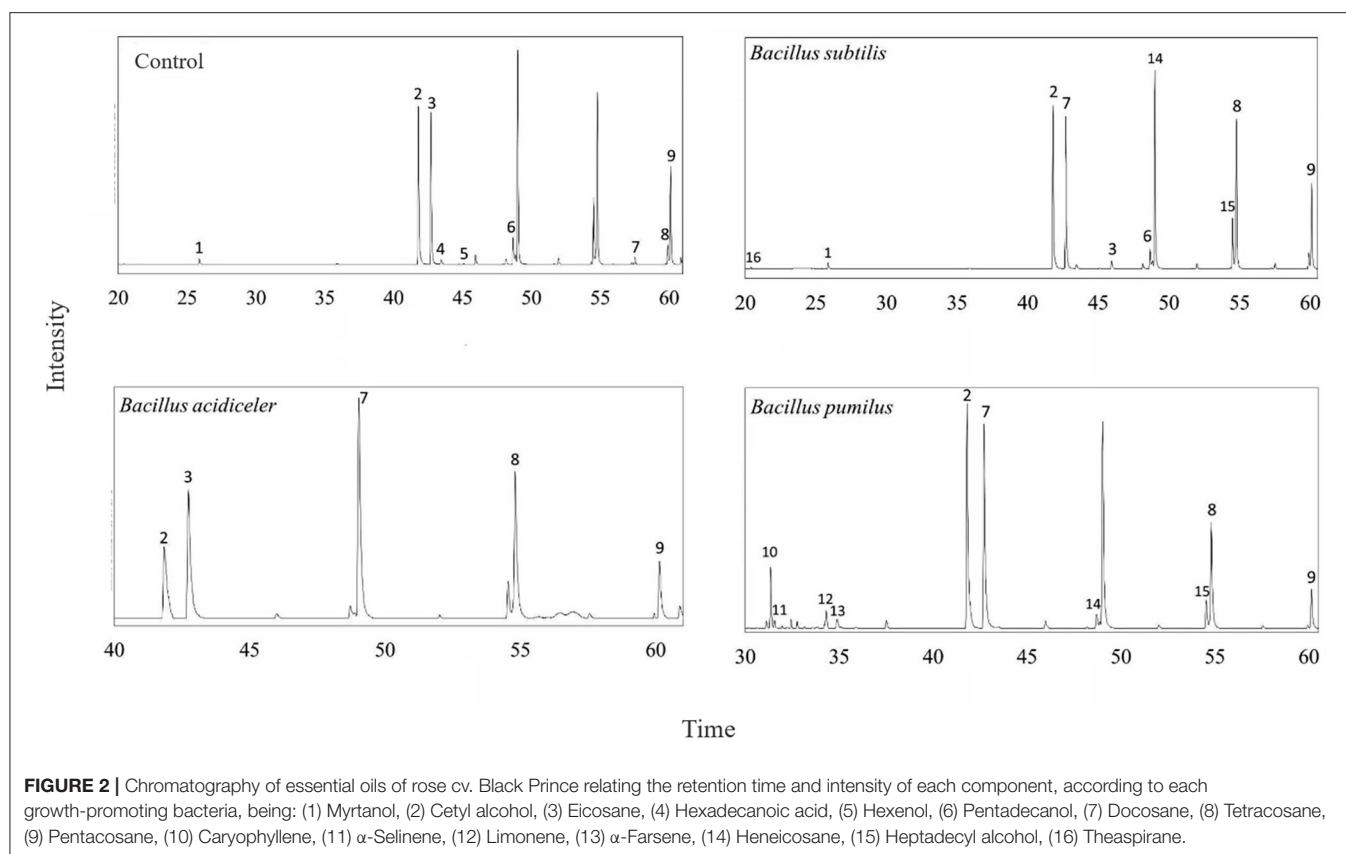
Bacteria	Yield	Content (%)
<i>Bacillus acidiceler</i>	0.0120 ± 0.0006 c	0.065 ± 0.004 b
<i>Bacillus subtilis</i>	0.0142 ± 0.0003 b	0.076 ± 0.004 a
<i>Bacillus pumilus</i>	0.0152 ± 0.0003 a	0.078 ± 0.003 a
Control	0.0137 ± 0.0005 b	0.074 ± 0.003 a
C. V. (%)	3.32	5.27

Means followed by the same letter in the columns do not differ by the Scott-Knott test ( $P \leq 0.05$ ).

**TABLE 6 |** Effect of growth-promoting bacteria on chemical composition of essential oil of roses cv. Black Prince extracted by hydro-distillation.

Constituents	RI tab*	Peak area %			
		Control	<i>B. acidiceler</i>	<i>B. pumilus</i>	<i>B. subtilis</i>
3- Hexenol	1095	0.38	–	–	–
Limonene	1132	–	–	1.60	–
Myrtanol	1258	2.66	–	–	1.25
$\alpha$ -Selinene	1498	–	–	0.68	–
$\alpha$ -Farnesene	1505	–	–	3.23	–
Theaspirane B	1550	–	–	–	0.39
Caryophyllene oxide	1582	–	–	6.89	–
Pentadecanol	1773	3.62	–	–	2.24
Cetyl alcohol	1874	36.51	18.60	25.30	21.86
6- Hexadecanoic acid	1959	2.28	–	–	–
Eicosane	2000	34.73	23.11	–	1.38
Heneicosane	2100	–	–	1.53	23.19
Docosane	2200	1.25	29.86	23.18	19.81
Heptadecyl alcohol	2290	–	–	2.04	5.08
Tetracosan	2400	2.81	20.93	22.81	16.76
Pentacosane	2500	15.71	7.48	12.69	8.01

RI, retention index. \*Adams mass-spectral retention index library (Adams, 2007).



plants treated with the other bacteria and the control (Table 5). However, it was not possible to obtain enough EO to perform the analysis on roses treated with the *S. equorum* bacterium through the methodology used for extraction.

The drying of the material can lead to decreases in the content of EOs and changes in its composition. Although freeze-drying is indicated due to less loss for EO amount, some species may have higher content of EOs when subjected to a convective drying process (Pirbalouti et al., 2013; Roslon et al., 2016).

In addition to phosphate solubilization and nitrogen fixation, growth-promoting microorganisms may be able to produce phytohormones, siderophores and antibiotics, and they are also capable of inducing systemic resistance in the plant (Sgroi et al., 2009). In addition, plants can also interact with these organisms by increasing their growth, since the increase in the amount of EOs is often related to the growth of plant organs due to greater absorption of nutrients; the quality of the oils can also be influenced by the activation of metabolic routes due to the interaction of the plant with microorganisms (Maji et al., 2013; Singh et al., 2019).

*B. subtilis* strains have the potential to stimulate plant biomass production even under adverse field conditions, such as in saline soils, and to increase chlorophyll production and soluble protein content (Wang et al., 2018). In addition, the application of *B. subtilis* strains positively influenced the growth and production of crops such as cotton, brachypodium grass (*Brachypodium distachyon*), arabidopsis (*Arabidopsis thaliana*)

and wheat (*Triticum aestivum*) (Ali, 2015; Gagné-Bourque et al., 2015; Wang et al., 2018; Andrees et al., 2019).

For the chemical composition of EO of roses, there was a significant difference according to each bacterium used in the treatment of plants (Table 6, Figure 2). The smallest number of components was found in the oils of plants treated with *B. acidiceler*. However, plants treated with *B. pumilus* and *B. subtilis* had higher number of compounds, in which the chemical constituents of carophyllene oxide,  $\alpha$ -selinene, limonene,  $\alpha$ -farnesene appeared only in plants treated with *B. pumilus* and teaspirane only in flowers treated with *B. subtilis*.

Limonene is related to the strong smell of orange, which constitutes the final citrus aspect of the aroma of the species under study. This compound has an insecticidal action and can confer resistance to insects in some plants, being widely used in flavor and fragrance industries (Thomas and Bessière, 1989; Sowndhararajan et al., 2015).  $\alpha$ -selinene, on the other hand, is a sesquiterpene that is not part of the smell composition of the oils where it is present, but it is part of the chemical composition of the EO of several plants such as guamirim (*Calyptanthus concinna*), citronella (*Cymbopogon nardus*), and tiririca (*Cyperus rotundus*) (Limberger et al., 2002; Lawal and Oyediji, 2009; Silva et al., 2011).

$\alpha$ -farnesene is a sesquiterpene related to the defense of plants to aphids, in addition to conferring the flavor of some vegetables, being found naturally coating fruits such as apples and pears.

In EOs, this compound is also related to the smell of floral-green or woody, being one of the major constituents of ginger oil (Harbone, 1997; Andrade et al., 2012).

Similar to  $\alpha$ -farnesene, carophyllene oxide is also a major constituent of EOs from some plants that may contain toxic compounds. This compound has the potential to inhibit the growth of Gram-positive bacteria, and is also toxic to ants and fungi associated with them. In addition, the carophyllene oxide found in EOs has already been associated with analgesic and anti-inflammatory action and can induce apoptosis in lymphoma and neuroblastoma cells (Norouzi-Arasi et al., 2006; Judzentienea et al., 2010; Sain et al., 2014). The presence of this compound may indicate an improvement in the medicinal properties of rose oil, as well as influencing their defense against leaf-cutting ants.

The alcohols and esters are the main constituents found in the EO of several varieties and species of roses, configuring more than 80% of the key components of its aromas in some of those plants, where the alcohols are the components used in the evaluation of the quality of the EO of roses (Xiao et al., 2018).

The compounds myrtilanol, cetyl alcohol, eicosane, hexadecanoic acid, hexenol, docosane, tetracosan, pentacosane, heneicosane, and heptadecyl alcohol are part of the volatile compounds of EO of rose species intended for the extraction of EOs. However, its presence and quantity in the oil can vary depending on the management, environmental conditions, methodology and time of extraction (Özel et al., 2004; Joichi et al., 2005; Xiao et al., 2018).

The promotion of plant growth has also been proven in strains of *B. pumilus*, showing its efficiency in the growth, development and production of plants, as well as in resistance to some environmental stresses, such as salinization, drought, and tolerance to heavy metals. Those strains also reduce reactive oxygen species, increasing the content of secondary metabolites and increasing the activity of antioxidant enzymes (Khan et al., 2016; Sirajuddin et al., 2016; Xie et al., 2019). The potential of these microorganisms to stimulate the production and accumulation of secondary metabolites may explain the increase in the EO content in roses and the different compounds present

in the chemical composition of the oil extracted from roses inoculated with *B. pumilus*.

## CONCLUSIONS

*B. acidiceler*, *B. pumilus* and *B. subtilis* improved the phytotechnical characteristics of roses. Among bacteria, *B. pumilus* increased the essential oil content as well as positively changed the chemical composition of the extracted essential oil from roses cv. Black Prince.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

NA and JD: research design, experiments administration, supervision, and writing. RB and MMAP: experiments conduction (bacterial analysis) and data discussion. BB, MC, and VB: experiments conduction (rose analysis) and data discussion. MP: visualization and data discussion. RR: writing and critical revision. All authors read and approved the final manuscript.

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# Mycorrhizal Interventions for Sustainable Potato Production in Africa

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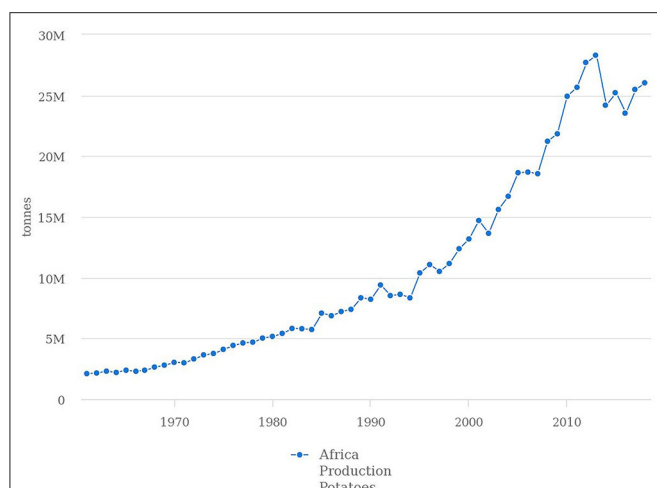
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The potato (*Solanum tuberosum* L.) is an important tuber crop with high dietary value that could potentially help to alleviate malnutrition and hunger in Africa. However, production is expensive, with high fertilizer and pesticide demands that lead to environmental pollution, and tillage practices that negatively affect soil structure. Microorganisms of different types have increasingly been found to be useful as biofertilizers, and arbuscular mycorrhizal (AM) fungi are an important crop symbiont. AM fungi have been shown to increase tolerance of crop plants to drought, salinity and disease by facilitating water and nutrient acquisition and by improving overall soil structure. However, the establishment and maintenance of the symbioses are greatly affected by agricultural practices. Here, we review the benefits that AM fungi confer in potato production, discuss the role and importance of mycorrhiza helper bacteria, and focus on how AM fungal diversity and abundance can be affected by conventional agricultural practices, such as those used in potato production. We suggest approaches for maintaining AM fungal abundance in potato production by highlighting the potential of conservation tillage practices augmented with cover crops and crop rotations. An approach that balances weed control, nutrient provision, and AM fungal helper bacterial populations, whilst promoting functional AM fungal populations for varying potato genotypes, will stimulate efficient mycorrhizal interventions.

**Keywords:** potato, symbiotic benefits, drought and salinity, soil management, mycorrhizospheric interactions, chemicals and fertilizers, sustainability

## INTRODUCTION

The potato (*Solanum tuberosum* L.), also known as the “Hidden Treasure,” is the most important non-cereal crop in the world (Ezekiel et al., 2013). In Africa, it is ranked 4th after wheat, maize and rice (Ezekiel et al., 2013; Dreyer, 2017). According to FAOSTAT (2020a), potato production within the continent rose by 90% between 2002 and 2018 (**Figure 1**), spanning 42 African countries on an estimated 1.9 million ha of arable land (FAOSTAT, 2020a). This contrasts with Europe, where there has been a downward trend in potato production from 221 million tons in 1961 to 105 million tons in 2018 (FAOSTAT, 2020a). In Africa, potato production is carried out by small-holder and commercial farmers, for household consumption and exportation, respectively (Gildemacher et al., 2009; Haverkort et al., 2013). The production and processing of potatoes provides employment for many in Africa, whilst the capacity to export them offers opportunities to earn foreign currency for economic development. In developing countries such as those in Africa, potatoes are mainly



**FIGURE 1 |** Potato production from 1961–2018, which shows an increased demand over the years. In 2018, 26,041,721 tons of potatoes were produced. The most dramatic change of 90% was realized between 2002 and 2018 (FAOSTAT, 2020a).

produced for their carbohydrate content. Potatoes can provide 130 Kcal of energy per individual every day (Ezekiel et al., 2013) with 100 g of baked potato (with its skin) providing 390 kJ of energy (Zaheer and Akhtar, 2016). Since one plant can produce many tubers, the potato provides more energy per plant compared to other staple crops (Ezekiel et al., 2013). The potato also contains no fat but has a considerable amount of protein, which is necessary for human growth and development (Wijesinha-Bettoni and Mouillé, 2019). The protein content is, however, dependent on variety, there being over 5000 potato varieties with varying degrees of nutritional value (Burlingame et al., 2009). This genetic diversity facilitates successful cultivation of potatoes in various agro-ecological regions across the continent (Dreyer, 2017), making it unique compared to other less diverse crops (Pathak et al., 2017).

Africa is home to 260 million undernourished people, 90% being in Sub-Saharan Africa (FAO, 2019). Potatoes provide vitamins C, B6 and E, iron (Fe), magnesium (Mg), phosphorus (P), potassium (K) and thiamine, which are all vital for human nutrition (Andre et al., 2014; Zaheer and Akhtar, 2016). They provide dietary fiber (through the potato skin), carotenoids, flavonoids, folate and phenolic compounds (Burlingame et al., 2009; Zaheer and Akhtar, 2016). These confer anti-diabetic and anti-cancer properties, in addition to helping to combat chronic skin inflammations and hepatic damage. They have also been reported to reduce the toxicity of cholesterol oxidation products, the incidence of hypertension, heart diseases and neurodegenerative diseases, among others (Duthie et al., 2000; Singh et al., 2008; Burlingame et al., 2009; Arun et al., 2015; Yang et al., 2015). As a major component of the diets of many

people of Africa and others worldwide, the potato can contribute toward the Sustainable Development Goal 2, which strives to “End hunger, achieve food security and improve nutrition and promote sustainable agriculture” by 2030 (FAO et al., 2019).

Various initiatives have been set up to promote efficient and high yield potato production, particularly in sub-Saharan Africa. Prominent among them is the CGIAR project for Root, Tuber and Banana (RTB) research, which was initiated to provide high-quality seed potato for planting (Harahagazwe et al., 2018). Other initiatives include non-profit organizations such as the African Potato Association and Potatoes South Africa.

Production of large quantities of high-quality potatoes to meet these goals requires the intensive application of fertilizers and pesticides. This threatens biodiversity by selecting for certain organisms at the expense of others, and human health due to point source pollution, particularly where runoff can reach water sources. Thus, current potato production systems are unsustainable with the expansion of Africa's population (United Nations Department of Economic Social Affairs Population Division., 2017). However, the soil already contains a diversity of beneficial microorganisms that can facilitate plant development through their role as biofertilizers (Barea et al., 2005), including bacteria, protozoa and fungi (Barea et al., 2005), many of which offer much more sustainable solutions. Primary amongst these are AM fungi, as they are major crop symbionts, however, as with all living organisms, they too are affected by human activity. An understanding of AM fungal symbiosis, and adaptation of management practices to ensure AM fungal biodiversity, is key to accruing their many invaluable agricultural benefits. This paper reviews current advances in understanding regarding the benefits that AM fungi can provide in potato production and discusses the maintenance of AM fungi through a more conservative agricultural approach for sustainable potato production.

## ARBUSCULAR MYCORRHIZAL FUNGI

Arbuscular mycorrhizal (AM) fungi are ubiquitous soil microbes forming obligatory biotrophic symbiotic associations with the roots of more than 80% of all vascular plant families (Besserer et al., 2006). Environmental history places their existence from the Ovicadan era and they are believed to have been the facilitators of land invasion by plants (Redecker et al., 2000). These fungi belong to the phylum Glomeromycota and order Glomerales (Pathak et al., 2017). AM fungal species are functionally diverse and their ability to form symbioses is largely dependent on their ability to acquire food from the host, whether they confer the required benefits to the host and whether the host genotype allows AM fungi to complete their life cycles (Feddermann et al., 2010; Pathak et al., 2017).

AM fungi are known to be ancient asexual organisms. The hyphae are aseptate and coenocytic (Parniske, 2008). They undergo anastomosis, which is the association of hyphae followed by genetic reshuffling that results in the development of new progeny (Chagnon, 2014). Anastomosis occurs intraspecifically and between related species (Parniske, 2008; Chagnon, 2014). It has been suggested that AM fungi can reproduce sexually,

**Abbreviations:** ABA, Absciscic acid; AM, Arbuscular mycorrhizal; CT, conventional tillage; NT, no-tillage; PAM, Periarbuscular membrane; PPA, Penetration Apparatus; ROS, Reactive Oxygen Species; RT, Reduced Tillage.

however, scientific research should be undertaken to validate this claim (Yildirim et al., 2020).

The AM fungal life cycle is composed of 3 main stages, namely the asymbiotic, presymbiotic and symbiotic stages.

## Asymbiotic Stage

AM fungi begin as resting spores, which, depending on species, can be from 50–100  $\mu\text{m}$  (or more) in diameter with thick cell walls. They lie in a conservatory state awaiting a host. Though resting, it has been proven that many metabolic and morphological processes occur during this stage. If the spores have the right moisture content and are under optimum temperatures, they can germinate for 2–3 weeks. They achieve this by utilizing their carbohydrate and triacylglyceride energy reserves (Requena et al., 2007). It was experimentally identified that trehalose is the main carbohydrate found in AM fungal spores and this is utilized during the first 5 days of spore germination, followed by the subsequent utilization of lipids (Bécard et al., 1991). During germination, the hyphae develop, forming germ tubes (Requena et al., 2007) (**Figure 2**). Numerous nuclei formed by cell division can be observed under the microscope at this stage (Bonfante and Perotto, 1995). If no host is recognized, germination arrests and the cytoplasm retracts with consequential hyphal retraction (Requena et al., 2007). This arrest occurs before the food reserves have been depleted, allowing the spores to proceed with germination a few more times to increase their chances of finding a host (Bago et al., 2000).

## Presymbiotic Stage

Once the spore or plant roots with spores are near a host plant's roots, various genetic, metabolic and signaling cascades are initiated. The plant releases the phytohormone strigolactone, which triggers AM fungal activity. In turn, AM fungi release lipochito-oligosaccharides commonly referred to as Myc factors (Harrison, 2012). These chemicals initiate an AM fungi-host genetic and metabolic coordination which encourages hyphal differentiation and extension toward the host roots (Chen et al., 2018).

## Symbiotic Phase

After the presymbiotic phase the hypha forms an appressorium, called the hyphopodium, on root epidermal cells (Parniske, 2008). The root epidermal cells begin an internal rearrangement process, pushing the nucleus to one corner and initiating the formation of a penetration apparatus (PPA) that induces invagination of the epidermal cell right below the hyphopodium (Siciliano et al., 2007). The PPA, consisting of the endoplasmic reticulum and microtubules, surrounds this invagination (Siciliano et al., 2007). The Myc factors likely influence the development of the PPA (Harrison, 2012). The hypha moves into the cortex cell through this invagination. Pumplun et al. (2010) and Feddermann et al. (2010) reported that Myc factors also induce the expression of *Vapyrin* and *PAM1* genes that allow cortex cell penetration. As the hypha continues to penetrate, it branches off into tree-like structures called the arbuscules (Parniske, 2008) (**Figure 2**). The cell wall of AM fungus is separated from the plant cell firstly by the periarbuscular space,

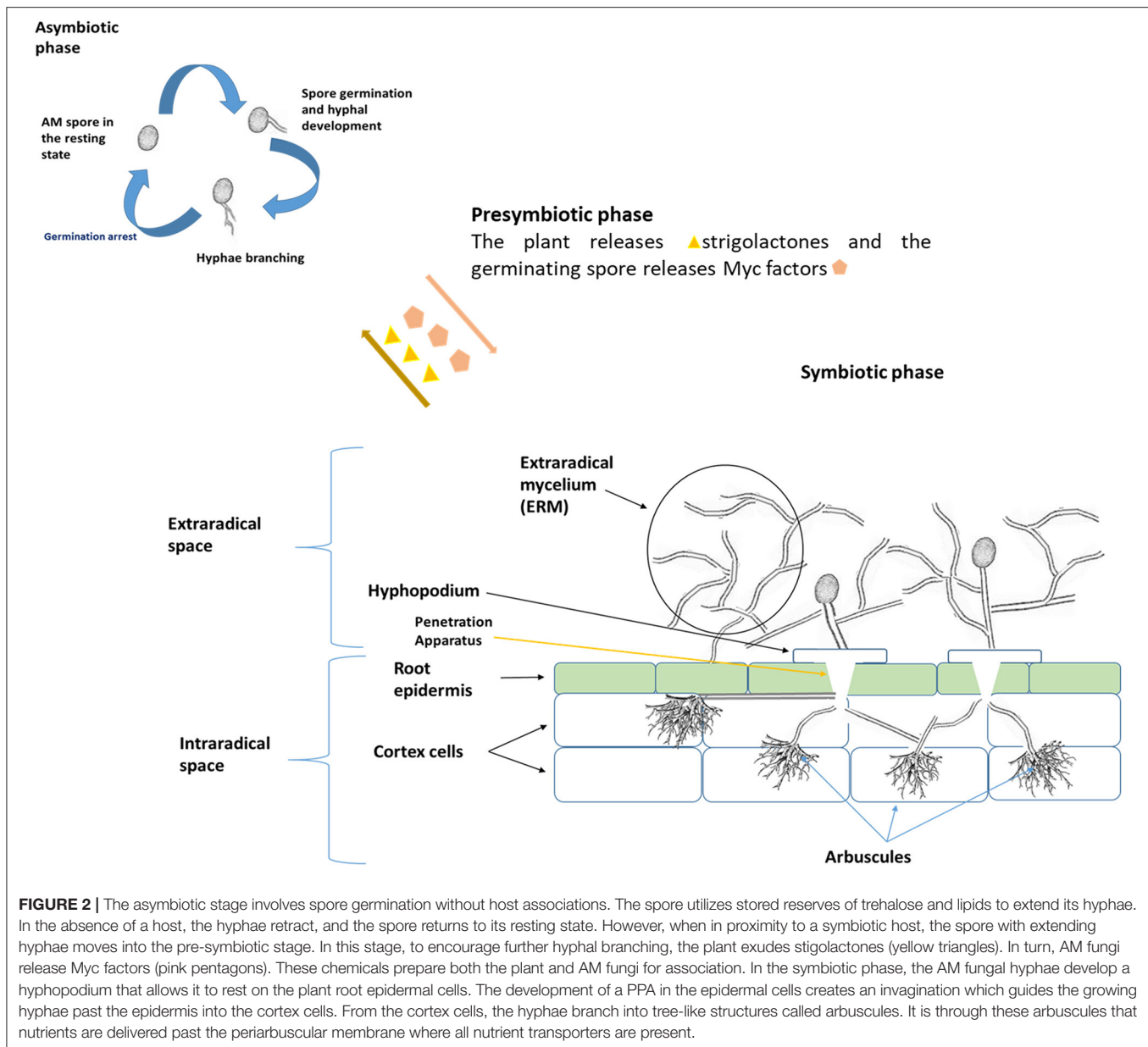
followed by the periarbuscular membrane (PAM) (Parniske, 2008). VAMP721d and VAMP721e proteins have been identified to play a major role in PAM development (Ivanov et al., 2012). This AM fungal and plant symbiosis induces the lysolipid lyso-phosphatidylcholine that in turn induces the expression of various transporter genes, such as the *Solanum tuberosum* Phosphate transporter 3 and 4 (*StPT3* and *StPT4*), for phosphate transport on the PAM (Drissner et al., 2007). In the symbiotic exchange, AM fungi acquire carbon and lipids for their own development. Continuous symbiosis leads to AM fungal growth and sporulation.

## SYMBIOTIC BENEFITS

### Enhanced Nutrient Uptake, Plant Growth, and Yield

Potato production relies heavily on the consistent use of fertilizers to provide all the 3 major nutrients, potassium (K), nitrogen (N), and phosphorus (P) (Jenkins and Mahmood, 2003). Lack of P results in stunted growth whilst a lack of N and K result in reduced tuber yield (Potatoes South Africa, 2020). Most researchers report that P is the more rate-limiting nutrient, as it influences the uptake of nutrients such as N (nitrogen uptake stops within days of P deficiency), iron (Fe), zinc (Zn), and magnesium (Mg) (Rufty et al., 1990; Skinner and Matthews, 1990; Fageria, 2001). Regardless of relative importance, all macronutrients diffuse slowly in the soil toward plant root hairs, creating a zone of depletion (Shuab et al., 2017). This coupled with the fact that potato plants have sparse and shallow root hair systems, results in a lack of optimum nutrients at any given time unless heavy fertilization ensues (Yamaguchi, 2002). The drawback is that fertilizers are expensive and often not available to the small-scale or subsistence farmers. In addition, heavy fertilizer application does not always guarantee high mineral nutrient efficiency (Brentrup and Pallière, 2010). AM fungi, on the other hand, can, through their extraradical mycelium, reach soil depths which root hairs cannot (Davies et al., 2005). Their thin hyphae increase the surface area for absorption and cover the gap between the potato root hairs and a nutrient sufficient zone (Davies et al., 2005). Under soil nutrient limiting conditions, AM fungi increase nutrient uptake and make it available to the plant from the arbuscular apoplast via the expression of numerous transporters located on the PAM (Genre et al., 2008). AM fungi are capable of absorbing P and presenting it as the more soluble form, orthophosphate ( $\text{Pi}$ ,  $\text{H}_2\text{PO}_4^-$ ), which the potato plant can absorb through AM fungal induced expression of the *StPT3* gene (Rausch et al., 2001; Smith and Smith, 2011). This absorption is facilitated by  $\text{H}^+$ ATPase activity (Gianinazzi-Pearson et al., 2000). AM fungi can also assist the potato in acquiring inorganic N present in the soil in the form of  $\text{NO}_3^-$  and  $\text{NH}_4^+$  through the nitrate reductase pathway and glutamine synthetase pathways, respectively (Jin et al., 2005; Shuab et al., 2017). They can also assimilate amino acids, such as arginine, via their extraradical mycelium and transport them intracellularly, where the amino acids are broken down via these pathways to make available N (Jin et al., 2005). Copper (Cu), Zn, Mg, Fe, and K are also readily





absorbed in AM plants (Shuab et al., 2017). The result of this nutrient absorption is enhanced potato growth and development. Inoculations with *Rhizophagus irregularis* and *Glomus proliferum* (\**Rhizophagus prolifer*—currently accepted AM fungal generic and species names according to Index Fungorum are shown in brackets were applicable) were shown to increase potato biomass, tuber ratio, tuber size and potato plant growth in low P conditions (Davies et al., 2005; Liu et al., 2018). Increased tuber size, which translated to increased yields over two seasons in mycorrhizal inoculated potato, was also observed (McArthur and Knowles, 1993; Douds et al., 2007). Potato tuber diameter, root length and shoot P content, as well as root and shoot fresh weight, increased when inoculated with *G. intraradices* (*R. intraradices*) and *G. mosseae* (*Funneliformis mosseae*) (Lone et al.,

2015). Similar results were observed by Vosátka and Gryndler (2000). The ability of AM fungi to assimilate nutrients lessens the requirement for fertilizer use.

Microtubers are becoming the most common potato planting material. AM fungi have been found to have a positive effect on their growth and development, increasing microtuber fresh weight, which reduces transplantation losses (Cheng et al., 2008). Once in soil substrate inoculated with AM fungi, the developed minituber produces an increased tuber number regardless of whether the plants are potted or field-grown (Cheng et al., 2008). The AM fungal association, therefore, increases seed potato production (Nurbaity et al., 2019). AM fungi continue to influence plant growth even after the fungi die, as fungal necromass has been shown to stimulate increases in crop biomass

(Jansa et al., 2020). It has been suggested that this may be caused by active AM fungal biomolecules present in the necromass and/or the stimulation of other plant development promoting microorganisms within the soil (Jansa et al., 2020).

Successful use of AM fungi in potato production was demonstrated in 231 field trials in Europe and North America (Hijri, 2016). Inoculation of potato seed with *Rhizophagus irregularis* DAOM 197198 produced a marketable yield of 42.2 tons/ha compared to 38.2 tons/ha in the uninoculated control, thus improving yield by 3.9 tons/ha (Hijri, 2016). However, the ability of the potato to access nutrients and to grow and produce high yields depends largely on the type of mycorrhiza and whether the inoculum is a pure strain or a mixture of strains (Cheng et al., 2008). In the Ecuadorian Andes, inoculation with *Rhizophagus irregularis* DAOM 197198 showed no significant impact on yield (Loján et al., 2017). This highlights the particular importance of AM fungal biogeography. Effective functionality is also highly influenced by inoculation techniques, interaction with indigenous background AM fungi and soil nutrient status, all of which may affect yield potential of potato crops significantly (Loján et al., 2017).

## Tolerance to Salinity

Soil salinity is determined by the concentration of soluble salts in the soil (Kapoor et al., 2013), and ions which contribute to high salinity include sodium ions ( $\text{Na}^+$ ), calcium ions ( $\text{Ca}^{2+}$ ), chloride ions ( $\text{Cl}^-$ ), bicarbonate anions ( $\text{HCO}_3^-$ ), carbonate ions ( $\text{CO}_3^{2-}$ ), strontium ions ( $\text{Sr}^{2+}$ ), silicon dioxide ( $\text{SiO}_2$ ), molybdenum (Mo), and barium ions ( $\text{Ba}^{2+}$ ), with NaCl being the most soluble salt (Hu and Schmidhalter, 2002; Munns and Tester, 2008; Kapoor et al., 2013). Sources of these salts mainly include ocean deposits and the weathering of rocks, as well as some sources of irrigation water (Munns and Tester, 2008). It is undeniable that water sources such as dams may also contain these salts in high concentrations, as many are close to industries where industrial effluent is not adequately purified before deposition into water bodies (Woolard and Irvine, 1995). Across Africa, farmers rely on irrigation and rainfall for potato production (Hijmans, 2003). Reliance on irrigation poses a threat because of the potential introduction of excessive salts into fields if the water is polluted, in addition to the accumulation of salts contributed by leachate post excessive fertilizer application (Han et al., 2015; Parihar et al., 2015).

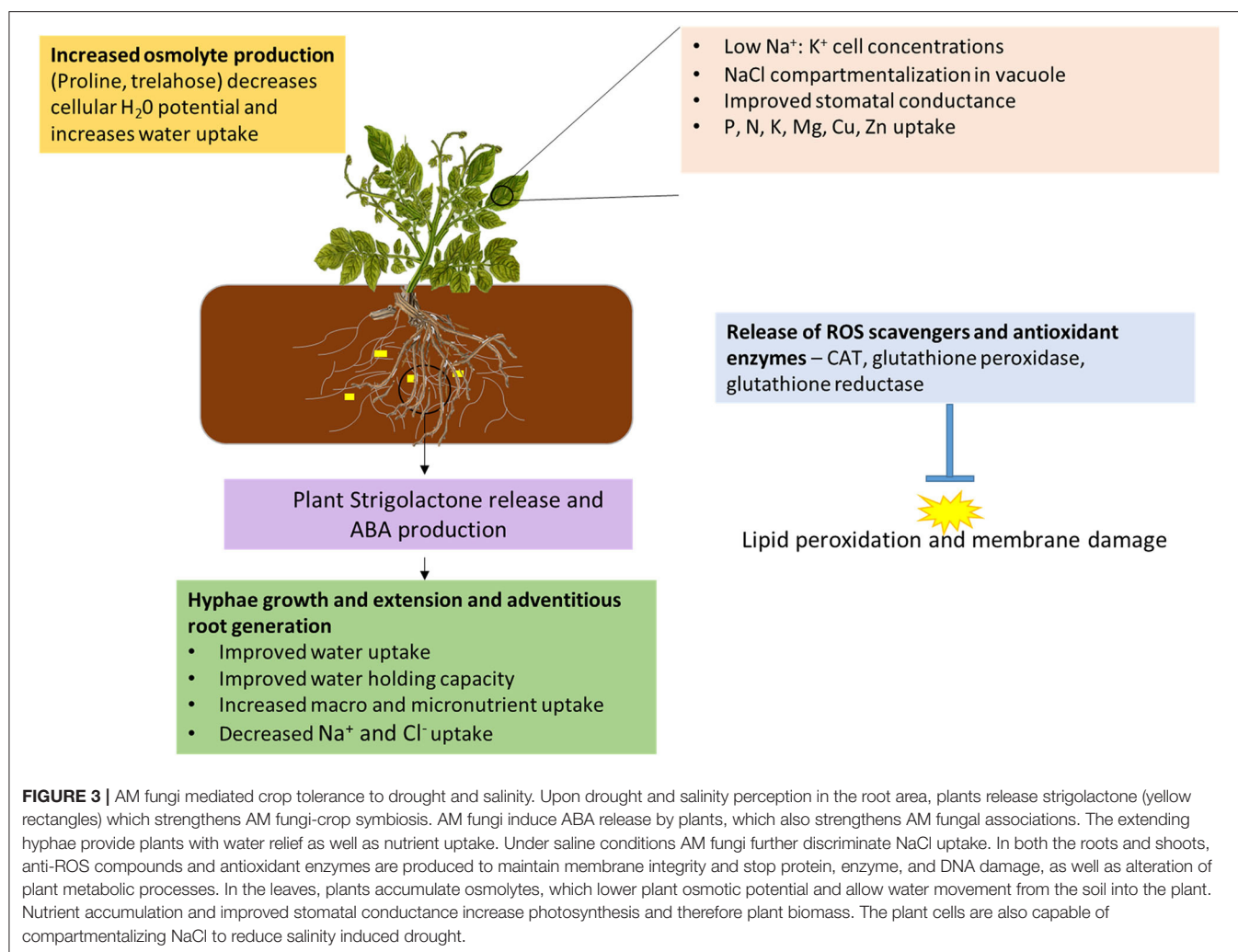
The osmotic imbalance induced by high salinity results in stunted growth, leading to a 20% decrease in yield in irrigated crops (Porcel et al., 2012). Salinity reduces photosynthesis, leads to cellular dehydration, nutritional disorders and alters protein and lipid metabolic processes (Evelin et al., 2012). These lead to increased senescence, early plant death, reduced shoot and root length, increased radical oxygen species and an overall reduction in biomass in the potato plant (Khenifi et al., 2011; Jaarsma et al., 2013). AM fungi have been shown in previous experiments to relieve plants of salinity stress by increasing osmotic regulation through plant accumulation of osmolytes such as internal metabolites (proline, betaines) and sugars (fructose, trehalose) to prevent salt dehydration (Porcel et al., 2012; Kapoor et al., 2013). As examples, inoculation with *Claroideoglossum*

*etunicatum* resulted in increased proline levels in maize roots under high salt conditions (Estrada et al., 2013b), and inoculation of *Zea mays* with *R. intraradices* led to increased stomatal conductance and reduced electrolyte leakage, which promoted photosynthesis (Estrada et al., 2013a). Under salt stress, AM fungi can increase uptake of N and Mg, where the latter is an important component of the chlorophyll molecule (Kapoor et al., 2013). It can also maintain  $\text{Na}^+:\text{K}^+$  leaf ratios, which are vital for photosynthesis (Evelin et al., 2019). Xu et al. (2018) showed that *Glomus tortuosum* (*Sieverdingia tortuosa*) in *Zea mays* increased chlorophyll content and stomatal conductance of the plant, which improved photosynthesis under saline conditions. AM fungi can selectively remove  $\text{Na}^+$  and compartmentalize it in vacuoles (Porcel et al., 2016). The ability of *Claroideoglossum etunicatum* to induce NaCl compartmentalization was also observed in rice, and this led to reduced shoot  $\text{Na}^+$  levels (Porcel et al., 2016). In addition, *Glomus intraradices* (*Rhizophagus intraradices*) was shown to increase the supply of macro and micro-nutrients, whilst avoiding the uptake of NaCl ions (Evelin et al., 2012). Uptake of P increases uptake of micronutrients such as Zn and Cu, and with Calcium (Ca), reduces damage to DNA by Reactive Oxygen Species (ROS) attack, and as these nutrients are vital components of molecular processes (Liu et al., 2000; Kapoor et al., 2012), thus increase plant biomass. The host plant upregulates strigolactone production which enhances AM fungal symbiosis and consequentially overall stress alleviation (Aroca et al., 2013).

## Tolerance to Drought

Increased temperatures attributable to global climate change have led to persistent drought spells in Africa, and thus to increased crop losses. This is of concern as the potato plant does not thrive under hot and dry conditions. This is because water makes up almost 90–95% of the plant tissue, whilst the tubers comprise 70–85% water (Potatoes South Africa, 2020). Averaging at 60 cm, the relatively small potato root system renders it unable to adapt to drought (Haverkort and Verhagen, 2008). Water is essentially required at every developmental stage of the potato (Potatoes South Africa, 2020), making the crop heavily reliant on irrigation and rainfall. It has been predicted that, without any climatic mitigation, potato yields will decline by ~50% across Africa by 2055 (Raymundo et al., 2018).

Drought stress results in tuber deformation, early senescence, and reduced growth yields (Potatoes South Africa, 2020). While water deficiency also interferes with the AM fungal cell cycle, some species tolerate water-deficient environments and can thrive (Stahl and Christensen, 1991). Plants tend to release strigolactone when under drought stress, and this acts as a signal to AM fungi (Aroca et al., 2013). Under drought conditions, AM fungi, through their hyphae, can extend into  $100\text{ m g}^{-1}$  of soil and scavenge for water and nutrients, subsequently making it available to the plant (Evelin et al., 2019). AM fungal interactions stimulate adventitious root development by reducing root tip meristem activity (Bahadur et al., 2019). This improved root architecture together with AM fungal hyphae increases water and nutrient availability, which stimulates plant growth. As water deficiency causes osmotic stress that limits cellular expansion for



growth, the accumulation of osmolytes can also relieve this stress (Bahadur et al., 2019). The accumulation of proline was noted in potatoes after inoculation with *R. intraradices* and *C. etunicatum* (Khosravifar et al., 2020). Reduced leaf water potential and increased tuber yield were also noted in this study. Other mechanisms of AM fungal induced drought tolerance include increased conductivity and transpiration, both of which improve water uptake (Kapoor et al., 2013). In addition, hormones such as abscisic acid (ABA) are released to help relieve the plant and further enhance AM symbiosis (Kumar and Verma, 2018). Inoculation of *Zea mays* with *Rhizophagus irregularis* induced plant rehydration, improved water efficiency, increased proline production, and increased root ABA production (Zhao et al., 2015; Quiroga et al., 2017). A further consequence of drought is the increased release of ROS, and AM fungi have been found to stimulate plant production of ROS scavengers and enzymatic antioxidants (Bahadur et al., 2019). Potatoes have been shown to deal with ROS through the induction of catalase (CAT), peroxidase and ascorbate peroxidase, though levels of these enzymes were highly dependent on the cultivar (Adavi et al., 2020). Fungal mycelia improve soil aggregation, which

enhances water holding capacity of the soil, thus making water available to the potato root hairs (Rillig and Mummey, 2006). The contribution of AM fungi toward drought and salinity tolerance in plants is summarized in **Figure 3**.

## Tolerance to Plant Pathogens and Diseases

The threat to potato production is a reality as increased temperatures and high humidity shorten microbial life cycles and allow potato pathogens to thrive. Potato virus Y (PVY) strain PVY<sup>NTN</sup>, Fusarium wilt, Verticillium, Late blight, Rhizoctonia, Early blight, bacterial wilt, common scab, and Erwinia blackleg are amongst the numerous viral, fungal and bacterial diseases that affect the potato, with fungal diseases being most prominent (Potatoes South Africa, 2020). The use of pesticides is costly both financially and environmentally, leading to pesticide residues and environmental pollution.

AM fungi can interfere with plant-pathogen interactions through the induction of beneficial microbe-associated molecular patterns (Van Wees et al., 2008). These involve the activation of induced systemic resistance at a mild level to prime

the crop (Van Wees et al., 2008). Various hormones such as jasmonic acid, salicylic acid, ethylene and abscisic acid, auxin and toxins cross communicate to prime for resistance (Wasternack and Hause, 2013). These provide the crop with tolerance to pathogens. Such observations were made by Gallou et al. (2011) through the upregulation of Pathogenesis related 1 and 2 (*PR1* and *PR2*) genes prior to *Phytophthora infestans* inoculation. “The *PR1* gene is linked to the jasmonic acid pathway in *Solanum* species, whilst the *PR2* gene expresses  $\beta$ -1,3-glucanase protein for the salicylic acid-dependent pathway” (Gallou et al., 2011). The Salicylate pathway triggers systemic resistance after infection with biotrophs, post initial attack, whilst the jasmonate pathway is induced by necrosis (Beckers and Spoel, 2006).

In other experiments, Fusarium dry rot, the most important postharvest potato disease caused by nine fusarium species, was reduced by 20–90% on potato minitubers in the presence of AM fungi, with effects being passed on to progeny (Niemira et al., 1996; Potatoes South Africa, 2020). The ability of AM fungi to reduce postharvest losses is critical. Fusarium species are also known to result in the production of mycotoxins which are harmful when consumed. The use of *Glomus irregulare* (*Rhizophagus irregularis*) inhibited the growth of *F. sambucinum* and consequential trichothecene 4, 15-diacetoxyscirpenol (mycotoxin) production, illustrating the potential use of AM fungi as an antimicrobial and anti-mycotoxin agent (Ismail et al., 2013). In a study by O’Herlihy et al. (2003), *Phytophthora infestans*, regarded as the most notorious potato pathogen, was reduced by growing mycorrhizal potatoes. The effects of AM fungi are not limited only to the roots, but also prevail aboveground. AM fungi colonization reduced the severity of shoot and crown rot disease caused by *Rhizoctonia solani*, whilst increasing nutrient uptake, tuber fresh weight and decreasing mortality (Yao et al., 2002). Similar observations were made by Larkin (2008) when a mix of AM fungi resulted in reduced stem canker and black scurf in the potato crop. AM fungi also act by alleviating  $H_2O_2$  in PVY stressed potato plants (Deja-Sikora et al., 2020). They can upregulate defense mechanisms upon herbivore attack against disease vectors such as aphids (Rizzo et al., 2020), as in the case with PVY. AM fungi have also been reported to contain silencing RNA, enabling negative regulation of pathogen gene expression and thus conferring plant protection (Silvestri et al., 2020).

AM fungi associate with other microorganisms in a synergistic fight against pathogens. *Pseudomonas* species associated with AM fungal spores have shown inhibition of *Verticillium dahliae* and *Erwinia carotovora*, both of which are potato pathogens (Bharadwaj et al., 2008). In the rhizosphere, AM fungal interactions with rhizobacteria in potato fields have been shown to lead to the production of antibiotics and enzymes that act as antimicrobials against potato pathogens (Sessitsch et al., 2004). Baradar et al. (2015) showed that the presence of rhizobacteria and iron decreased *R. solani* disease progression and increased potato biomass. AM fungi also interact with other soil pathogens such as nematodes. By influencing nematode

hatching, they could provide an effective way to reduce disease severity or stop infection caused by nematodes (Schouteden et al., 2015).

It seems that the response of AM fungi to stress is to improve the general health of the host plant through various processes, to enable it to fight against biotic and abiotic stress. This may be verified by the fact that systemic acquired resistance hormones also interact with auxins and gibberellins during biotic stress (Wasternack and Hause, 2013). An important factor to note, however, is that AM fungi exhibit increased effectiveness when the soil into which they are inoculated is free from disease. An experiment which combined solarization and use of AM fungi reported a reduction in diseased tubers compared to when either solarisation or AM fungi were applied alone (Ngakou et al., 2006). It is also vital to determine which AM fungal species interacts best with which potato cultivar, as these have been observed to be important variables (Yao et al., 2002). AM fungal effectiveness in disease control is also dependent on pathogen virulence and mycorrhizal potential (Azcón-Aguilar and Barea, 1997). AM fungi and the benefits they provide to the potato plant are summarized in **Table 1** below.

## Improved Soil Health

Soil structure is a complex encompassment of soil aggregates, pore space, as well as biota (Rillig and Mummey, 2006). AM fungi contribute to the development of soil particles by driving their hyphae into soil beds and breaking them down into smaller pieces (Miller and Jastrow, 2000). Their soil penetration allows nutrients and water to be drawn from deep within the earth (Davies et al., 2005). AM fungi do not compete for soil C as they are supplied with C as a trade-off from their symbiotic hosts, allowing soil C enrichment (Rillig, 2004). Aggregates also comprise non-organic matter, and the proportion of soil metals affect soil structure and fertility. AM fungi can play an important role in limiting the concentration of xenobiotics and heavy metals in the soil through bioremediation (Li et al., 2018). They also add to the pool of decomposing matter through their senescing hyphae (Miller and Jastrow, 2000). AM fungal interactions with other microorganisms in the rhizosphere and mycorrhizosphere enrich the microbiome and increase nutrient availability in the soil, for example, by facilitating the proliferation of  $N_2$ -fixing bacteria, which increases N in the soil (Jeffries et al., 2003; Johansson et al., 2004).

AM fungi release glomalin, a sticky substance that forms hydrophobic interactions with the soil particles (Miller and Jastrow, 2000; Rillig and Steinberg, 2002; Vlček and Pohanka, 2020). This causes aggregation of clay, silt and other particles in the soil and brings together nutrients and biota (Miller and Jastrow, 2000). The unique fungal extraradical mycelia act together like a net that firmly holds this enriched structure together (Rillig and Mummey, 2006). The mycelial net increases soil water holding capacity, limits leaching and indirectly improves soil aeration (Rillig and Mummey, 2006). This enriched soil structure (Miller and Jastrow, 2000) and improved soil health around the root area, induced by the presence of AM fungi, ultimately translates to healthier potato crop growth and helps to achieve maximum yield.



**TABLE 1 |** Summary of AM fungal species and the benefits they confer to the potato plant.

AM fungal species*	Nutrient uptake	Plant growth	Tuber number/size	Yield	Drought	Disease protection	Pot trial	Field trial	References
<i>G. mosseae</i> ( <i>Funnelliformis mosseae</i> )		Increased fresh weight	Increased size	54.2% increase minituber yield			Pot and seedling plates		Cheng et al., 2008
Consortium of <i>G. mosseae</i> and <i>G. versiforme</i> ( <i>Funnelliformis mosseae</i> and <i>Glomus versiforme</i> )			Increased size	20.9% increase				Yes	
<i>G. intraradices</i> ( <i>R. intraradices</i> )	N, Cu Fe, Na, B, P	Increased shoot and root dry matter	Increased dry matter				Yes		Davies et al., 2005
<i>R. irregularis</i>						Reduced oxidative effects of Potato virus Y	<i>in vitro</i>		Deja-Sikora et al., 2020
Consortium of <i>G. intraradices</i> , <i>G. mosseae</i> , <i>G. clariodeum</i> , <i>Gigaspora gigantea</i> ( <i>R. intraradices</i> <i>Funnelliformis mosseae</i> )			Increased size	Total of 45% increase of tuber fresh weight				Yes	Douds et al., 2007
<i>G. intraradices</i> ( <i>R. intraradices</i> )			Increased size						
<i>R. irregularis</i> DAOM 197198			Yes, >5cm	Slight increase of 3.9 tons/ha				Yes	Hijri, 2016
<i>G. irregulare</i> DAOM 197198 and 234328 ( <i>R. irregularis</i> DAOM 197198 and 234328)						<i>F. sambucinum</i> mycotoxin	Yes		Ismail et al., 2013
Single and dual inoculations of <i>R. intraradices</i> ; <i>C. etunicatum</i>		Increased leaf area index, increased shoot dry weight	Increased tuber dry weight	Max. 36%	Increased Proline			Yes	Khosravifar et al., 2020
Consortium of <i>Glomus aggregatum</i> , <i>G. clarum</i> , <i>G. deserticola</i> , <i>G. intraradices</i> , <i>G. monosporus</i> , <i>G. mosseae</i> , <i>Gigaspora margarita</i> , and <i>Paraglomus brazilianum</i> ( <i>R. aggregatus</i> , <i>R. clarum</i> , <i>Septoglomus deserticola</i> , <i>R. intraradices</i> , <i>G. monosporus</i> , <i>Funnelliformis mosseae</i> , <i>Gigaspora margarita</i> , and <i>Paraglomus brasiliianum</i> )				Minor (4–7%)		<i>R. solani</i> black scurf and stem canker		Yes	Larkin, 2008
Single inoculations of <i>R. irregularis</i> and <i>G. proliferum</i> ( <i>R. prolifer</i> )	P, N	Higher photosynthetic rate, increased total plant biomass			Improved water use efficiency		Yes		Liu et al., 2018
Consortium of <i>G. mosseae</i> ; <i>G. intraradices</i> ( <i>Funnelliformis mosseae</i> ; <i>R. intraradices</i> )		Increased leaf size and number, plant height root dry weight, shoot fresh weight					Yes		Lone et al., 2015

(Continued)

TABLE 1 | Continued

AM fungal species*	Nutrient uptake	Plant growth	Tuber number/size	Yield	Drought	Disease protection	Pot trial	Field trial	References
Single inoculations of <i>G. intraradices</i> ; <i>G. mosseae</i> ; <i>G. dimorphicum</i> ( <i>R. intraradices</i> ; <i>Funnelliformis mosseae</i> ; <i>Funnelliformis dimorphicus</i> )	K, Mg, N	Increased leaf expansion					Yes		McArthur and Knowles, 1993
<i>Glomus intraradix</i> ( <i>R. intraradices</i> )						<i>F. sambucinum</i> dry rot	Greenhouse beds		Niemira et al., 1996 Nurbaity et al., 2019
<i>AM</i> (non-specified)			Increased stolon to tuber numbers, increased tuber weight						
<i>R. intraradices</i>						herbivore attack priming			Rizzo et al., 2020
<i>G. magarita</i>						noncoding RNA			Silvestri et al., 2020
Single inoculations of <i>G. etunicatum</i> and <i>G. fistulosum</i> ( <i>C. etunicatum</i> and <i>Claroideoglomus claroideum</i> )			Increased tuber fresh weight				Yes		Vosátka and Gryndler, 2000
Single inoculations of <i>G. etunicatum</i> ; ( <i>C. etunicatum</i> )		Increased shoot fresh weight and root dry weight	Increased tuber number			<i>R. solani</i> shoot and crown rot	Yes		Yao et al., 2002
<i>G. intraradices</i> ( <i>R. intraradices</i> )									

\*Both current and old names of AM fungal genera and species have been used in this table. The names used in the original articles are not bracketed, whilst the current accepted names according to Index Fungorum have been put in brackets for each species.

## SOIL MANAGEMENT AND MAINTAINING MYCORRHIZAL BIODIVERSITY

### Soil Tillage

Various crop management systems significantly affect the AM fungal composition and association, shaping AM fungal communities. Tillage is a practice often used by farmers and involves the mechanical turning of soil by disc plows to de-weed, increase aeration, reduce pathogens and enhance decomposition in agricultural systems (Kabir, 2005). Tillage can be conventional or conservative (Kabir, 2005). Conventional tillage (CT) consists mainly of numerous tillage cycles which are followed by fertilizer and pesticide application (Kabir, 2005). Costa et al. (2017) demonstrated that deep tillage, which goes beyond conventional tillage by 50 cm, improves potato yield by reducing soil compaction, allowing aeration, nutrient flow and tuber expansion. Whilst this practice initially appears beneficial, it destroys soil structure long term. It affects the physical, chemical and biological cohesion associated with the AM fungal network (Wang et al., 2020). This is because AM fungi are mainly found in the topsoil where they make symbiotic associations with their hosts (Kabir, 2005). Soil disturbance can dislodge the fungi from their hosts. It interrupts the physical interaction between mycelium and soil particles. Conventional tillage reduces mycelium density as well as field glomalin (Kabir et al., 1998; Avio et al., 2013). It alters AM fungal diversity within the soil as some AM fungal species are permanently eradicated (Castillo et al., 2006; Brito et al., 2012; Sälé et al., 2015). This undoubtedly shifts the microbiome, as with the movement of organic and inorganic material within the soil, macro and micronutrient supply will be diluted (Borie et al., 2006; Roger-Estrade et al., 2010).

Importantly, it appears that AM fungi are differentially vulnerable to disturbance (Brito et al., 2012). This implies that tillage induces some kind of selection pressure, and it is therefore vital to understand what degree of disturbance each tillage practice creates (Brito et al., 2012). AM fungal populations can be sustained by practicing conservation tillage, which involves less intensive, reduced tillage (RT), or no tillage at all (NT). This practice maintains crop soil surface cover, which can act as mulch for improved soil fertility (Kabir, 2005). A lack of disturbance allows AM fungi to proliferate, leading to increased hyphal lengths, spore richness, and increased glomalin concentrations (Borie et al., 2006; Sälé et al., 2015).

Overall, it is important to look holistically at the advantages brought about by the practice of conservation agriculture. The success of conservation tillage is highly dependent on the ability to control weeds, pests and ensure efficient nutrient delivery (Morse, 1999). Whilst AM fungal populations are increased, there is a need to understand how integrated methods can be used to control weeds and pests. If reduced tillage is used, a type of tilling mechanism is required that does not affect AM fungi, but aims for the success of conservation agriculture (Roger-Estrade et al., 2010). Most conservation tillage systems are augmented by crop rotations, the use of cover crops and a more organic approach to farming to improve the success rate of the practice. Cover crops control weeds and can be a source of organic matter after their

destruction (Morse, 1999). Some experiments utilizing RT with manure and cover crops have been shown to increase potato yield when compared to CT, without any quality compromises (Larney et al., 2016). Hou and Li (2018) demonstrated that NT combined with straw mulch increased potato marketable yield and tuber production. In another experiment, NT was carried out in low lying areas after a rice rotation and drainage (Sarangi et al., 2018). Potatoes grown on this drained paddy soil had enough moisture to sustain germination. The use of straw as cover eliminated weeds whilst the use of compost manure enriched the soil and reduced fertilizer application. The straw also served as mulch, which reduced irrigation requirements. No pesticides were used, and these methods led to the production of high-quality potato tubers (Sarangi et al., 2018).

## Agrochemical Applications

### Pesticide Application

A sustainable approach to agriculture requires an appreciation of the effects of pesticides and fertilizers on the environment, human health and the microbiome. Though pesticide usage in Africa is only 2%, this percentage is high considering the percentage of utilized cultivatable land (FAOSTAT, 2020b). Most pesticides are abused, often without the use of proper formulations. Fumigation affects soil microorganisms (Collins et al., 2006). Only about 0.1% of pesticides reach the target pests, whilst the rest pollutes the soil, influencing microorganism profiles including that of AM fungi (Meena et al., 2020).

There are various reports regarding the effect of pesticides on AM fungi. In a review, Hage-Ahmed et al. (2019) reported that some AM fungal spores only germinate after the active pesticide ingredient has worn off, whilst other AM fungal species seem to have developed abilities to cope with pesticide residues. However, the harmful effects of pesticides have been observed even in lower pesticide concentrations than those applied in the field (de Novais et al., 2019). Kjølner and Rosendahl (2000) and Kling and Jakobsen (1997) showed that fungicides had a deleterious effect on alkaline phosphatase and succinate dehydrogenase activity in extraradical hyphae. The malfunctioning of these enzymes affects AM survival as well as P supply to the plant. Fungicides also induce abnormal hyphal branching, reduce the hyphal length and diminish anastomosis, which is pivotal in the life cycle of AM fungi, and consequentially AM fungal-plant symbiosis (de Novais et al., 2019). In experiments involving pesticides against *Rhizotonia solani*, azoxystrobin affected AM fungal extraradical development and spore formation whilst flutolanil affected arbuscular formation (Buysens et al., 2015). In these experiments, the lack of spore formation hindered any sort of AM fungus-host symbiosis, whilst a lack of arbuscules compromises the plant by limiting the potential for transfer of nutrients to the plant. In the same study, however, the use of pencycuron targeted only the pathogen, with no effect on AM fungi (Buysens et al., 2015). This experiment is a clear demonstration of the different effects of the various active ingredients. Ipsilantis et al. (2012) showed that even biopesticides such as azadirachtin (extracted from neem seeds) had persistent deleterious effects in the field. This means that any antagonistic effects against other favorable

soil microorganisms, such as AM fungi, should be considered during biopesticide formulation. Fungicide coated seeds of maize had no adverse effects on AM fungal colonization, suggesting that this could be a strategy for employing fungicides (depending on the mechanism of action of the fungicide) without affecting AM fungal diversity (Cameron et al., 2017).

The application of herbicides and their interaction with AM fungi can be varied. AM fungi were reported to be able to remove atrazine from the soil and relieve maize from chemical stress (Huang et al., 2007). However, when nicosulfuron was tested, the AM fungal population decreased due to a cumulative effect of the active ingredient in the soil (Karpouzas et al., 2014). Glyphosate-based herbicides were also shown to have a deleterious effect on AM fungi infection rates in maize grown in pasteurized soil (Savin et al., 2009). Therefore, the mode and the rate of application of chemicals, the active ingredient(s), the chemistry and the microbiome all have significant impacts on the maintenance of AM fungal populations (Hage-Ahmed et al., 2019).

### Fertilizer Application

Only about 50% of N and about 15–20% of P fertilizers, respectively, are taken up by crops (Smil, 1999; Roy et al., 2006). This means that fertilizer is applied in excess of demand. Excessive fertilization may not affect AM fungal diversity or structure, but it limits AM fungal colonization, as the crop has sufficient available nutrients for growth (Higo et al., 2020). Savin et al. (2009) reported that applying N and P reduced AM fungal infection in maize. Reports of low AM infectivity in potatoes upon fertilizer application were also reported by Loit et al. (2018). Lin et al. (2012) showed that, contrary to initial thinking, fertilizers affected AM fungal diversity. AM fungal populations can, however, be maintained by using lower fertilizer concentrations. Ziane et al. (2017) reported that 50% NPK application and AM fungal inoculation allowed crops to reach optimal yield. Ultimately, organic farming can be used to avoid the adverse effects of synthetic fertilizers and at the same time promote AM fungal populations, as it was observed that organic farming increased microbial richness (Lupatini et al., 2017; Loit et al., 2018). In addition, pyrolysis in organic farming produces compounds that act positively on AM fungal growth and development, as well as promoting the growth of other beneficial microbes (Gryndler et al., 2009).

### Monoculture, Selection of Cultivars, and Fallow Periods

Monoculture of the potato crop drastically reduces crop yield. This is because the practice severely reduces soil microbial diversity, decreasing soil fertility, and potentially increasing the incidence of disease (Liu et al., 2014). To successfully improve or maintain high crop yield, it is advisable to practice crop rotations/intercropping. Legumes encourage the proliferation of nitrogen-fixing bacteria, which increases soil N availability, whilst AM fungi associating crops influence AM fungal community composition with the aforementioned benefits. Regarding AM fungal diversity and crop rotations, it is important to note that AM fungal host compatibility depends on the host genotype and

therefore differs from crop to crop (Feddermann et al., 2010; Wu et al., 2013). Arbuscular mycorrhizal response increases from grasses to legumes, then to wheat, with maize being the most responsive, whilst the non-mycorrhizal plants in the Brassicaceae and Chenopodiaceae families are unresponsive (Plenchette et al., 2005). For instance, intercropping maize–potato increases AM fungal biomass, faba-bean–potato, increases the presence of functional AM fungal species, and rotating potatoes with peas, beans and alfalfa generally increases mycorrhizal infectivity (Plenchette et al., 2005; Ma et al., 2016). On the other hand, although *Trifolium pratense* is used as a nitrogen-fixing crop, its rhizospheric AM fungal infectivity in potato roots is low, whilst the non-leguminous *Leucanthemum vulgare* Lam. promotes the growth of AM fungi that efficiently colonize potato roots (Bharadwaj et al., 2007). Therefore, the use of strongly mycorrhizal plants can increase potato yields under crop rotation. Shared diseases should also be considered in the selection of cultivars, as crop rotation with crops that share the same diseases allows disease proliferation and increases crop losses from one rotation to the next.

Fallowing is a term for the use of a resting period that allows soil recovery, and this can be undertaken after periods of monoculture. Whilst perennial farming is detrimental to the soil, it has been suggested that extensive fallow periods are often detrimental to AM fungi (Plenchette et al., 2005). This is probably because of decreased AM fungal activity for long periods in the absence of a host plant and reduced microbes to maintain AM fungal activity (Plenchette et al., 2005). However, once crops are reintroduced in the correct order, AM fungal activity can be increased. Short periods of fallowing with cover crops may also be an alternative to crop rotations and intercropping for monoculture crops, to maintain AM fungal diversity.

### Mycorrhizospheric Interactions With Other Soil Microbes

Arbuscular mycorrhizal fungi influence the microbiome by changing soil pH and exuding stimulatory or inhibitory substances that create an environment for AM fungal establishment and plant colonization over time (Johansson et al., 2004; Larkin, 2008; Budi et al., 2012). Bacteria found in association with AM fungi have been isolated from AM fungal spore surfaces, hyphae and cytoplasm, with Gram-positive bacteria having strict associations with the spore surface (Lecomte et al., 2011; Cruz and Ishii, 2012; Selvakumar et al., 2016). These bacteria degrade the hyaline wall layer through the release of hydrolytic enzymes and inhibit AM fungal anti-germination compounds (Roesti et al., 2005). This facilitates spore germination (Roesti et al., 2005), initiating the AM fungus life cycle. They are also capable of forming biofilms by stacking themselves around the spore and hyphae, as well as on the surface of plant root epidermal cells, protecting the fungus and the plant (Manchanda et al., 2017). An example of this protection is the induction of bacterial *aiiA* gene expression against the activation of *E. carotovora* virulent genes in infected potato plants (Dong et al., 2000). Bacteria also exude chitinases and cellulases, which assist penetration of root epidermis by AM



**TABLE 2 |** Summarized list of AM fungi and their associating bacteria in the potato soil microbiome.

AM fungi*	AM fungal associated bacteria	Synergistic effect	Experimental condition	Reference
<i>G. mosseae</i> ; <i>G. intraradices</i> ( <i>Funneliformis mosseae</i> ; <i>R. intraradices</i> )	<i>P. fluorescens</i> F <sub>140</sub> , <i>P. fluorescens</i> T <sub>17-4</sub>	Increased plant biomass and chlorophyll content	Greenhouse pots	Baradar et al., 2015
<i>G. mosseae</i> and <i>G. intraradices</i> ( <i>Funneliformis mosseae</i> ; <i>R. intraradices</i> )	<i>Stenotrophomonas maltophilia</i> ; <i>Pseudomonas putida</i> biotype B; <i>Bacillus subtilis</i> ; <i>P. putida</i> biotype A; <i>S. maltophilia</i>	Disease resistance and IAA production	<i>in vitro</i>	Bharadwaj et al., 2008
<i>Glomus mosseae</i> and <i>G. fasciculatum</i> ( <i>Funneliformis mosseae</i> ; <i>Rhizophagus fasciculatus</i> )	<i>Pseudomonas</i> P173 and P168, <i>Bacillus subtilis</i> and <i>B. megaterium</i>	Increased P uptake and increased chlorophyll content	Greenhouse pot	Hassani et al., 2014
<i>Glomus intraradices</i> ( <i>R. intraradices</i> )	<i>Pseudomonas</i> CHAO-4, <i>Azotobacter</i> DSM-281, <i>Bacillus</i> PTCC-1020	Increased plant growth and increased yields	Green house pot	Otroshy et al., 2013
<i>Glomus fistulosum</i> ( <i>Claroideoglomus claroideum</i> )	<i>Bacillus subtilis</i>	Increased minituber number and weight	Greenhouse	Vosátka and Gryndler, 2000

\*Both current and old names of AM fungal genera and species have been used in this table. The names used in the original articles are not bracketed, whilst the current accepted names according to Index Fungorum have been put in brackets for each species.

fungal hyphae, and confer defense against pathogens (Bonfante and Perotto, 1995; Bharadwaj et al., 2008). Examples of spore associating bacteria that conferred resistance to *R. solani*, *E. carotovora*, *Verticillium dahliae* and *Phytophthora infestans* were found on *G. mosseae* (*Funneliformis mosseae*) and *G. intraradices* (*R. intraradices*) and include *Bacillus subtilis*, *P. putida* biotype A, and *S. maltophilia*, among others (Bharadwaj et al., 2008). In symbiotic response to this association, AM fungi supply chitin, polysaccharides, carboxylates, formate, and acetate for bacterial growth (Toljander et al., 2007 and Selvakumar et al., 2016; Zhang et al., 2016). It was also found that AM fungi induced bacterial proteolytic activity to assist the bacteria in the utilization of amino acids (Selvakumar et al., 2016).

Arbuscular mycorrhizal fungi interact synergistically with rhizobia and other phosphate solubilising bacteria that make P available for AM fungal uptake and transport to the plant (Toro et al., 1997). The solubilising bacteria can moderate their population to accommodate AM fungi, allowing AM fungal establishment on host plants (Toro et al., 1997). *Glomus mosseae* (*Funneliformis mosseae*) and *G. fasciculatum* (*Rhizophagus fasciculatus*) interaction with P solubilizing *Pseudomonas* and *Bacillus* resulted in an overall increase in P absorption and increased potato plant chlorophyll content (Hassani et al., 2014). The P absorbed by AM fungi activates nodule formation and nitrogenase expression in N<sub>2</sub>-fixing bacteria (Barea et al., 1992). This, in turn, makes N available for plant uptake, resulting in increased plant biomass and macronutrient accumulation in the host and therefore a high carbon pool for the fungi (Barea et al., 1992) and other microorganisms. Experiments with *Azotobacter* DSM-281, *Bacillus* PTCC-1020 and *Glomus intraradices* (*Rhizophagus intraradices*) in potato production led to increased minituber number and size, which translated to increased yields (Otroshy et al., 2013). Overall, AM fungi and microbiome associations lead to crop improvement.

Vosátka and Gryndler (2000) observed that *Glomus fistulosum* (*Claroideoglomus claroideum*) and *Bacillus subtilis* associations resulted in increased minituber numbers and total minituber weight. *Glomus mosseae* (*Funneliformis mosseae*) with *P. fluorescens* F<sub>140</sub> and *G. intraradices* (*R. intraradices*) with *P. fluorescens* T<sub>17-4</sub> lead to increased potato root colonization and increased potato plant biomass (Baradar et al., 2015). *Stenotrophomonas maltophilia* and *Pseudomonas putida* biotype B induced indole acetic acid production, which leads to increased potato plant growth (Bharadwaj et al., 2008). AM fungi also associate with other non-mycorrhizal saprophytic fungi and these synergize the availability of soil nutrients for plant growth and enhance plant immunity (Saldajeno and Hyakumachi, 2011). These multipartite associations greatly contribute to plant development and growth. **Table 2** below is a summary of AM fungi and associated bacteria and their synergistic effect on potato development.

The ability of a plant to have and maintain multiple microbial associations maximizes the benefits accruing to the plant. To conserve these interactions, it is important to understand which type of AM fungi associate with any specific staple crop, and which microorganisms associate harmoniously with the dominant AM fungal species for each crop. It is not sufficient simply to know the existing microorganisms, but it is also necessary to evaluate which cropping systems and crop associations enhance and maintain such synergies.

## RECOMMENDATIONS FOR COMMERCIAL AND SMALL-SCALE CROP PRODUCTION

While the purchase of AM fungus inoculum is expensive, managing existing populations may be the best approach for acquiring benefits conferred by symbionts. Conventional

practices which utilize tillage, monoculture, pesticides, and intense fertilization interfere with soil microbial dynamics, negatively affecting AM fungal symbiosis, resulting in diluted micronutrient supplies and leading to poor soil structure. Adopting conservation agricultural systems would favor AM fungal and microbial proliferation in the soil. We suggest the use of conservation systems that utilize organic fertilizer (for example seaweed extracts and animal manure), cover crops, crop rotations and no-tillage. Crops with high AM fungal responses such as maize, wheat and legumes should be utilized in crop rotations with potatoes. We also highlight that symbiosis is affected by both AM fungal and potato genotype, and thus it would not be enough to have a practice that focuses only on the AM fungal population. Thus, it would be appropriate for the potato-growing industry to collaborate with academia in researching the extent to which different conservation farming practices affect potato AM fungal populations, and thus influence functional AM fungi against potato genotypes. This would encourage natural selection for functionally beneficial AM fungi, either individually or in a consortium, for any potato genotype. Since agro-ecological zones vary across Africa, differentially influencing symbiosis, we encourage in-country research for the identification of applicable conservation agricultural practices suited to these ecological zones. Furthermore, since AM fungi and other soil microorganisms such as bacteria are interdependent, suitable conservation practices should allow greater exploitation of such interactions.

## CONCLUSION

Potato production is vital for food security within the African continent and increasing population numbers will soon give rise to further increase in demand. This pressure will most likely result in intensified conventional farming practices. However, from this review of current research into the growing of potatoes, it is clear that the use of arbuscular mycorrhizal fungi and

other potential symbionts can help to promote sustainable agriculture, whilst at the same time producing high quality and high yielding potato crops, presenting a potentially highly advantageous, less damaging and more sustainable alternative. This would be reliant on the ability to maintain mycorrhizal populations and associates, requiring the practice of conservation agriculture that will increase the diversity and populations of AM fungal species, while positively selecting for AM fungi most functional with chosen potato cultivars. This can be done using crop rotations with favorable crop species, less tillage and the use of organic manure, which will increase AM fungal proliferation and that of AM fungus associated microorganisms, among others. Ultimately, the conservation practice should be able to balance nutrient provision, weed control and water usage, whilst promoting functional AM fungi and maintaining soil microbial dynamics. To benefit from AM fungal intervention, research in Africa should focus on the individual ecological zones to identify the conservation agriculture approach and the varying potato genotypes and symbionts best suited to local conditions. Such systems employing effective mycorrhizal interventions could promote sustainable potato production across Africa.

## AUTHOR CONTRIBUTIONS

VC drafted the review and JD read, edited, and approved the review.

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# What Did We Learn From Plant Growth-Promoting Rhizobacteria (PGPR)-Grass Associations Studies Through Proteomic and Metabolomic Approaches?

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Plant growth stimulation by microorganisms that interact in a mutually beneficial manner remains poorly understood. Understanding the nature of plant-bacteria interactions may open new routes for plant productivity enhancement, especially cereal crops consumed by humans. Proteomic and metabolomic analyses are particularly useful for elucidating these mechanisms. A complete depiction of these mechanisms will prompt researchers to develop more efficient plant-bacteria associations. The success of microorganisms as biofertilizers may replace the current massive use of chemical fertilizers, mitigating many environmental and economic issues. In this review, we discuss the recent advances and current state of the art in proteomics and metabolomics studies involving grass-bacteria associations. We also discuss essential subjects involved in the bacterial plant-growth promotion, such, nitrogen fixation, plant stress, defense responses, and siderophore production.

**Keywords:** plant-bacteria association, Plant growth-promoting rhizobacteria (PGPR), grass, nitrogen fixation, proteomics, metabolomics

## INTRODUCTION

World food demand is increasing due to population growth and dietary changes. From an estimated 7.7 billion people worldwide in 2019, was projected that the global population could grow to around 8.5 billion in 2030, 9.7 billion in 2050, and 10.9 billion in 2100 (United Nations, New York, 2019). Consequently, by 2050, annual world demand for maize (*Zea mays* L.), rice (*Oryza sativa* L.), and wheat (*Triticum aestivum* L.) is expected to reach 3.3 billion tons or 800 million tons more than 2014's combined harvest (Reeves et al., 2016). Cereals provide 42.5% of human dietary calories consumption (Reeves et al., 2016). To meet this crop production demand in the next decades, more nitrogen, phosphorus, and potassium, are expected to be applied to fields. Cereal agriculture, such as wheat, rice and maize, account for ~60% of the global fertilizer used, and they are expected to account for half of the fertilizer consumed in 2050 (Alexandratos and Bruinsma, 2012).

Applying fertilizers to the soil is accompanied by negative environmental impacts. The first problem is a loss of substantial levels of fertilizer, especially nitrogen fertilizer, through natural erosion or leaching soil, which may result in aquatic habitats eutrophication (Carpenter et al., 1998). Also, nitrogen oxide gases, such as the nitrous oxide (N<sub>2</sub>O), generated by the microbial



transformation in the soil, contribute to the greenhouse effect (Hall et al., 1996). Industrial fertilizer production *per se* is an additional contributor to the “green problem.” Nitrogen fertilizers are produced in large-scale through the Haber–Bosch process, in which atmospheric  $N_2$  reacts with  $H_2$  or methane at very high pressure (200 atm) and temperature (450°C) over an iron catalyst yielding ammonia or urea. The electricity or natural gas consumed to generate ammonia by this process composes 90% of the total cost (Erisman et al., 2008). Furthermore, other fertilizers, such as phosphorus and potassium, are refined from rocks or sediment; however, the global reserves are susceptible to depletion (Galloway and Cowling, 2002; Erisman et al., 2008; Curatti and Rubio, 2014). As an alternative, sustainable agricultural policies have contributed alleviating the impact on the environment (Tilman et al., 2002).

Potential sustainable agriculture includes microbial inoculants or biofertilizers that aid in environmentally sustainable crop production (Johansson et al., 2004; Singh et al., 2011; Bashan et al., 2014; Bhardwaj et al., 2014; Backer et al., 2018). Plant growth-promoting rhizobacteria (PGPR) are those that beneficially interact with plants and promote their growth (Lugtenberg and Kamilova, 2009; Babalola, 2010; Dutta and Podile, 2010; Bhattacharyya and Jha, 2012; Fibach-Paldi et al., 2012; Vacheron et al., 2013; Nadeem et al., 2014). These bacteria can be found in the rhizosphere at the root surface or inside the plant. The bacteria that colonize inside of plants are commonly referred to as endophytic. Several species from the genera *Pseudomonas*, *Azospirillum*, *Herbaspirillum*, *Azotobacter*, *Klebsiella*, *Burkholderia*, *Bacillus*, *Serratia*, *Rhizobium*, and *Bradyrhizobium*, among others, can promote plant growth (Johansson et al., 2004; Singh et al., 2011; Vacheron et al., 2013; Nadeem et al., 2014; Fukami et al., 2018). *Poaceae* family, also called *Gramineae* or true grasses, are commonly associated with cyanobacteria, *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, *Azoarcus*, *Herbaspirillum*, and *Burkholderia*, in associative or endophytic forms (Pérez-Montaña et al., 2014).

There was an increase of ~23% in total biomass and 65% in grain yield after inoculation of rice with PGPR (Tariq et al., 2007). Rice independently inoculated with *Azospirillum brasilense* R1, *A. lipoferum* RSWT1 and *Pseudomonas* sp. Kyl exhibited increased grain weight. Significant increases in the total biomass of rice and sugarcane plants were also observed in plants inoculated with *Herbaspirillum seropedicae* (Boddey et al., 1995; Baldani et al., 2000; James, 2000; Gyaneshwar et al., 2002). Wheat and maize inoculated with a mix of *A. brasilense* Ab-V5 and Ab-V6 exhibited a 31 and 27% increased yield, respectively (Hungria et al., 2010). In a Cerrado Oxisol with a low capacity to supply N, the same mix increased maize grain yield in more than 30% (Martins et al., 2018). *H. seropedicae* and *H. rubrisubalbicans* are components of the commercial inoculant for sugarcane currently recommended by the Brazilian Agricultural Research Company (EMBRAPA) (Oliveira et al., 2006, 2009).

Interestingly, another Brazilian study showed that inoculation of sugar cane varieties grown in two different soils with a consortium of diazotrophic bacteria did not show significant differences concerning N fertilization, but concluded that the

growth promotion was due to the positive environmental impact of biofertilizer (Schultz et al., 2017).

Despite the widespread use of biofertilizers in cereal crops, the molecular mechanisms are not fully understood. Omics techniques such as transcriptomics, metabolomics, and proteomics have considerable potential to discover novel mechanisms on plant-bacteria interaction due to the capacity to analyse pools of molecules fast and simultaneously. For instance, proteomics is the appropriated technique to explore differential levels of protein expression and modification before and after plant inoculation with bacteria. In this review, we provide state of the art on beneficial grass-bacteria association studies through proteomic and metabolomic analysis and discuss how these data can pave the way to isolate or develop better biofertilizer strains.

## PROTEOMIC STRATEGIES FOR STUDYING THE GRASS-PGPR ASSOCIATION

Proteomics analysis is a technique with great potential for globally understanding processes involving simultaneous modulation of several proteins expression. This analysis can be applied to a range of systems from simpler ones such as cultured cells to more complex systems, such as a population of organisms in a particular niche (metaproteomics) or an association between different organisms (e.g., plant-microbe association). Proteomics relies on three pillars, as follows: (i) extraction of proteins, (ii) fragmentation of proteins in peptides and identification of peptides by mass spectrometry, and (iii) quantification of protein expression and post-translational modifications.

For instance, proteomics has been used to study the *Rhizobia* and legumes interaction (Cheng et al., 2010; Khatabi et al., 2019), the effects of abiotic stress in plants (Ghosh and Xu, 2014; Prinsi et al., 2018), and the pathogenicity of fungi and certain bacteria (Quirino et al., 2010). However, to our knowledge, only 14 proteomics studies have focused on beneficial grass-bacteria interactions (Table 1); most of the studies were performed using rice, likely because its genome sequence is known (Goff et al., 2002; Yu et al., 2002).

Genome sequencing of the *Oryza sativa* ssp. *japonica* cultivar Nipponbare (IRGSP, 2005) was completed, and its genome colinearity with other grass species was identified (Keller and Feuillet, 2000; Bennetzen and Ma, 2003); therefore, rice occupies a prominent position among grasses as an experimental model. In addition, rice is economically significant due to its high global consumption. Thus, several proteomic analyses on rice have focused on abiotic and biotic stresses (Kim et al., 2014), which facilitate a better understanding of different molecular mechanisms triggered by stress factors.

Maize and wheat genomes were also sequenced. The Maize GDB (Maize Genetics Executive Committee) is a global repository of information on genetics, genomics, and breeding research, being a crucial tool to advance the understanding of beneficial plant-maize (Schnable et al., 2009; Portwood et al., 2019). In 2018, the International Wheat Genome Sequencing Consortium (IWGSC) published a detailed analysis of the whole



**TABLE 1** | Progress in grass-bacteria interactions based on proteomics approaches.

Year	Achievement
2019	Differential proteomic analysis of maize seedlings colonized by <i>Azospirillum brasilense</i> (Sp7) detected up-regulated proteins involved in metabolism/energy and pathogenic cell lysis (Lade et al.)
2018	Identification of up-regulated proteins related to photosynthesis and metabolism and down regulated proteins related to redox homeostasis in the leaf of maize seedlings treated with <i>Azospirillum brasilense</i> Sp7 (Lade et al.)
2017	<i>Pseudomonas</i> sp. TLC 6-6.5-4 alone induced the most of total upregulated proteins in roots sorghum when compared to arbuscular mycorrhiza alone and associated with the PGPR (Dhawi et al.)
2017	Comparative proteomic analysis of wheat under NaCl stress colonized by <i>Enterobacter cloacae</i> SBP-8 and uninoculated revealed changes in the metabolism of the plant (Singh)
2017	Identification of bacterial proteins involved in nitrogen fixation and poly(3-hydroxybutyrate) and down-regulated of the rice roots protein with antioxidant activity colonized by <i>Herbaspirillum rubrisubalbicans</i> (Valdameri et al.)
2015	Comparative proteomic analysis of <i>Zea mays</i> roots inoculated with <i>Azospirillum brasilense</i> Strain FP2 detected 46 differently expressed protein, but only three proteins were identified (Faleiro et al.)
2013	Identification of methionine recycling induction in rice roots colonized by <i>Herbaspirillum seropedicae</i> strain SmR1 and bacterial proteins involved in nitrogen metabolism (Alberton et al.)
2013	Detection and identification of high abundance of a bacterial adhesin in <i>Zea mays</i> roots inoculated with <i>Azospirillum brasilense</i> Strain FP2 (Cangahuala-Inocente et al.)
2013	Proteomic and transcriptomic analysis of <i>Miscanthus sinensis</i> with the endophyte <i>Herbaspirillum frisingense</i> GSF30 identified up-regulated photosynthesis related proteins and proteins involved in stress metabolism (Straub et al.)
2013	Comparative proteomic study of rice inoculated with <i>Bacillus cereus</i> identified up-regulated proteins involved in plant growth and defense related proteins (Wang et al.)
2011	The first and unique quantitative shotgun Proteome analysis of PGPR <i>Gluconacetobacter diazotrophicus</i> interacting with sugarcane identified differentially expressed proteins mostly involved in defense mechanism, and bacterial proteins (Lery et al.)
2010	The unique proteomic analysis of different tissues of rice inoculated with a rhizobial endophyte <i>Sinorhizobium meliloti</i> 1021 identified up-regulated photosynthesis related proteins in leaf and leaf sheath and only defense related proteins in root (Chi et al.)
2009	Proteomic analysis of PGPR <i>Pseudomonas fluorescens</i> strain KH-1 during interaction with rice leaf sheaths identified differentially expressed proteins involved in metabolism and defense (Kandasamy et al.)
2006	The first proteomic study of interaction between different cultivars of rice with a nitrogen-fixing endophyte bacteria <i>Azoarcus</i> sp. and addition of jasmonic acid identified related to defense mechanisms (Miché et al.)

reference sequence of the bread wheat (*Triticum aestivum*) genome (Alaux et al., 2018; IWGSC, 2018). The maize and wheat genomes are key tools for advances in proteomic studies on bacteria-grass associations. The genomes for the PGPR models used in the seven studies were fully sequenced, which is extremely useful for large protein identification projects by peptide mass fingerprint (PMF).

Most studies employed 2D electrophoresis for protein separation (Table 2). According to Fey and Larsen, “the 2D gel electrophoresis is the technology that everyone loves to hate” (Fey and Larsen, 2001). The main reason that 2D gel electrophoresis remains the preferred method for protein separation is its ability to separate many proteins and isoforms from complex samples with high resolution and high reproducibility, and it facilitates both label-free quantitative analyses and post-translational modifications (Cheng et al., 2010). Furthermore, the known technical limitations, as the analysis of the highly acidic and alkaline proteins, low-abundance and hydrophobic proteins are not obstacles, but challenges that have led to improvements in analyses for these types of proteins (Görg et al., 2009).

For protein identification, nine out of 14 studies used MALDI-TOF MS (Matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry) (Table 2). The other four studies

applied LC/MS (liquid chromatography/mass spectrometry) methods. Accordingly, 2-D electrophoresis/MALDI-TOF is the proteomic strategy most employed for studying plant-PGPR interactions (Afroz et al., 2013). Surprisingly, no study so far has reported a proteomic shotgun analysis to evaluate a grass-PGPR association. A shotgun proteomics approach refers to the identification of proteins from complex mixtures of peptides generated by protein digestion with proteases employing a combination of high-performance liquid chromatography combined with mass spectrometry (Figure 1). This approach typically identifies many more proteins compared with 2D electrophoresis, is fast and adaptable to analyse several samples simultaneously. However, the high complexity of proteins coming from the host and bacteria might be challenging to separate adequately in the chromatographic step. In any case, the establishment of shotgun methods for the analysis of grass-bacteria interaction may yield important advances to the field.

Of the studies in Table 2, the *Saccharum* spp. and *Gluconacetobacter diazotrophicus* PAL5 proteome study was the only study performed by combining  $^{15}\text{N}/^{14}\text{N}$  metabolic protein labeling, one-dimensional gel electrophoresis (1D), and LC-electrospray ionization quantitative time-of-flight (ESI-Q-TOF) (Lery et al., 2011). The authors indicated that  $^{15}\text{N}/^{14}\text{N}$

**TABLE 2 |** Proteomics studies on PGPR-grass associations.

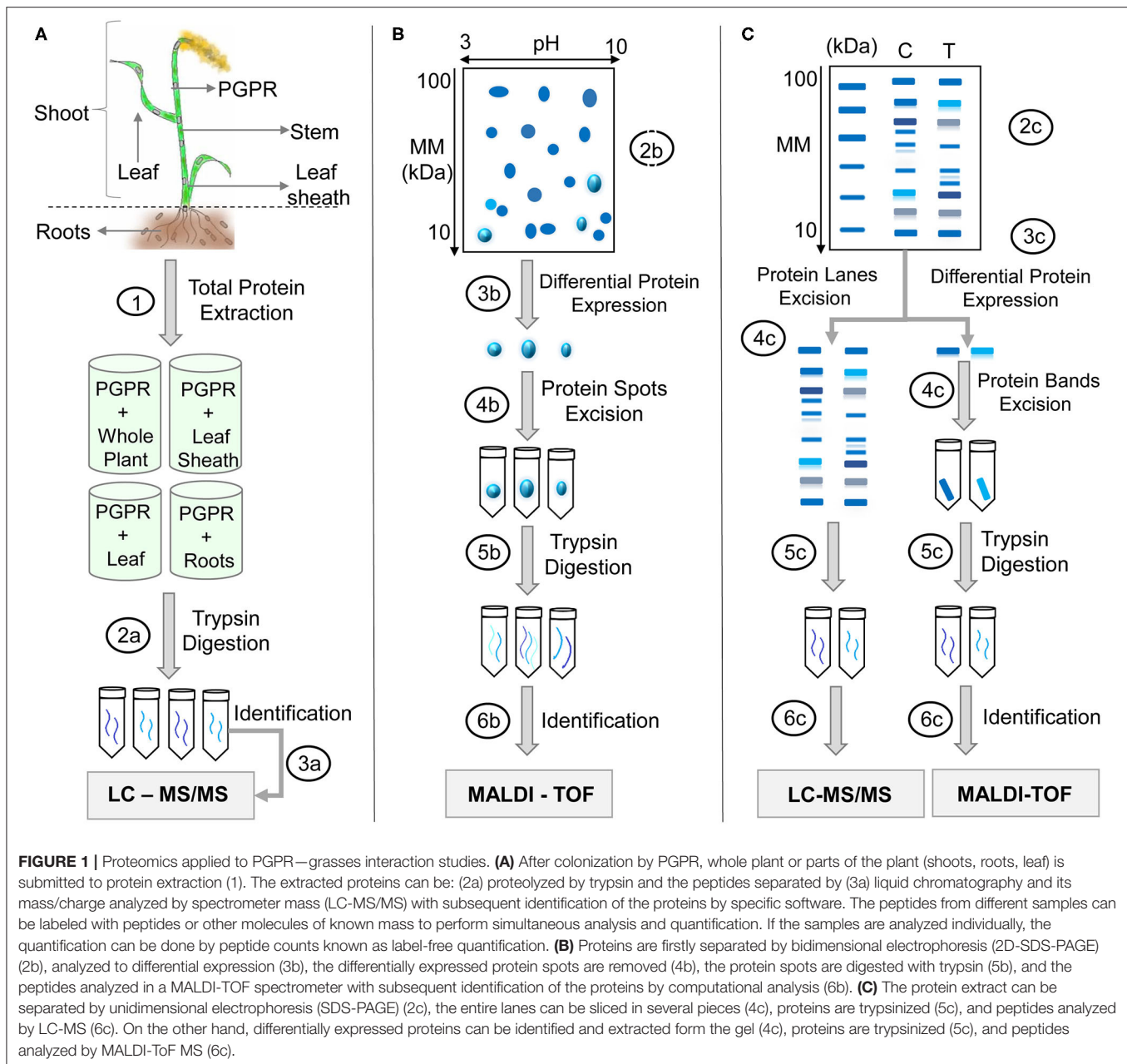
Grass	Bacterium	Tissues	Extraction	Methods	IEF	SDS-PAGE	Protein staining	Proteins detected	Proteins regulated	References
<i>Oryza sativa</i>	<i>Azoarcus</i> sp. BH72	Roots	A	2D-PAGE/MALDI-ToF or Nano LC MS/MS	IPG strips pH 3–10 NL 18 cm	12.5%	CBB R-250	1,000	47	Miché et al., 2006
<i>Oryza sativa</i>	<i>Pseudomonas fluorescens</i> KH-1	Leaf sheath	C	2D-PAGE/2D Nano LC MS/MS	IPG strips pH 4–7 17 cm	12%	Silver	–	23	Kandasamy et al., 2009
<i>Oryza sativa</i>	<i>Sinorhizobium meliloti</i> 1021	Roots/leaf sheath/leaf	A	2D-PAGE/MALDI-ToF	IEF rod gels pH 3.5–10 13 cm/3 mm	15% resolving gels/5% stacking gels	CBB R-250	1,000	21/19/12	Chi et al., 2010
<i>Saccharum</i> spp.	<i>Gluconacetobacter diazotrophicus</i> PAL5	Roots	B	1D-PAGE/ESI-Q-ToF	–	12.5% (mini gel)	CBB GR-250	>400 (identified)	34/43 <sup>b</sup> 7/8 <sup>c</sup>	Lery et al., 2011
<i>Oryza sativa</i>	<i>Herbaspirillum seropedicae</i> SmR1	Roots	A	2D-PAGE/MALDI-ToF	IPG strips pH 4–7 L 13 cm	11.5%	Blue silver	>500	26	Alberton et al., 2013
<i>Zea mays</i>	<i>Azospirillum brasilense</i> FP2	Roots	A	2D-PAGE/MALDI-ToF	IPG strips pH 3–10 L 13 cm	12.5%	CBB GR-250	>500	87	Cangahuala-Inocente et al., 2013
<i>Miscanthus sinensis</i>	<i>Herbaspirillum frisingense</i> GSF30	Whole plant	D	2D-PAGE/? <sup>a</sup>	IPG strips pH 3–11 24 cm	12%	Modified colloidal CBB	–	14	Straub et al., 2013
<i>Oryza sativa</i>	<i>Bacillus cereus</i> NMSL88	Roots and leaf	C	2D-PAGE/MALDI-ToF	IPG strips pH 4–7 L 17 cm	10%	Silver	>700	31	Wang et al., 2013
<i>Zea mays</i>	<i>Azospirillum brasilense</i> FP2	Roots	C	2D-PAGE/MALDI-ToF	IPG strips pH 4–7 L 13 cm	12.5%	CBB G-250	993	46	Faleiro et al., 2015
<i>Sorghum bicolor</i>	<i>Pseudomonas</i> sp. TLC 6-6.5-4	Roots	C	Tryptic Digest/LC-MS/MS	–	–	–	730	96	Dhawi et al., 2017
<i>Triticum aestivum</i> L.	<i>Enterobacter cloacae</i> SBP-8	Whole Plant	C	Tryptic Digest/LC-MS/MS	–	–	–	307 (identified)	75	Singh et al., 2017
<i>Oryza sativa</i>	<i>Herbaspirillum rubrisubalbicans</i> M1	Roots	A	2D-PAGE/MALDI-ToF	IPG strips pH 4–7 L 13 cm	11.5%	Blue silver	699	36	Valdameri et al., 2017
<i>Zea mays</i>	<i>Azospirillum brasilense</i> Sp7	Leaf	C	2D-PAGE/MALDI-ToF	IPG strips pH 5–8 18 cm	12.5%	Flamingo <sup>TM</sup> Gel fluorochrome	<sup>e</sup>	43	Lade et al., 2018
<i>Zea mays</i>	<i>Azospirillum brasilense</i> Sp7	Leaf	C	2D-PAGE/MALDI-ToF	IPG strips pH 5–8 18 cm	12.5%	Flamingo <sup>TM</sup> Gel fluorochrome	<sup>e</sup>	42	Lade et al., 2019

Methods for protein extraction: A—extraction in sucrose and phenol-containing buffer followed by precipitation with ammonium acetate in methanol; B—plant total protein extraction kit. The kit includes a plant-specific protease inhibitor cocktail and new chaotropic reagent with increased solubilizing power for extracting more hydrophobic proteins; C—trichloroacetic acid (TCA)–acetone precipitation; D—acetone precipitation. <sup>a</sup>MS method was not described. <sup>b</sup>Bacterial proteins differentially expressed by *G. diazotrophicus* co-cultivated with sugarcane SP70-1143/Chunee, respectively. <sup>c</sup>Proteins expressed by sugarcane SP70-1143/Chunee during interaction with *G. diazotrophicus*, respectively. <sup>e</sup>The number of proteins extracted was not reported.

protein labeling was used for the first time during co-culture of a bacterium and its plant host. About 542 proteins were identified, and 78 proteins presented differential expression levels (Table 2), indicating this is a powerful strategy for such studies.

Regarding the protein extraction protocol employed (Table 2), one study used a commercial extraction kit (Lery et al., 2011), while the other studies used protocols based on phenol/sucrose buffer extraction followed by TCA/acetone precipitation (Isaacson et al., 2006). A version of the integrated

protein extraction protocol for crop proteomics analyses was proposed by Wu et al. (2014). The protocol combines three steps: (1) TCA/acetone precipitation to remove non-protein compounds, (2) SDS extraction, which resolubilizes the proteins precipitated by TCA/acetone, and (3) phenol extraction that promotes the denaturation and solubilization of proteins into the organic phase separating them from water-soluble compounds. The use of this protocol may mitigate problems during isoelectric focusing and electrophoresis, which are



**FIGURE 1 |** Proteomics applied to PGPR—grasses interaction studies. **(A)** After colonization by PGPR, whole plant or parts of the plant (shoots, roots, leaf) is submitted to protein extraction (1). The extracted proteins can be: (2a) proteolyzed by trypsin and the peptides separated by (3a) liquid chromatography and its mass/charge analyzed by spectrometer mass (LC-MS/MS) with subsequent identification of the proteins by specific software. The peptides from different samples can be labeled with peptides or other molecules of known mass to perform simultaneous analysis and quantification. If the samples are analyzed individually, the quantification can be done by peptide counts known as label-free quantification. **(B)** Proteins are firstly separated by bidimensional electrophoresis (2D-SDS-PAGE) (2b), analyzed to differential expression (3b), the differentially expressed protein spots are removed (4b), the protein spots are digested with trypsin (5b), and the peptides analyzed in a MALDI-TOF spectrometer with subsequent identification of the proteins by computational analysis (6b). **(C)** The protein extract can be separated by unidimensional electrophoresis (SDS-PAGE) (2c), the entire lanes can be sliced in several pieces (4c), proteins are trypsinized (5c), and peptides analyzed by LC-MS (6c). On the other hand, differentially expressed proteins can be identified and extracted from the gel (4c), proteins are trypsinized (5c), and peptides analyzed by MALDI-ToF MS (6c).

commonly associated with non-protein compounds released from plant tissues during extraction. Considering that the protein extraction protocols are laborious and time-consuming, a modified TCA/acetone precipitation was established for plant proteomics. This protocol can be performed in 45 min, avoiding chemical protein modification and degradation as occur with the classical TCA/acetone or acetone method (Niu et al., 2018, 2019).

In most studies in Table 1, only proteins from plants were identified. For the *Saccharum* spp. and *G. diazotrophicus* interaction proteome (Lery et al., 2011), two sugarcane genotypes (SP70-1143 and Chune) were inoculated. The *G. diazotrophicus* co-cultivated with SP70-1143 and Chune genotypes resulted in 34, and 43 bacterial proteins differently expressed, respectively.

The up-regulated proteins were involved in metabolism and signaling pathways. Proteins from *Herbaspirillum seropedicae* SmR1 colonizing rice roots were also identified, highlighting proteins involved in nitrogen fixation and assimilation (Alberton et al., 2013). Among 87 differentially expressed proteins of maize roots (DKB240 variety) inoculated with *A. brasilense* FP2, four spots were identified as bacterial proteins: malate dehydrogenase (2 spots),  $H^+$ -transporting F-type ATPase beta (1 spot) and major outer membrane protein OmaA precursor (1 spot). The latter acts as adhesin involved in root adsorption and cell aggregation (Cangahuala-Inocente et al., 2013). Michè and collaborators detected potential bacterial proteins in the *Azoarcus* sp. and rice proteome study, but these proteins were not identified because

the bacterial genome had not been completed (Miché et al., 2006). For the grass proteins, most of the studies identified defense proteins. In the proteomic study of sorghum colonized by *Pseudomonas* sp. TLC 6-6.5, 54 bacterial proteins were detected (Dhawi et al., 2017).

## HORMONAL MODULATION IN PLANT-PGPR INTERACTIONS; WHAT DOES PROTEOMICS TELL US?

The phytohormones produced by PGPR are probably responsible for increasing plant growth and yield (Droque et al., 2012). In such complex networks, several compounds, such as auxins, gibberellins, ethylene, jasmonates, cytokinins, abscisic acid, brassinosteroids and salicylic acid, regulate root hair proliferation and elongation. Phytohormones also coordinate plant responses to environmental factors, leading to nutrient uptake enhancement from the soil and activate defense mechanisms against pathogens (Dodd et al., 2010; Dicke and van Loon, 2014). Reviews on phytohormone biosynthesis, regulation, and function are (Mano and Nemoto, 2012; Duca et al., 2014; Tsukanova et al., 2017; Lymperopoulos et al., 2018). Therefore, we briefly describe the existing models for certain phytohormones that explain the participation of these molecules in PGPR-plant interactions.

Certain PGPR stimulate root proliferation through phytostimulant auxin biosynthesis; indole-3-acetic acid (IAA) is the most well-known auxin and enhancing mineral uptake from the soil (Masciarelli et al., 2013) and root exudation, which stimulates root colonization (Spaepen and Vanderleyden, 2011). However, higher concentrations of bacterial IAA can also inhibit root growth (Dobbelaere et al., 1999). Bacterial and plant IAA biosynthesis can be tryptophan dependent or independent (Duca et al., 2014), and although some intermediates differ, most bacterial pathways are similar to the pathways described in plants (Woodward, 2005; Spaepen et al., 2007; Karnwal, 2009).

High concentrations of auxin promote plant growth through ethylene biosynthesis activation (Hardoim et al., 2008). The gaseous phytohormone ethylene regulates physiological processes throughout the plant life cycle. However, during biotic and abiotic stress conditions, production of this hormone is exacerbated, which leads to deleterious consequences in plants (Bleecker and Kende, 2000). Ethylene is synthesized from S-adenosyl-L-methionine (AdoMet) in two steps. (i) 1-Amino-cyclopropane-1-carboxylic acid (ACC) synthase (ACS) catalyzes the conversion of S-adenosyl-methionine (AdoMet) into ACC and the by-product 5'-methylthioadenosine (MTA). (ii) ACC oxidase (ACO) catalyzes the conversion of ACC and O<sub>2</sub> into ethylene, CO<sub>2</sub> and cyanide (Rzewuski and Sauter, 2008). The 5'-methylthioadenosine (MTA) is recycled back into methionine through a salvage pathway. Glick and colleagues proposed a modulation mechanism for plant ethylene biosynthesis by PGPR (Glick et al., 1998). Briefly, plant-exuded tryptophan is taken up by bacteria for the IAA synthesis. IAA secreted by bacteria and also synthesized by plants can stimulate the activity of ACC synthase in plants, converting Adomet into ACC, which can be

taken up by the bacteria and hydrolyzed by ACC deaminase into ammonia and  $\alpha$ -ketobutyrate. Through IAA bacterial activation, the plant synthesizes more ACC, and the bacteria utilize it as a nitrogen and carbon source; consequently, the lower levels of ethylene favor plant development (Figure 2).

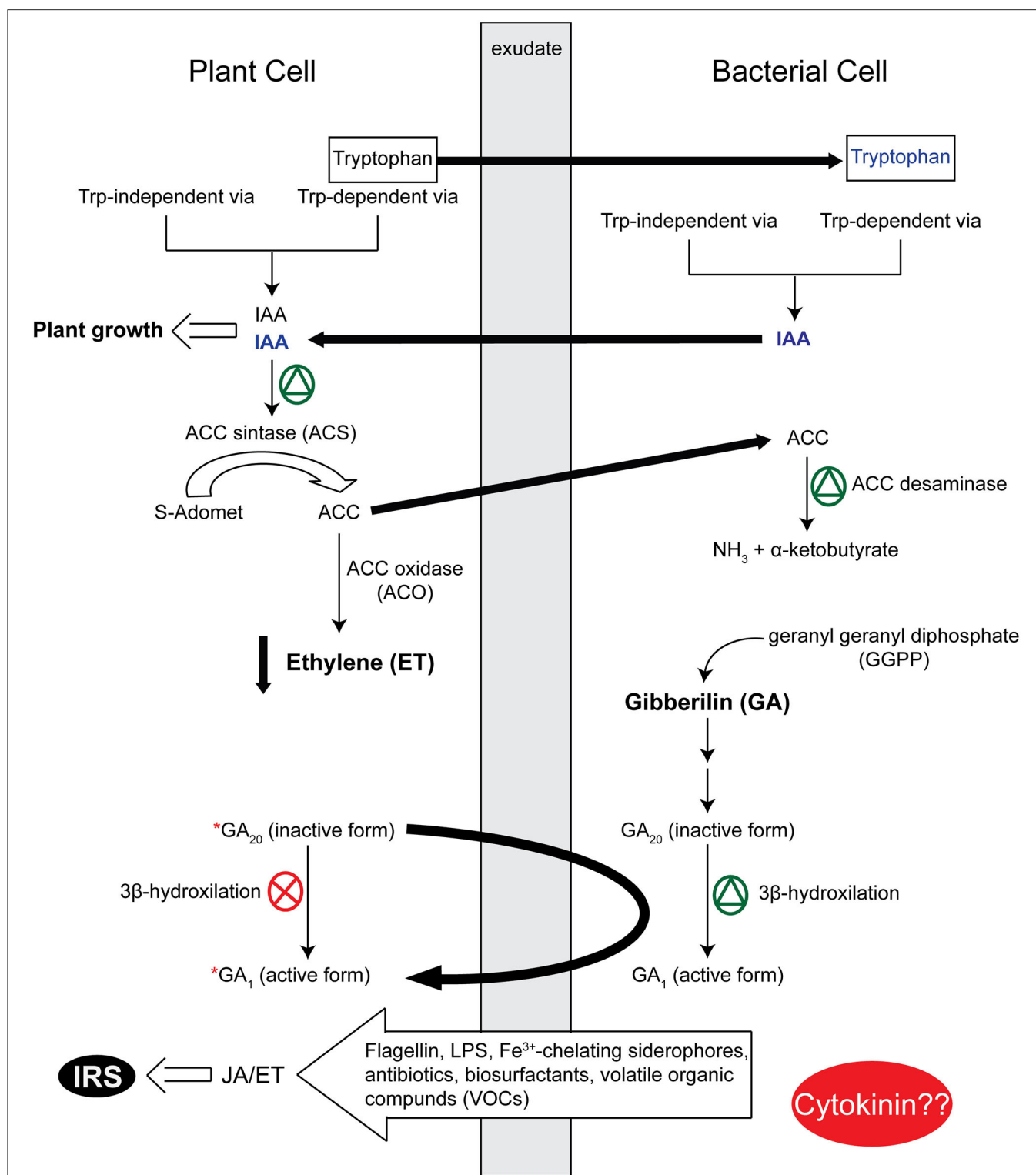
Gibberellins (GAs) are also involved in PGPR-plant interaction. Present in plant, fungi, and bacteria (MacMillan, 2001), ~136 GAs have been identified (see <http://www.phytohormones.info/gibberellins.htm>). However, little is known on how gibberellins participate in this interaction (Rademacher, 1994; Bottini et al., 2004). Studies on *Azospirillum* sp. in gibberellin-deficient mutants of rice demonstrated that *Azospirillum* could convert the inactive form of gibberellins in plants (GA<sub>20</sub>) into the active form (GA<sub>1</sub>) through 3 $\beta$ -hydroxylation, which, consequently, promotes plant growth (Cassán, F., et al., 2001; Cassán et al., 2001) (Figure 2). The inoculation of seed-borne endophytic *B. amyloliquefaciens* RWL-1 in rice plants promoted a significant increase in the content of GA, which led to faster growth of shoot and roots of inoculated rice plants (Shahzad et al., 2016).

Other phytohormones produced by PGPR, such as with cytokinin, aid in plant growth promotion, but their underlying mechanisms remain unclear. Lettuce plants (*Lactuca sativa* L., cv *Lolla Rossa*) inoculated with *Bacillus* sp. strain IB-22 had larger shoots than control plants, but the root length decreased, and consequently, the root to shoot ratio (Arkhipova et al., 2007). According to Vacheron and colleagues, the contribution of cytokinin by PGPR to root system architecture modifications remains speculative (Vacheron et al., 2013) (Figure 2).

Jasmonic acid (JA) and the derivative jasmonates are hormones important to the plant immune system acting in the induced systemic resistance (IRS), which reduces pathogen invasion and colonization (Pozo et al., 2008) (Figure 2). In rice, ISR mediated by *Pseudomonas fluorescens* WCS374 against *Magnaporthe oryzae* depends on jasmonate and the ethylene-regulated signaling pathway (De Vleeschauwer et al., 2008).

Few proteins related to phytohormone biosynthesis and signaling pathways have been identified through proteomics analyses on PGPR-grass interactions. Proteomics analyses of the interaction between *Sinorhizobium meliloti* 1021 and rice demonstrated that one auxin-induced protein was up-regulated in leaves and that IAA-amino acid hydrolase is down-regulated in roots; thus, IAA biosynthesis may be induced by rhizobia through a tryptophan-independent pathway (Chi et al., 2010). Although it was not a study using grass as host, proteomic analysis of *Pseudomonas putida* UW4 exposed to canola exudates showed the expression of an ACC deaminase 1.64-fold higher compared with the control condition (without exudates). In order to confirm the ACC deaminase activity of *P. putida* UW4, ACC, and IAA concentrations were measured on the root exudates of canola treated with the wild-type bacteria and their ACC deaminase negative mutants. Lower ACC and higher IAA concentrations were detected in the exudates of the canola root treated with the wild *P. putida* UW4, confirming the action of ACC bacterial deaminase on the ACC levels of the plant (Cheng et al., 2009).





**FIGURE 2** | A schematic representation of phytohormone modulation by plant-bacteria interactions. Indol-3-acetic acid (IAA) produced by bacteria and plants through tryptophan-dependent or/and tryptophan-independent pathways activates ACC synthase in plants. ACC synthase stimulates ACC production; ACC is hydrolyzed by bacterial ACC deaminase in  $\text{NH}_3$  and  $\alpha$ -ketobutyrate, decreasing ethylene levels in plants (Glick et al., 1998; Glick, 2012). After inoculation with the *Azospirillum lipoferum* strain USA5b in mutant rice plants that present genetic lesions in the 3 $\beta$ -hydroxylation step in gibberillin synthesis, the active form of gibberillin ( $\text{GA}_1$ ) was detected, which indicates active gibberillin synthesis in bacteria (Cassán, F., et al., 2001; Cassán et al., 2001). The response to jasmonic acid (JA), its derivatives and ethylene is necessary for induced systemic resistance (IRS), and different bacterial determinants are responsible for initiating priming in these hormones. Little information is available on bacterial cytokinin role in plant-bacteria interactions. ▲ activation; ⊗ inhibition.

*G. diazotrophicus* co-cultivated in the presence of sugarcane plantlets induced the expression of the GreA transcription factor. According to the authors, *greA* is close to the *ilvA*, which encodes a threonine dehydratase, indicating that *greA* and *ilvA* might be co-regulated. *IlvA* is an enzyme of the isoleucine biosynthesis pathway. Isoleucine is conjugated with JA to affect plant development and interactions with microorganisms (dos Santos et al., 2010). The rice root proteome upon treatment with JA and the *Azoarcus* sp. strain BH72 exhibited induction of a putative ACC oxidase only in the JA-treatment condition, indicating that the ethylene and JA pathways interact with each other (Miché et al., 2006). Although most responses mediated by ethylene and JA are independent, specific pathways, such as induction of the plant defensin gene, *PDF1.2*, require both hormones (Penninckx et al., 1998; Pieterse et al., 2000; Wang et al., 2002).

Xyloglucan endotransglucosylase (XTH) was 2.8-fold more expressed in rice leaves colonized by *Bacillus cereus* NMSL88 (Wang et al., 2002) and was also up-regulated in wheat colonized by *Enterobacter cloacae* SBP-8 (Singh et al., 2017). This enzyme is involved in xyloglucan metabolism in the primary cell wall through xyloglucan endo-cleavage and transferring the new reducing ends to other polymeric or oligomeric xyloglucan molecules, which are important to plant growth and development (Fry et al., 1992; Cosgrove, 2005). In the rice leaf sheath, the XTH gene was up-regulated by GA<sub>3</sub>, one of the active forms of gibberellin (Jan et al., 2004).

Colonization of the grass *Miscanthus sinensis* by *Herbaspirillum frisingense* over 3 h up-regulated the genes *BGAF-1*, *BGAF-2*, and dirigent protein gene (*DIR*) in roots and shoots involved in jasmonate biosynthesis and signaling (Straub et al., 2013). However, after 3 weeks of colonization, these genes were repressed, especially in the roots. *BGAF* is a  $\beta$ -glucosidase-aggregating factor that interacts with maize  $\beta$ -glucosidase, forming large insoluble complexes (Blanchard et al., 2001; Kittur et al., 2007). This protein is induced by JA and contains two domains: a jacalin-related lectin and a disease-response or dirigent protein (Blanchard et al., 2001; Kittur et al., 2007); one of its key functions in plants is the defense response against insects and pathogens (Williams et al., 2002).

*H. frisingense* induced low expression of the auxin response factors *ARF1*, *ARF6*, *IAA18*, and *IAA20* in the *Miscanthus sinensis* shoots and roots. At the same time, ethylene modulation was demonstrated by up-regulation of the ethylene receptors (*ETR1*, *SCER1a*, and *SCER1b*) and downregulation of the ethylene response factor (*SCERF1-like*) in shoots and roots, which suggests ACC deaminase activity from *H. frisingense*. These results were obtained by transcriptomic and quantitative real-time PCR (qRT-PCR) analyses. The proteins identified by differential proteomics did not match those found by RNA transcripts quantification (Straub et al., 2013).

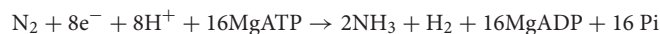
Conversely, proteome analysis of rice roots colonized by *H. seropedicae* SmR1 showed up-regulation of proteins involved in methionine recycling and the 1-aminocyclopropane-1-carboxylic acid (ACC) synthase which synthesizes ACC, the precursor for ethylene biosynthesis. In addition, the ethylene levels were lower in plants inoculated with *H. seropedicae*. Although the ACC

deaminase and IAA pathway enzymes were not identified, this result suggests that *H. seropedicae* modulates the ethylene levels in rice roots, decreasing the stress response promoted by this hormone (Alberston et al., 2013).

In summary, PGPR can affect plant growth through phytohormones. However, few pathways related to this interaction were elucidated, which indicates that more studies are necessary to demonstrate how the phytohormones produced by PGPR alter plant development. Although few proteins related to phytohormone biosynthesis and degradation have been identified in the different proteomes, we can infer from proteomics data that differential expression of the defense and metabolism proteins is likely due to the complex phytohormone network.

## PROTEOME AND BIOLOGICAL NITROGEN FIXATION IN GRASS

Nitrogen is an essential component for all living organisms, and its availability is one of the major factors that limit plant growth. The most abundant nitrogen source is dinitrogen gas (N<sub>2</sub>), which composes 78% of the atmosphere; however, this form is effectively inert. Only certain bacteria and archaea can catalyze biological nitrogen fixation and convert N<sub>2</sub> into a plant-assimilable form (Dixon and Kahn, 2004). These microorganisms are diazotrophs and encode nitrogenase, which is the metalloenzyme complex that reduces N<sub>2</sub> to NH<sub>3</sub> through the following chemical reaction (Burgess and Lowe, 1996):



Nitrogenase is composed of two components that use the electron transfer cascade to reduce its substrate. The dinitrogenase reductase (NifH or Fe protein) transfers electrons in an ATP-dependent manner to the second nitrogenase component, the dinitrogenase (NifDK or MoFe protein), which contains the active site FeMo-cofactor (Seefeldt et al., 2009). In addition to the nitrogenase structural components encoded by *nifHDK*, diazotrophs have three essential FeMo-cofactor biosynthetic components encoded by *nifENB* (dos Santos et al., 2012) and a variable set of genes to optimize nitrogenase biosynthesis, activity and regulation. Essentially, biological nitrogen fixation is a natural fertilization process, and its optimization plays a significant role in sustainable agriculture, reducing the economic and environmental impacts of nitrogen inputs for increasing crop productivity (Beatty and Good, 2011; Oldroyd and Dixon, 2014).

The first evidence of nitrogen fixation with plant-associated bacteria was demonstrated through the symbiotic interaction between rhizobia and legume nodules (Smith et al., 2004). Since then, the interaction between legumes and bacteria that participate in nitrogen-fixing symbiosis has been extensively studied (Cheng et al., 2010). Briefly, a series of proteomic studies showed that the key transcription factors responsible for differentiating nitrogen-fixing nodules, then proteins involved in nitrogen metabolism, mainly NifHDK and regulatory PII proteins, are expressed to initiate nitrogen fixation a few days after inoculation (for more details, please refer to Salavati et al.,

2013 and Muneer et al., 2012). Recent studies show that the most important crops worldwide, such as rice, wheat, and maize, interact with nitrogen-fixing bacteria, and in addition to the nitrogen supplied by the soil, biological nitrogen fixation significantly contributes to nitrogen accumulation in plants (James, 2000). Although rhizobia-legume symbiosis is the most important and likely the most efficient nitrogen-fixing system, nitrogen fixation in crops consumed by humans has received particular attention in the past few decades (Franchete et al., 2009; Beatty and Good, 2011).

Nitrogen biologically fixed by endophytic/endosymbiotic diazotrophs can be transferred to grass, which is inferred mainly by plant dry weight, nitrogen content, and nitrogenase gene expression of their hosts (Egener et al., 1999; Roncato-Maccari et al., 2003; Camilios-Neto et al., 2014). However, few proteomics studies have identified bacterial proteins related to nitrogen fixation or nitrogen metabolism during grass colonization. Only two proteomics studies have identified proteins involved in nitrogen fixation and ammonium assimilation, NifH and glutamine synthetase (GS), respectively, which are up-regulated in rice roots colonized by the *H. seropedicae* SmR1 (Alberton et al., 2013) or the *H. rubrisubalbicans* M1Sm300 (Valdameri et al., 2017). These findings suggest that the cells are under nitrogen-fixing conditions after 7 days of inoculation. *G. diazotrophicus* GS was also up-regulated upon interaction with rooted sugarcane plantlets after 1 day of inoculation (Lery et al., 2011). Differently, this GS expression seems unrelated to fixed nitrogen availability because it was observed with low and high nitrogen input. In contrast, it could be involved in osmoregulatory responses and plant development (Sleator, 2001; Miflin and Habash, 2002).

Interaction with nitrogen-fixing bacteria also affects the regulation of host proteins involved in nitrogen metabolism. *G. diazotrophicus* inoculation of sugarcane roots led to host's glutamate-ammonia lyase up-regulation, which suggests that nitrogen metabolism was stimulated (Lery et al., 2011). The assimilatory ammonium enzymes, cytosolic GS, and glutamate dehydrogenase (GDH), were down-regulated upon *S. meliloti* 1021 inoculation of rice seedlings, leaf sheaths, and roots, respectively (Chi et al., 2010). GS catalyzes ATP-dependent conversion of glutamate to glutamine using ammonium as a substrate, which is followed by a condensation reaction involving 2-oxoglutarate (2-OG) and glutamine to produce glutamate, which is catalyzed by glutamate synthase (GOGAT). However, in an alternative pathway, GDH catalyzes the reversible deamination of glutamate into 2-OG. Together, the down-regulation of GS and GDH suggests that glutamate accumulates, and the lower 2-OG levels support down-regulated aspartate aminotransferase. To maintain glutamate homeostasis, this precursor might be used for proline biosynthesis in agreement with the up-regulation of a putative peptidyl-prolyl isomerase under the same conditions (Wang et al., 2007; Broyart et al., 2010). GS plays a key role in controlling plant productivity, and researchers have suggested using GS as a molecular marker for nitrogen utilization and uptake efficiency (Swarbreck et al., 2011). Thus, addressing the regulatory mechanism for GS protein content and activity regulation, isoforms, and localization

through proteomics approaches could lead to strategies for higher nitrogen efficiency, allowing for less use of nitrogen fertilizers without lowering crop yield.

Other proteins involved in nitrogen metabolism have not been identified through proteomics analyses of PGPR-grass interactions, likely because low-abundance bacterial proteins are challenging to detect in complex protein mixtures. In addition, Fe-S metallo-centers in nitrogenase can be irreversibly damaged by O<sub>2</sub>, which leads to protein degradation and prevents identification through proteomics after a lengthy sample preparation procedure (Hartmann and Burris, 1987; Fisher and Newton, 2005).

## STRESS AND DEFENSE RESPONSES IN GRASS-PGPR INTERACTIONS AS SEEN BY A PROTEOMICS APPROACH

Plant defense mechanisms induced by pathogens in non-compatible interactions has been intensely studied (Senthil-Kumar and Mysore, 2013; Kannoja et al., 2019). However, PGPR has been described to elicit or activate defense mechanisms in host plants, which suggests that even beneficial interactions can trigger plant-defense cell signaling (Goh et al., 2013). Proteomics approaches can be used to understand the molecular basis underlying the modulation of plant proteins in the presence of PGPR, which requires additional studies. Here, we selected stress and defense-related proteins identified in more than one single, comparative proteomic analysis of grass-PGPR to address common pathways.

### Stress Response

Peroxidases (POX) are widely recognized as important antioxidant proteins involved in cellular protection against reactive oxygen species (ROS) (Sharma et al., 2012). Two POX isoforms were detected up-regulated in two *Oryza sativa* cultivars, IR36 and IR42. The colonization study showed that IR36 was successfully and IR42 poorly colonized by the endophyte *Azoarcus* sp., respectively, (Miché et al., 2006). Interestingly, POX isoforms were not regulated in both cultivars upon inoculation with *Azoarcus* sp., but when the two cultivars were treated with jasmonic acid, the two POX were up-regulated. Furthermore, POX was also up-regulated in rice roots colonized by *B. cereus* or *S. meliloti* 1021 (Chi et al., 2010; Wang et al., 2013). In addition to POX, catalase is a key antioxidant enzyme and was up-regulated in rice leaves and sheaths inoculated with *S. meliloti* 1021 (Chi et al., 2010).

Interestingly, rice root colonization by *H. seropedicae* down-regulated two putative L-ascorbate peroxidases (APX), which is related to a lower antioxidant defense (Alberton et al., 2013). In contrast, proteomic analysis of *G. diazotrophicus*-sugarcane interaction showed an opposite response, wherein APX was exclusively expressed by sugarcane SP70-1143 during its interaction with *G. diazotrophicus* (Lery et al., 2011). Furthermore, the differential proteomics analysis in *M. sinensis* seedlings grown in the presence or absence of *H. frisingense* revealed that APX1 was up-regulated and, in stark contrast,

APX2 was down-regulated in inoculated plants (Straub et al., 2013). Given the contradictory data obtained using proteomic approaches, the direct effect of APX regulation in grass inoculated with PGPR must be further investigated. It is likely interplay of different isoforms, cultivar-dependent response and time-dependent expression of APX.

Treatment of rice (cv. IR36) seedlings with JA or inoculation with *Azoarcus* sp. showed two isoforms of the rice salt-stress induced protein (SalT) up-regulated (Miché et al., 2006). Besides, SalT was also up-regulated in rice roots inoculated with *H. seropedicae* (Alberton et al., 2013). Up-regulated proteins involved in plant defense, including the salt stress root protein RS1, were also observed in the interaction between *Bacillus cereus* and rice (cv. Kyou818) (Wang et al., 2013). Together, these data suggest that SalT is up-regulated in rice inoculated with different PGPR. Based on these proteomics studies, SalT may be suggested as a molecular marker for beneficial grass-bacteria interactions. In addition, a BlastP analysis using the *Oryza sativa* Indica cultivar-group SalT (Uniprot ML OsI\_001780) as query against the protein database showed that SalT homologs are widely distributed among grasses, including rice, sorghum, wheat, maize, sugarcane, *Setaria* grass, Tausch's goatgrass, and Ravenna grass, with at least a 38% identity and an expected value (E-value) of  $1 \times 10^{-23}$ .

Wheat colonized by *Enterobacter cloacae* SBP-8 and submitted to salt stress (200 mM NaCl) enhanced the "calcium channel protein" which is part of the ROS-responsive  $\text{Ca}^{2+}$  channel, mediating the salinity-induced  $\text{Ca}^{2+}$  influx in the leaves (Singh et al., 2017).  $\text{Ca}^{2+}$  is a plant second messenger released under salt stress (Choi et al., 2014). For a review of the beneficial effect of PGPR on the development of plants under salt stress, refer to Bhat et al. (2020).

## Defense Response

PGPR can benefit plant development by activating defense mechanisms against attackers. It is already known that PGPR can elicit induced systemic resistance (ISR) against infection by pathogens (fungi, viruses, nematodes, and bacteria) (Jettiyanon and Kloepper, 2002; Walters et al., 2013). The ISR elicited by PGPR is mainly dependent on jasmonic acid-ethylene (JA-ET) signaling (Pieterse et al., 2014; Kannoja et al., 2019).

*Azoarcus* inoculation promoted weak induction of two different PR proteins in *Oryza sativa* cv. IR36, PR-10b, and RSOsPR10 (Miché et al., 2006). One putative exoglucanase precursor, classified as an important PR protein, was induced in rice seedlings inoculated with *S. meliloti* 1021 (Chi et al., 2010). *Azoarcus* also induced overexpression of a PR protein (Prb1) in cv. IR42 (Miché et al., 2006).

Probenazole-induced protein (PBZ1) is a PR protein with an unknown biological function, although its ribonuclease activity has been described (Bantignies et al., 2000). The PBZ1 expression levels were not regulated upon inoculation of rice with *Azoarcus* sp., but JA induced PBZ1 up-regulation (Miché et al., 2006). Interestingly, rice inoculated with *H. seropedicae* exhibited lower PBZ1 mRNA expression levels in roots (Brusamarello-Santos et al., 2012, 2019). These results suggest that specific rice defense

targets, such as PBZ1, present opposing behaviors depending on the bacteria and treatment used.

This opposite expression pattern of PBZ1 in rice induced by *Azoarcus* sp. and *H. seropedicae* was also observed for glutathione *S*-transferase (GST) because it was not regulated and up-regulated by rice inoculation, respectively (Miché et al., 2006; Alberton et al., 2013). Similarly, up-regulated GST was observed in rice plants upon JA treatment or inoculation with *Bacillus cereus* and *P. fluorescens* (Miché et al., 2006; Kandasamy et al., 2009; Wang et al., 2013). GST was also up-regulated in maize leaves colonized by *Azospirillum brasilense* Sp7 for 16 days (Lade et al., 2018). The receptor-like protein kinase (RLK) DUF26 is involved in both symbiosis and pathogen defense and was up-regulated in rice seedlings inoculated with *Bacillus cereus* (Wang et al., 2013). In addition, a putative receptor-like kinase was up-regulated in rice roots following either *Azoarcus* inoculation or JA treatment (Wang et al., 2013).

*S*-adenosylmethionine synthetase (SAMS, also known as methionine adenosyltransferase) is involved in methionine recycling and under stress is related to alleviating biotic and abiotic induced-damage; SAMS was up-regulated in rice leaf sheaths inoculated with *S. meliloti* 1021 (Chi et al., 2010). Two spots corresponding to SAMS and SMAS2 were up-regulated in rice roots inoculated with *H. seropedicae* (Spaepen et al., 2007). Interestingly, SAMS was up-regulated in wheat grown under salt stress. The inoculation with *Enterobacter cloacae* SBP-8 reduced SAMS expression under the same salt stress condition (Singh et al., 2017). Proteins involved in methionine recycling, including methylthioribose kinase and acireductone dioxygenase 1, were also induced by *H. seropedicae* inoculation. Under the same conditions but analyzed using a different approach, the mRNA that codes enolase, which is also involved in methionine recycling, was up-regulated in rice upon *H. seropedicae* colonization (Alberton et al., 2013). Accordingly, the spots that corresponded to enolase were up-regulated in rice inoculated with *S. meliloti* 1021 (Chi et al., 2010). These data suggest that some beneficial bacteria might induce methionine recycling, which is involved in ethylene hormone biosynthesis in the plant host.

Two proteomic studies on the interaction between *Azospirillum brasilense* Sp7 and maize pointed out the maize beta-glucosidase 2 up-regulation (Lade et al., 2018, 2019). Beta-glucosidase 2 catalyzes the cleavage hydroxamic acid glucosides, specifically the glucoside of 4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA), a secondary metabolite plant against pathogens (Babcock and Esen, 1994; Frey et al., 1997). After 16 days of inoculation with *A. brasilense* Sp7, beta-glucosidase 2 from maize leaf was 2-fold more expressed than non-inoculated maize plants (Lade et al., 2018). In another study, when the maize plants were co-treated with *A. brasilense* Sp7 and the maize dwarf mosaic virus (MDMV), after 16 days, the Sp7 promoted up-regulation around 1.8 of the beta-glucosidase 2 (Lade et al., 2019). Lade et al. (2018, 2019) concluded that *Azospirillum brasilense* Sp7 activates the systemic resistance (IRS). Tobacco plants expressing  $\beta$ -glucosidase from *Trichoderma reesei* showed increased internode



length, height, biomass, leaf area, and trichome density than control plants and all these developmental parameters are regulated by gibberellins (Jin et al., 2011). Therefore, beta-glucosidase possibly acted on inactive glucosyl-gibberellin conjugates, releasing the active form of this hormone, as well as of the hormones zeatin and indole-3-acetic acid. In addition, plants expressing beta-glucosidase also showed resistance against whitefly (*Bemisia tabaci*) and aphid (*Myzus persicae*) attacks, due to protection provided by the sugar esters exudated from globular trichomes of the leaves (Jin et al., 2011).

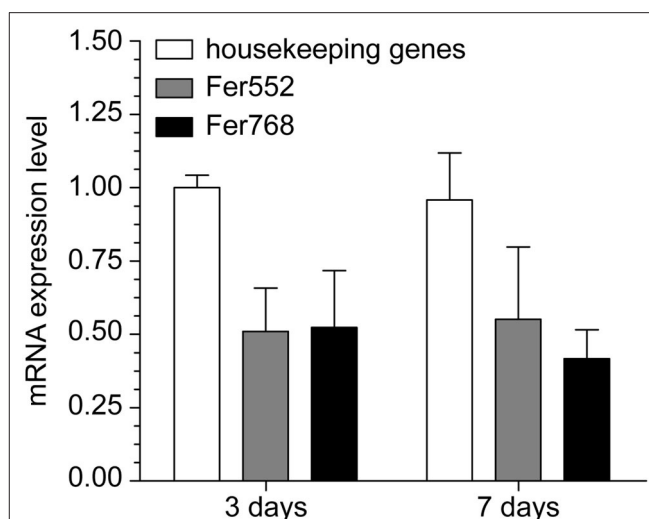
## SIDEROPHORE PRODUCTION BY PGPR

Rice inoculation with *H. seropedicae* showed that plant proteins involved in methionine recycling and mugineic acid biosynthesis were up-regulated using a proteomics approach and RT-qPCR, respectively. One of the up-regulated genes that code for key enzymes in the phytosiderophore synthesis pathway was nicotianamine synthase 1 (*NAS1*); the mRNA level was ~100-fold higher in inoculated plants (Alberton et al., 2013). The *NAS1* protein was also up-regulated in rice seedlings upon inoculation with *Bacillus cereus*, as demonstrated by a comparative proteomics analysis (Wang et al., 2013). Siderophore produced by PGPR may improve host plant iron absorption using an Fe-siderophore complex (Dimkpa et al., 2009). Given that *H. seropedicae* and *B. cereus* increased the *NAS1* expression levels, we hypothesized that inoculated plants might naturally accumulate iron increasing the nutritional value, which is a strategy recently introduced in transgenic rice overexpressing the *NAS1* gene (Dimkpa et al., 2009).

To address this hypothesis, we measured by RT-qPCR the mRNA levels for two rice ferritin isoforms, which is one of the most important proteins involved in iron storage, to determine whether the rice ferritin levels increased in rice roots upon inoculation with *H. seropedicae*. Surprisingly, the mRNA expression levels for both rice ferritin genes (*Fer552* and *Fer768*) were repressed by *H. seropedicae* inoculation (Figure 3). This finding is inconsistent with our first assumption and leads to two assumptions for further assessment: (i) ferritins might be not involved in iron storage under this condition and (ii) the bacteria reduced the iron availability, which induced the *NAS1* mRNA levels to improve iron uptake by the plant. Brusamarello-Santos et al. (2019) detected a bacterioferritin gene induced (2.2-fold) in the study of the interaction between *H. seropedicae* *SmR1* and roots rice. However, other genes related to siderophore biosynthesis were not identified.

## THE METABOLOMIC VIEW OF THE INTERACTION BETWEEN PGPR AND GRASSES

While proteomics identifies and quantifies proteins and their modifications, metabolomics identifies and quantifies



**FIGURE 3** | Rice gene expression levels determined using reverse-transcription quantitative-PCR. The levels of mRNA that code two ferritins exhibited differential expression in rice roots inoculated with *Herbaspirillum seropedicae*. *Fer552* corresponds to similar to ferritin 1, chloroplast precursor (EC 1.16.3.1) (*ZmFer1*), RAPDB Os11t0106700-01, primers forward 5' CCGCGCGCGCGCGCTACC 3' and reverse 5' CCTTCCCTTCCCGCGCGCC. *Fer768* corresponds to similar to ferritin 1, chloroplast precursor (EC 1.16.3.1) (*ZmFer1*), RAPDB Os12t0106000-01, primers forward 5' ATTCCTTGAGGAGCAGGTG 3' and reverse 5' GCTTCTTCCTCAAGCAGCTTC 3'. The expression levels were normalized using three housekeeping genes (actin 1, tubulin beta-2 chain, and protein kinase). Three independent RNA samples from inoculated and uninoculated rice roots were used, and each sample was analyzed in triplicate.

metabolites concentration variations. Metabolites are small molecules (<1,500 kDa) and the end products of gene expression. Therefore, the metabolomic picture of a cell or tissue can be directly correlated to the phenotype (Tugizimana et al., 2018). Both proteomics and metabolomics employ the previous extraction with solvents to obtain the analytes, which will be identified and quantified by mass spectrometers. Metabolomic studies also can use Nuclear Magnetic Resonance (NMR) spectroscopy, as in the study of the interaction between plant growth-promoting bacteria and mycorrhiza with *Setaria italica*, a livestock grass and a potential source of carbon for biofuel production as well (Dhawi et al., 2018). In this study, the endomycorrhizal consortium alone, composed by *Glomus intraradices*, *G. mosseae*, *G. aggregatum*, and *G. etunicatum*, or combined with the PGPR *Pseudomonas* TLC6-6-5.4, showed a positive effect on biomass, plant height and chlorophyll content compared to non-inoculated plants. The same was determined for plants inoculated with the ectomycorrhizal consortium, composed by *Rhizopogon villosulus*, *R. luteolus*, *R. amylogogon*, *R. fulvigleba*, *Pisolithus tinctorius*, *Scleroderma cepa*, and *S. citrinum*. In inoculated plants, the fructose and glucose concentrations were decreased, having a negative or no correlation with the *S. italica* biomass amount, while gallate, gluconate, and malate increased positively correlated with plant height and chlorophyll amount (Dhawi et al., 2018).

*Pseudomonas* TLC6-6-5.4 and the endomycorrhizal blend were inoculated in maize seedlings grown in stamp sands. After 62 days of the seeds germination, the metabolites of the plants were extracted and analyzed by GC-MS (gas chromatography-mass spectrometry), showing upregulation of glyoxylate and dicarboxylate metabolism. In addition, PGPR alone or associated with endomycorrhizal mix increases the concentrations of mannitol, palmitic acid, lysine, stearic acid, and sucrose. In the plants inoculated only with the endomycorrhizal consortium, sucrose concentration was significantly decreased (Dhawi et al., 2015). Regardless of the metabolic variations, the maize biomass was increased in the group inoculated with the combination of endomycorrhiza and PGPR (Dhawi et al., 2015).

Metabolomic analyses of plant-bacteria association have been useful to study the mechanisms by which some bacteria elicit plant resistance against fungi. For instance, the inoculation of rice with *Rhizobium leguminosarum* bv *phaseoli* RRE6 and *R. leguminosarum* bv *trifolii* ANU 843 increased the concentrations of phenolic acids, such as cinnamic, ferulic, gallic, and tannic acids, increasing the resistance against the pathogen *Rhizoctonia solani* (Mishra et al., 2006). Noteworthy, the *R. leguminosarum* RRE6 was the most efficient strain eliciting resistance and phenolic compounds synthesis.

The plant inoculation with PGPR can also activate antimicrobial production. Maize inoculated with *Azospirillum lipoferum* CRT1, and *Azospirillum brasilense* strains CFN-535 and UAP-154 were transferred to non-sterile soil under greenhouse conditions. After 10 days of inoculation, metabolomic analyses showed that the concentrations of benzoxazinoids were increased (Walker et al., 2011). Benzoxazinoids are secondary metabolites of plant defense and allelopathy. Maize inoculated with *Azospirillum lipoferum* CRT1 and transferred to the field showed higher benzoxazinoids amounts than non-inoculated control (Walker et al., 2012). These findings show that metabolomics is a useful tool to screen large libraries of plants and microbes associations, searching for up-regulation of relevant compounds for plant resistance against pathogens and abiotic stresses. Indeed, such kind of technology will be critical to find better and competitive biofertilizers.

Metabolomics has been showing to be a useful tool to compare strains of different genetic background and deepen our understanding of plant-bacteria association. The metabolic profiles of maize roots inoculated with nitrogen-fixing bacteria (*Herbaspirillum seropedicae* SmR1 and *Azospirillum brasilense* FP2) presented more alterations than those inoculated with non-nitrogen-fixing mutants (*Herbaspirillum seropedicae* SmR54 and *Azospirillum brasilense* FP10). In the roots of the maize variety FV252, the concentrations of mannitol, trehalose, isocitrate, amino adipate, malonate, gluconate, cysteine, threonate, and trans-aconitate were increased for the group inoculated with nitrogen-fixing strains of *H. seropedicae* or *A. brasilense*. For instance, the concentration of mannitol in roots inoculated with *H. seropedicae* SmR1 was 50-fold higher in comparison to roots inoculated with *H. seropedicae* SmR54. Similarly, a 33-fold increasing in mannitol concentration was measured in roots inoculated with *Azospirillum brasilense* FP2. Interestingly, the metabolic changes promoted by the diazotrophic strains in

FV252 were significantly reduced in roots of the FV2 variety, indicating that early events of interaction and plant recognition are essential for metabolic adaptability (Brusamarello-Santos et al., 2017).

The metabolic response of different cultivars of rice (Cigalon and Nipponbare) was tracked after inoculation with the epiphyte *Azospirillum lipoferum* 4B or the endophyte *Azospirillum* sp. B510. Both strains colonize the rhizoplane of both rice varieties, while only *Azospirillum* sp. B510 was found within the roots of Cigalon and Nipponbare. Although *A. lipoferum* 4B colonizes only the root surface, it stimulated accumulation of tryptophan in roots of both cultivars. Tryptophan is the precursor of auxin synthesis like indole acetic acid (IAA) in bacteria and plants. On the other hand, *A. lipoferum* 4B induced modifications in the secondary metabolism only in Cigalon roots, while B510 promoted secondary metabolic variations on shoots and roots of both cultivars (Chamam et al., 2013). The different combinations of the interactions of the two rice cultivars and two bacteria revealed that the endophytic strain B510 seemed to have little effect on root morphology of both cultivars compared to the non-endophytic strain 4B. However, in Nipponbare, its original host cultivar, the B510 induced the highest increase of shoot and root biomass. Interestingly, 4B induced variations in flavonoids concentrations in both cultivars and to hydroxycinnamic acid-based compounds in Cigalon, particularly p-coumaric acid and feruloyl quinic acid. p-Coumaric and ferulic acids are among the main phenolic compounds of rice (Dixon et al., 2002; Chung et al., 2018). Phenolic compounds are important for resistance against pathogens and allelopathy in rice (Chung et al., 2018). For instance, feruloyl quinic acid confers plant resistance against *Burkholderia andropogonis* (Mareya et al., 2019). Some C-glycosylated flavones, like luteolin-6-C-arabinoside-8-C-glucoside, isoorientin-200-glucoside, apigenin-6-C-arabinoside-8-C-glucoside, 6-C-diglucose coumaroyl isoscoparin, and isoscoparin-200-glucoside had their concentrations varying in the shoots of colonized Cigalon and Nipponbare. *Azospirillum* sp. B510 increased the concentration of luteolin-6-C-arabinoside-8-C-glucoside in shoots of Nipponbare, as well as the concentration of apigenin-6-C-arabinoside-8-C-glucoside in shoots of Cigalon. As these flavonoids are described having antioxidant and antimicrobial activity, the B510 strain might induce systemic resistance in rice (Yasuda et al., 2009; Compant et al., 2010).

Except for the metabolomic analysis performed with HPLC in the work of Mishra et al. (2006) and with NMR by Dhawi et al. (2018), the other metabolomic studies employed LC-MS or GC-MS. Agtuca et al. (2020) studied the metabolome of *Setaria viridis* roots colonized by *Herbaspirillum seropedicae* SmR1 (Fix<sup>+</sup>) and the SmR54 mutant (Fix<sup>-</sup>) using laser-ablation electrospray ionization (LAESI) (Agtuca et al., 2020). LAESI employs a mid-infrared to ablate material from a sample surface, and then, the ablated material is ionized by an electrospray source (Etalo et al., 2018). The LAESI-MS allowed the detection of several metabolites from different pathways. Metabolites from the purine, zeatin (cytokinin), and riboflavin biosynthesis pathways were significantly more abundant in roots inoculated with SmR1 and SmR54 than when uninoculated. However, the diazotrophic

strain SmR1 enriched a large number of different types of metabolites in the *Setaria roots* (Agtuca et al., 2020). Roots inoculated with SmR54 or uninoculated have a decreasing in the concentration of metabolites involved in nitrogen, starch, and sucrose metabolisms, possibly due to N limitation. Interestingly, indole-alkaloid biosynthesis metabolites were more abundant in the roots colonized by SmR54, perhaps reflecting a plant defense response (Agtuca et al., 2020).

## CONCLUDING REMARKS

Only few proteomic and metabolomic studies have addressed the PGPR interactions with grass so far. However, the inverse is true when the two organisms are evaluated separately. Thus, why are there few proteomic and metabolic studies on PGPR-grass interactions? One limitation in PGPR and grass proteomics studies is the lack of genomic information for plant models, especially for grass. However, high throughput genome sequencing platforms have significantly increased the genomic databank and, consequently, led to more proteomics studies. For the metabolomics studies, likely the low concentration of some metabolites might be challenging to measure confidently with the current techniques. Besides, the extraction of metabolites is usually lengthy and do not guarantee that the quenching of metabolites during the process is sufficient to avoid misinterpretation of the data. Indeed, improved techniques based on LAESI or MALDI-Imaging that can extract and measure the metabolites *in situ* with high sensitivity will be a breakthrough in the area.

Another important point is the “transcriptomic supremacy.” The recent trend focuses on transcriptomic analyses to obtain global data on plant-bacteria interactions, including the grass-bacteria interactions, for which the number of studies is remarkably more significant. High throughput gene expression analysis, such as cDNA microarrays and transcriptome analyses through next-generation sequencing

(RNA-seq), has aided in elucidating plant responses to bacteria colonization. However, proteomics has certain advantages over transcriptome analyses because it analyses protein levels, avoiding misinterpretation from post-transcriptional and translational regulation when only mRNA levels are considered. Therefore, proteomics analyses are still essential for understanding the complex molecular mechanisms involved in plant-bacteria associations, specifically, the beneficial PGPR and grass interactions. Of course, as the concentrations of the metabolites into the cell are dependent on the transcriptomic and proteomic status, metabolomics analysis turns indispensable to have a complete understanding of the grasses-microbe interactions.

Another question is whether the PGPR and grass interactions are an uninteresting topic for proteomics and metabolomics research. The answer seems to be no. The beneficial PGPR and grass interactions involve numerous pathways, which are a scientific topic that must be solved using—omics techniques. In addition, the considerable agriculture interest in grass farming and replacing chemical fertilizer for more sustainable forms of fertilization suggest that employing proteomics/metabolomics to study PGPR-grass interactions will be a fruitful subject for omics research in the next years.

## AUTHOR CONTRIBUTIONS

All authors reviewed the literature and contribute for the manuscript writing and revision.

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# Inoculation With Growth-Promoting Bacteria *Azospirillum brasilense* and Its Effects on Productivity and Nutritional Accumulation of Wheat Cultivars

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Agricultural practices that allow a productive increase in a sustainable manner are becoming increasingly necessary to feed an ever-growing global population. The inoculation with *Azospirillum brasilense* has the potential to reduce the use of synthetic mineral fertilizers with efficient capacity to promote plant growth and increase nutrition. Therefore, this research was developed to investigate the potential use of *A. brasilense* to increase the accumulation of macro- and micronutrients and its influence on grain yield, plant height, and spike population in two wheat cultivars (CD1104 and CD150), under irrigated conditions in the Brazilian Cerrado. The study was carried out in a Rhodic Hapludox under a no-tillage system. The experiment was designed in randomized blocks with six replications, arranged in a 2 × 2 factorial scheme: two cultivars (CD150 and CD1104) and two levels of inoculation (control and with *A. brasilense*). The inoculation with *A. brasilense* provided greater accumulation of micronutrients in the aerial part of the wheat. In the cultivar CD1104, B and Cu had an accumulation 27.7 and 57.4% higher compared with those of the control without bacteria. In the cultivar CD150, *Azospirillum* increased the accumulation of B, Fe, and Mn by 43.8, 49.9, and 22%, respectively, and reduced Cu by 21.9%, compared with those of the control. The cultivar CD150 has greater efficiency to accumulate N (+35.5 kg N ha<sup>-1</sup>) as compared with the cultivar CD1104. Interactions between inoculation and cultivars resulted in greater accumulation of S and K in the shoot of the wheat cultivar CD150, as well greater accumulation of Cu in CD1104. In growth assessments, inoculation or cultivars did not statistically influence wheat grain yield and spike population. However, for plant height, the CD1104 genotype has 13.1% bigger plant height on average than that of the CD150 genotype. Inoculation can contribute more sustainably to wheat nutrition.

**Keywords:** cereal, no-tillage system, *Triticum aestivum* L., crop nutrition, plant-growth-promotion, sustainable-agriculture



## INTRODUCTION

Wheat (*Triticum aestivum* L.) stands and ranks second in world cereal production with volume of around 760 million tons (CONAB, 2019). It is an annual cycle plant, being considered a winter and spring cereal with great economic importance (Galindo et al., 2019a). The world average wheat consumption as food was 98.7 kg person<sup>-1</sup> year<sup>-1</sup> in 2019 (USDA – United States Department of Agriculture, 2020). The climatic extremes and food security predicted and estimated that the agriculture sector will face the challenge of expanding food production by 70% in the year 2050 to meet the increased demand without significant price impacts and shortages in the world (FAO, 2019; ONU, 2019).

Nitrogen (N) is one of the most necessary nutrients in high doses to meet the nutrient limitation and current wheat productivity demands (Teixeira Filho et al., 2012; Galindo et al., 2016). The complex nature of N dynamics in the soil and the use of non-ideal management practices in several crops (Abalos et al., 2014) lead to a large amount of N losses from leaching and volatilization that can affect water bodies and pollute the environment (Bindraban et al., 2015). Given its high cost in the production process and the need for agriculture that maintains ecosystems and biodiversity, the search for solutions that combine production increases and increasingly sustainable agricultural practices is necessary.

In this sense, inoculation and application of plant growth-promoting bacteria (PGPB) especially *Azospirillum brasilense* is an important strategy in cereal cultivation. Researches about *A. brasilense* report enhanced plant growth by a number of mechanisms, including but not limited to the synthesis and secretion of hormones that increase the root system (Pankievicz et al., 2015). Greater accumulation and availability of nutrients (Galindo et al., 2016; Rosa et al., 2020) and greater tolerance to stresses (such as drought, salinity), plant vigor (Forni et al., 2017), chlorophyll content, and stomatal conductance (Bulegon et al., 2017), as well as increases in grain productivity (Munareto, 2016; Galindo et al., 2019a), were also reported.

The triticulture countries like Brazil and United States have the potential to reduce N fertilizers by 30–40 kg ha<sup>-1</sup> through inoculation with *A. brasilense* (Fukami et al., 2016, 2017). This is due to the capability of inoculation to develop a well-penetrated root system, thus exploring more soil volume and resulting in higher nutrient absorption (Galindo et al., 2018). Ardakani et al. (2011) reported a synergistic effect between inoculation and nutrient absorption, increasing the absorption efficiency of N, P, and K in wheat by inoculation with *A. brasilense*. Nevertheless, Teixeira Filho et al. (2017) observed that inoculation with *A. brasilense* strains AbV5 and AbV6 increased the concentrations of Ca, Mg, Mn, and Zn in wheat grains. The authors also concluded that inoculation is a low-cost, easy-to-apply, and pollutant-free technique that fits the desired sustainable context in reality.

The most frequent technique of inoculation is via seeds; however, spraying during sowing has also demonstrated similar possible results (Munareto, 2016). Several studies indicated positive impacts on crop productivity via seed inoculation

(Teixeira Filho et al., 2017), as in the sowing furrow (Morais et al., 2016), or via the leaf (Ferreira et al., 2014; Munareto, 2016). Munareto (2016) highlighted that inoculation with *A. brasilense* via the leaf is an efficient strategy to increase productivity of wheat cultivars in the Brazilian Cerrado region. It should also be noted that the efficiency of inoculation via seeds can be affected by the use of chemicals during treatment, such as fungicides and insecticides, which reinforces the importance of researches and analysis to use supplementary forms of inoculation.

Inoculation with *A. brasilense* can take place in three ways as also described above (Teixeira Filho and Galindo, 2019); however, few studies have evaluated the effectiveness of application via the leaf, and using this method as an alternative to increase the uptake of N and other nutrients in wheat crop is more ecological and less costly, reducing the risks or incompatibilities with chemical seed treatments and enabling even greater productivity. Although one or more benefits in the literature have been found, increased accumulation of nutrients and increased productivity of wheat have not always been observed, highlighting the need for further studies. Based on the above discussion, this study was aimed to evaluate the efficiency of inoculation with *A. brasilense* via the leaf in wheat crop in an irrigated area, verifying its effect on biometrics, grain yield, and nutritional accumulation of wheat cultivars and studying the effect of PGBP on crop nutrient uptake.

## MATERIALS AND METHODS

### Location of the Experimental Design

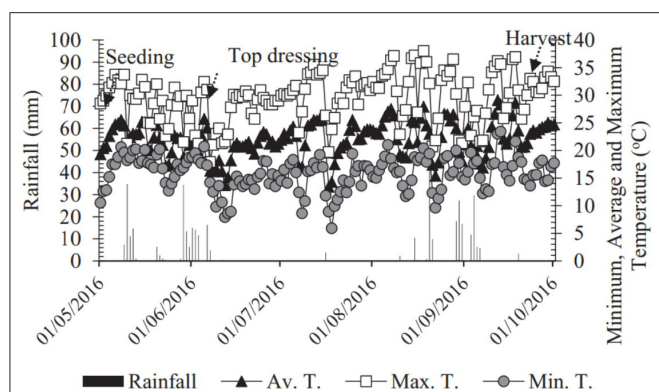
The study was carried out under field conditions at the Teaching, Research and Extension Farm of São Paulo State University (UNESP) in the city of Selvíria, state of Mato Grosso do Sul, Brazil (20°20'43" S; 51°24'7" W, at 355 m above sea level), in 2016. The soil of the experimental area is classified as Dystrophic Red Latosol, clay texture (EMBRAPA, 2018), and was classified as clayey Oxisol (Rhodic Hapludox) according to the Soil Survey Staff (2014). The experimental area was cultivated with annual crops (cereals and legumes) for more than 30 years, with the last 12 years using the no-till system (direct planting system). The last harvest sequence was corn before wheat sowing.

The climatic classification of the region is Aw-Köppen (Alvares et al., 2013) with an average annual rainfall of 1,232 mm and average annual temperature of 24.5°C (Hernandez et al., 1995). The maximum and minimum temperatures and relative humidity of the air and rain indexes were all verified during the study and summarized in **Figure 1**. The experimental area has a sprinkler irrigation system via a central pivot with 14 mm application layer for 72-h watering shifts.

Twenty soil samples were collected randomly before the beginning of the field trial and mixed well, forming a subsample, which was used to determine the chemical attributes according to Raji et al. (2001). The results are shown in **Table 1**.

### Experimental Setup

Wheat cultivars CD150 and CD11104 (Coodetec®) are the most used wheat varieties in Latin America (especially in Brazil and



**FIGURE 1 |** Rainfall and maximum, average, and minimum temperatures obtained from the weather station located at the Education and Research Farm of UNESP during wheat cultivation in the period May 2016 to October 2016.

**TABLE 1 |** Soil chemical attributes in 0–0.20 m layer before the beginning of the wheat field trial.

Soil chemical attributes	0–0.20 m layer
P (resin)	41 mg dm <sup>-3</sup>
S (SO <sub>4</sub> )	2.9 mg dm <sup>-3</sup>
Organic matter	32 g dm <sup>-3</sup>
pH (CaCl <sub>2</sub> )	5.51
K	3.30 mmolc dm <sup>-3</sup>
Ca	41.3 mmolc dm <sup>-3</sup>
Mg	25.6 mmolc dm <sup>-3</sup>
H+Al	34.5 mmolc dm <sup>-3</sup>
B (hot water)	0.24 mg dm <sup>-3</sup>
Cu (DTPA)	5.9 mg dm <sup>-3</sup>
Fe (DTPA)	28.2 mg dm <sup>-3</sup>
Mn (DTPA)	83.9 mg dm <sup>-3</sup>
Zn (DTPA)	1.6 mg dm <sup>-3</sup>
Base saturation	66%

DTPA = chelating solution diethylene triamine penta acetic acid.  $n = 20$ .

Paraguay) with good performance aptitude for the experimental region. Wheat sowing was carried out on 16th May 2016 and harvest on 5th September 2016 (totaling 116 days cycle) with sowing density of 400 viable seeds m<sup>-2</sup> (99% purity and 93% germination) in both varieties. The planting furrows were fertilized with formulated NPK (08-28-16) at the dose of 275 kg ha<sup>-1</sup>. Chemical seed treatment occurred 24 h before sowing, using fungicide and insecticide [thiophanate-methyl + pyraclostrobin fungicide (56 g + 6 g active ingredient—ai—per 100 kg seeds) and insecticide fipronil (62 g ai per 100 kg seeds)] in both cultivars.

The experiment was designed in completely randomized blocks with six replications, arranged in a 2 × 2 factorial scheme. The treatment combination was composed of two wheat cultivars (CD150 and CD1104) and inoculation with PGPB (control—without inoculation and inoculation with *A. brasilense* via the leaf). Each experimental plot was composed of 12 lines of 5 m

in length spaced 0.17 m between the lines. The useful area of each plot was eight central lines with the exclusion of 0.5 m from each end. The size of each plot was 10.2 m<sup>2</sup>.

Plant emergence occurred 6 days after sowing, on 22nd May 2016. The inoculation with *A. brasilense* (strains Abv5 and Abv6, with a guarantee of  $2 \times 10^8$  CFU ml<sup>-1</sup>) was carried out via leaf spray at 9 days after crop emergence (DAE). The spray was done in the early morning under a clear sky (no cloudiness) using a CO<sub>2</sub>-type pump sprayer with a full cone nozzle at a dose of 200 ml ha<sup>-1</sup> of the commercial inoculant in a solution volume of 120 L ha<sup>-1</sup>. Nitrogen fertilization in coverage was carried out at 28 and 43 DAE, using 70 kg N ha<sup>-1</sup> (applied as urea) in each stage. A total of 140 kg N ha<sup>-1</sup> in coverage was applied and incorporated by central pivot irrigation on the same day.

In crop management, weed control was carried out in the total area by applying Pendimetalina (4 L ha<sup>-1</sup>) right after sowing, methyl methulfuron (3.3 g ai ha<sup>-1</sup>) at 20 DAE and 2,4-dichlorophenoxy (1.2 L ha<sup>-1</sup>) at 35 DAE. To prevent disease in the spike, trifloxystrobin + tebuconazole (0.1 L ha<sup>-1</sup>) was sprayed at 75 and 90 DAE, respectively. There was no need of insecticide application in the experimental area.

## Assessments

The evaluation of nutritional accumulation in wheat shoot was carried out at the full flowering stage (50% wheat plants with fully expanded spike) by collecting the aerial part (aboveground) of the wheat plants (1 × 0.17 m) from the central lines of each experimental unit. The collected materials were placed in a forced air ventilation oven at 65°C until uniform mass (72 h) is obtained and weighed with an analytical precision balance (0.001 g) to obtain the dry mass of the plants. Afterwards, the samples were ground in a Wiley mill with a 1-mm (20 mesh) sieve, and the nutrients were determined as described by Malavolta et al. (1997). Using the concentrations of these nutrients and the dry matter of the collected plants, we calculated the accumulation of nutrients in wheat shoot and extrapolated the data into kg ha<sup>-1</sup>.

At the end of the wheat crop cycle, at the time of harvest, the following attributes were determined: (a) plant height: as being the distance (m) from the ground level to the apex in a cluster of 15 plants plot<sup>-1</sup>; (b) spike population: determined by the spikes counted in 0.51 m<sup>2</sup> (0.17 × 3 m of the line) in the central rows of each plot; and (c) grain yield: by manual harvesting of all plants in the useful area of each plot [eight central lines, excluding 0.5 m at the ends being calculated after mechanical threshing and data obtained were corrected to 13% humidity (wet basis) and transformed into 60 kg bags per ha<sup>-1</sup>].

## Statistical Analysis

The Shapiro–Wilk normality test was used to analyze the data set, followed by the variance and covariance test ( $F$  test,  $p < 0.05$ ). The comparison of treatment means was performed by Tukey test ( $p < 0.05$ ). Statistical calculations were performed using the Minitab v17.0 software and graphics were made using SigmaPlot v12.5 software.

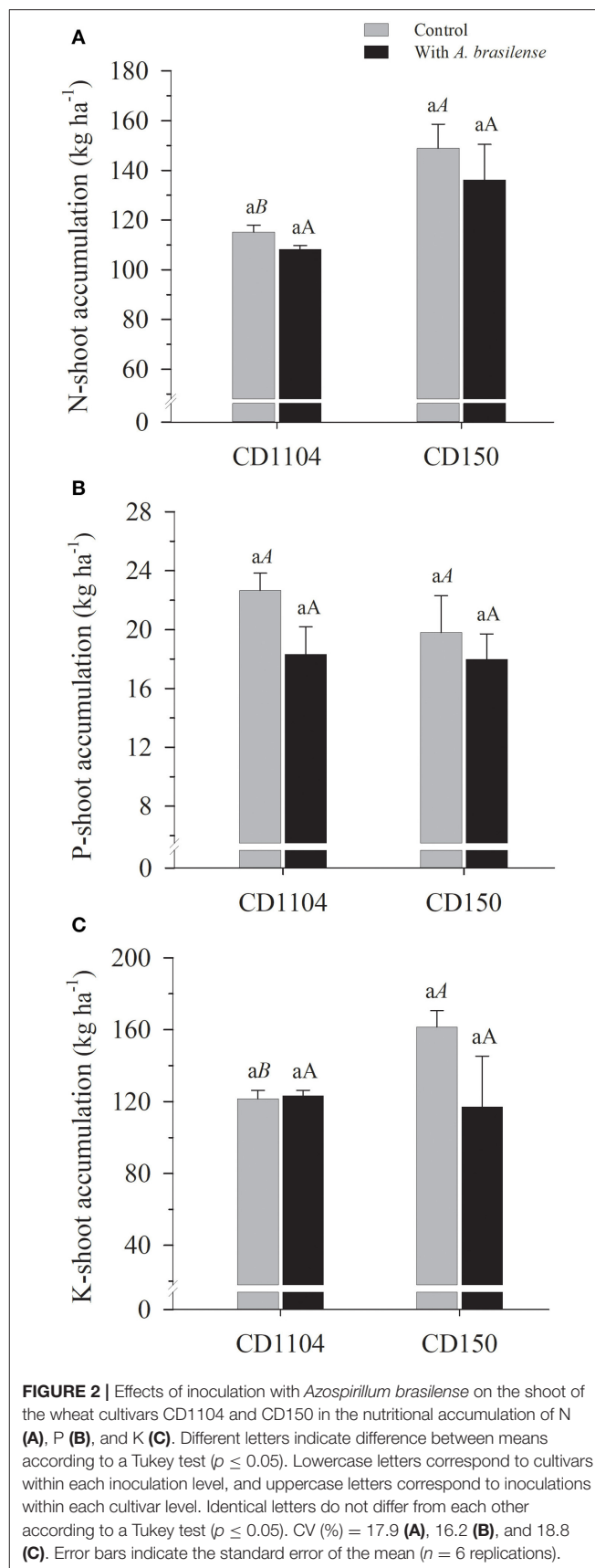
## RESULTS

Interactions between wheat cultivars and inoculation with *A. brasilense* were observed in the accumulation of K in the shoot of the wheat crop (**Supplementary Table 1**;  $p < 0.05$ ). Regarding the nutritional accumulation of primary macronutrients (N, P, and K), with the exception of P, positive responses were verified for accumulations of N and K (**Figures 2A–C**). The wheat genotype CD150 resulted in greater N accumulation in wheat shoot with an average difference of 33.1% in relation to the CD1104 genotype. Higher accumulation of K in the shoot was observed by analyzing the interaction between cultivars and inoculation with *A. brasilense*, where the control (without) resulted in greater accumulation of K by 36% in the genotype CD150 in relation to CD1104 (**Table 2, Figure 2C**).

Interactions between wheat cultivars and inoculation with *A. brasilense* were observed for the accumulation of S in the shoot (**Supplementary Table 1**;  $p < 0.01$ ). Responses in the accumulation of secondary macronutrients such as Ca and Mg were observed to be not significant among both genotypes and inoculation with *A. brasilense* (**Figures 3A,B; Supplementary Table 1**). Significant responses in the accumulation of S were observed by the interaction between cultivars and inoculation. The inoculation with *A. brasilense* efficiently increased the accumulation of S in the wheat genotype CD150 in relation to genotype CD1104. This corresponds to a difference of 23.1% (**Table 2, Figure 3C**).

The inoculation with *A. brasilense* also influenced the accumulation of micronutrients in the wheat shoot (**Supplementary Table 2**;  $p < 0.01$ ). With the exception of Zn, greater accumulation of B and Cu in response to inoculation with *A. brasilense* for the CD1104 genotype, in the order of 73.1 and 158.1%, respectively, was observed, in comparison with the control (without inoculation). Meanwhile, for CD150, *A. brasilense* increased the accumulation of B, Fe, and Mn in the order of 43.8, 49.9, and 22%, respectively, while reducing the accumulation of Cu in the order of 21.9%, for this genotype, in comparison with the control (**Figures 4, 5A, Supplementary Table 2**).

We also observed that the wheat cultivars differed on the accumulation of micronutrients in the shoot (**Figures 4, 5, Supplementary Table 2**). The cultivars influenced the accumulation of B and Mn in the shoot (**Supplementary Table 2**). The results of the CD1104 genotype demonstrated greater accumulation capacity of B with an average of 65.16 g accumulated B ha<sup>-1</sup>, whereas genotype CD150 resulted in the accumulation of 41.37 g B ha<sup>-1</sup> in the wheat shoot (**Figure 4A**), with a difference of 57.5% greater accumulation of B in the CD1104 genotype. Inverse behavior was observed for the accumulation of Mn, where the CD150 genotype resulted in higher Mn accumulation (737.42 g Mn ha<sup>-1</sup>) with 13.3% greater accumulation than the CD1104 genotype (650.57 g Mn ha<sup>-1</sup>) (**Figure 5A**). With respect to Cu accumulation, inoculation with *A. brasilense* promoted the accumulation of Cu in the wheat genotype CD1104 by 57.5% in relation to the control (**Figure 4B**). It was also interesting that inoculation with *A.*



**FIGURE 2** | Effects of inoculation with *Azospirillum brasilense* on the shoot of the wheat cultivars CD1104 and CD150 in the nutritional accumulation of N (**A**), P (**B**), and K (**C**). Different letters indicate difference between means according to a Tukey test ( $p \leq 0.05$ ). Lowercase letters correspond to cultivars within each inoculation level, and uppercase letters correspond to inoculations within each cultivar level. Identical letters do not differ from each other according to a Tukey test ( $p \leq 0.05$ ). CV (%) = 17.9 (**A**), 16.2 (**B**), and 18.8 (**C**). Error bars indicate the standard error of the mean ( $n = 6$  replications).



**TABLE 2 |** Interaction between wheat cultivars and inoculation with *Azospirillum brasilense* for K, S, and Cu shoot accumulation.

Shoot accumulation(kg ha <sup>-1</sup> )	K		S		Cu	
	CD1104	CD150	CD1104	CD150	CD1104	CD150
Control	118.57 bA	161.25 aA	12.86 aA	12.33 aA	100.71 bB	133.69 aA
With <i>A. brasilense</i>	119.56 aA	128.30 aA	11.13 bA	13.70 aA	158.56 aA	109.66 bB
Standard error	12.42		0.53		0.74	

Lowercase letters correspond to cultivars within each inoculation level, and uppercase letters correspond to inoculations within each cultivar level.

*brasilense* decreased the accumulation of Cu in genotype CD150 by 18% in relation to its control. Interactions between cultivars and inoculation were not observed for the accumulation of Cu in the wheat shoot (Supplementary Table 2,  $p < 0.01$ ).

The wheat grain yield (Figure 6A) and spike population (Figure 6B) were not significantly influenced by wheat genotypes or by the *A. brasilense* inoculation in the present study. Analyzing the wheat genotypes, significant differences were observed in the height of the wheat genotypes (Figure 6C; Supplementary Table 3). The CD1104 genotype has 13.1% bigger plant height on average than the CD150 genotype (Figure 6C). This behavior was also visually characterized in the field which can be observed in the records of Supplementary Figure 1.

## DISCUSSION

Interestingly, the CD150 genotype, despite being a predecessor of CD1104 in the wheat cultivar released line (COODETEC, 2017), was reported to have a higher capacity of N uptake and input in the wheat shoot (Figure 2A). Being a constituent of amino acids, proteins, chlorophyll, and several secondary metabolites, N is the most interfering nutrient in the productivity of crops, especially non-atmospheric N<sub>2</sub> fixers (Marschner, 2012). It is among the mineral nutrients that has been noted to have higher concentrations in plant tissues and grains and makes it the most required nutrient in wheat crop productivity (Galindo et al., 2019b). The average accumulation of nutrients in the shoot at the wheat flowering stage of both cultivars resulted in a decreasing trend of K > N > P > Ca > S > Mg. Phosphorous (P) has several important functions in plants, is a constituent element of the ATP molecule, and has a role in the supply and storage of energy in metabolic processes, such as the synthesis of organic compounds and the absorption of nutrients, in addition to being involved in the phosphorylation and electron transport reactions of photosynthesis (Hawkesford et al., 2012). Calcium (Ca) is an important enzyme activator having an indispensable role in the formation of pollen grains and growth of the pollen tube, and it is also a constituent of cell wall together with Mg. Being a central ion of the chlorophyll molecule, Mg is involved in the activation of Rubisco in the Calvin cycle (Taiz et al., 2017).

Potassium (K) is a non-structural component that participates in more than 60 enzymatic reactions in plants, such as playing an important function in the osmotic potential of cells, regulating stomata opening and closing, and helping in the transport of

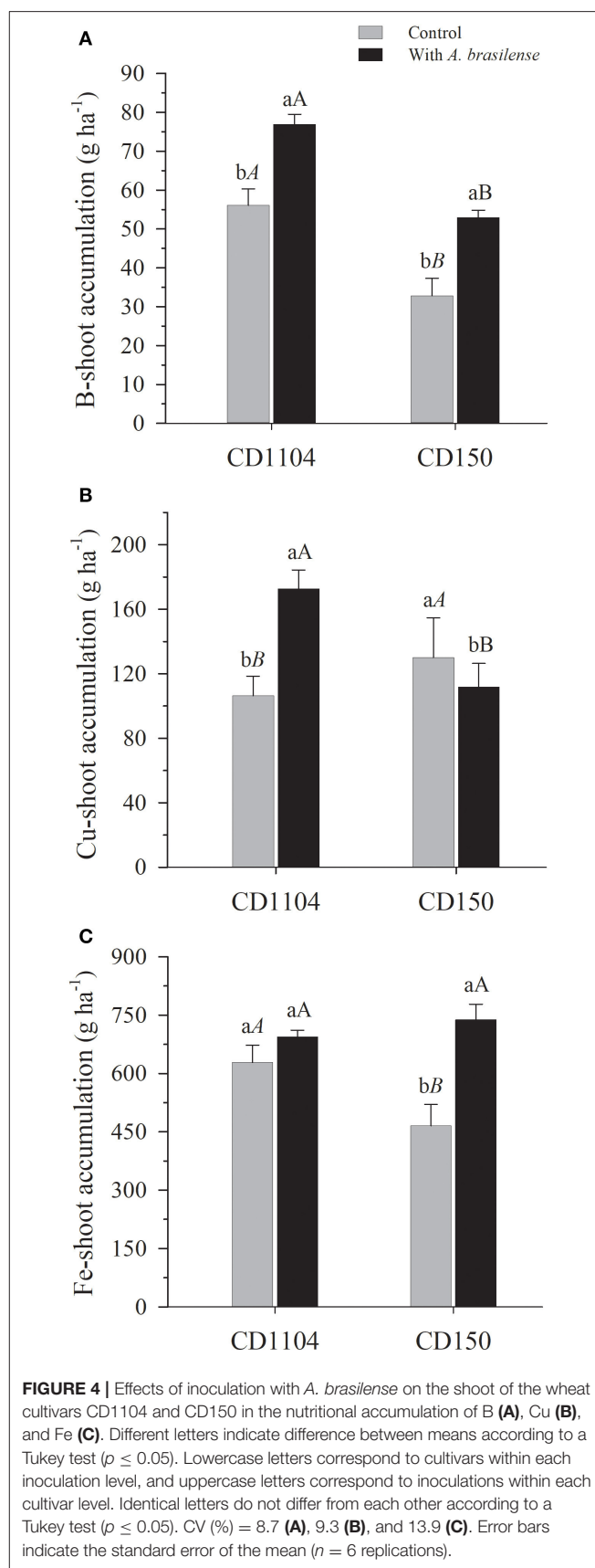
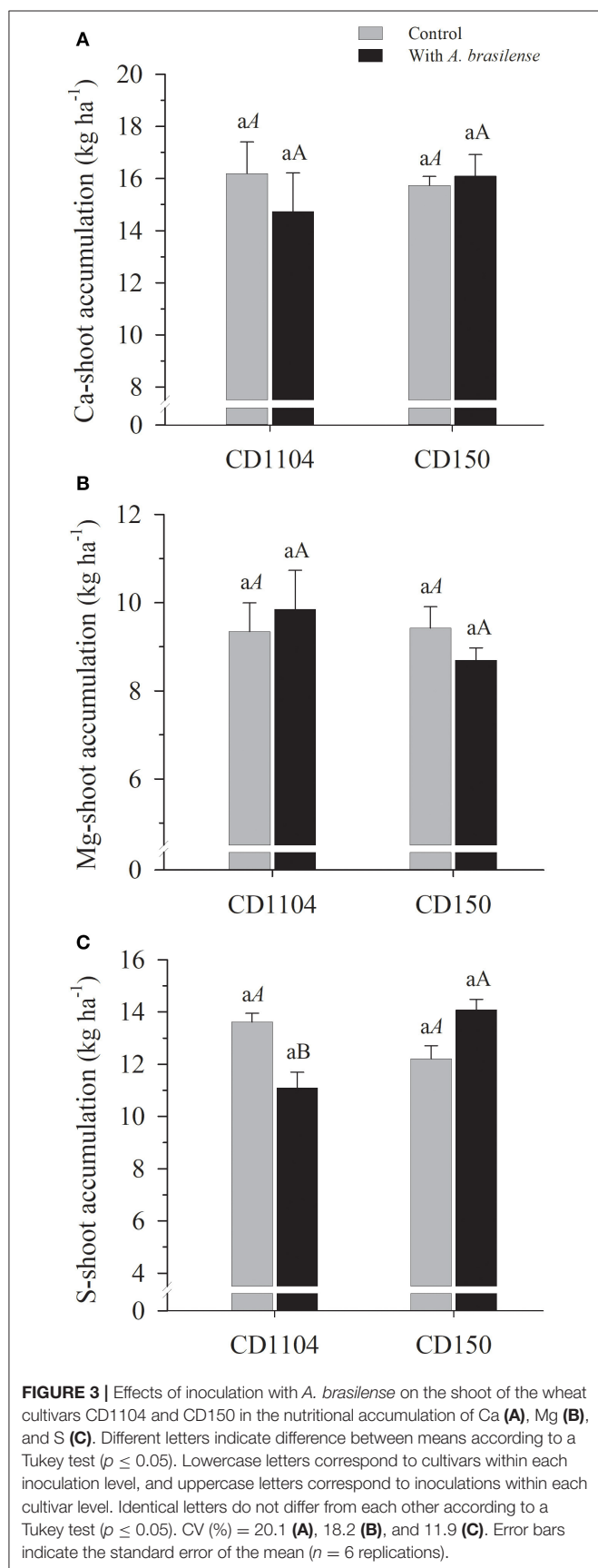
carbohydrates (Marschner, 2012). Teixeira Filho et al. (2017) evaluated *A. brasilense* strains (AbV5–AbV6) in wheat and observed its response in the higher contents of Ca and Mg exported by grains leading to high grain productivity, although accumulation of K was not influenced. The data obtained for K in the present study (Table 2, Figure 2C) demonstrates that the response patterns of this particular bacterium–PGPB association are not only dependent on the ideal conditions, such as temperature, viable CFUs, and PGPB affinity, for establishing inoculation, but also on physiological–molecular interrelationships, intrinsic to plant genetic material (cultivars). Thus, cultivars are able to respond in different ways to the same bacterial strain in the same environment (climate and soil) under the same experimental conditions.

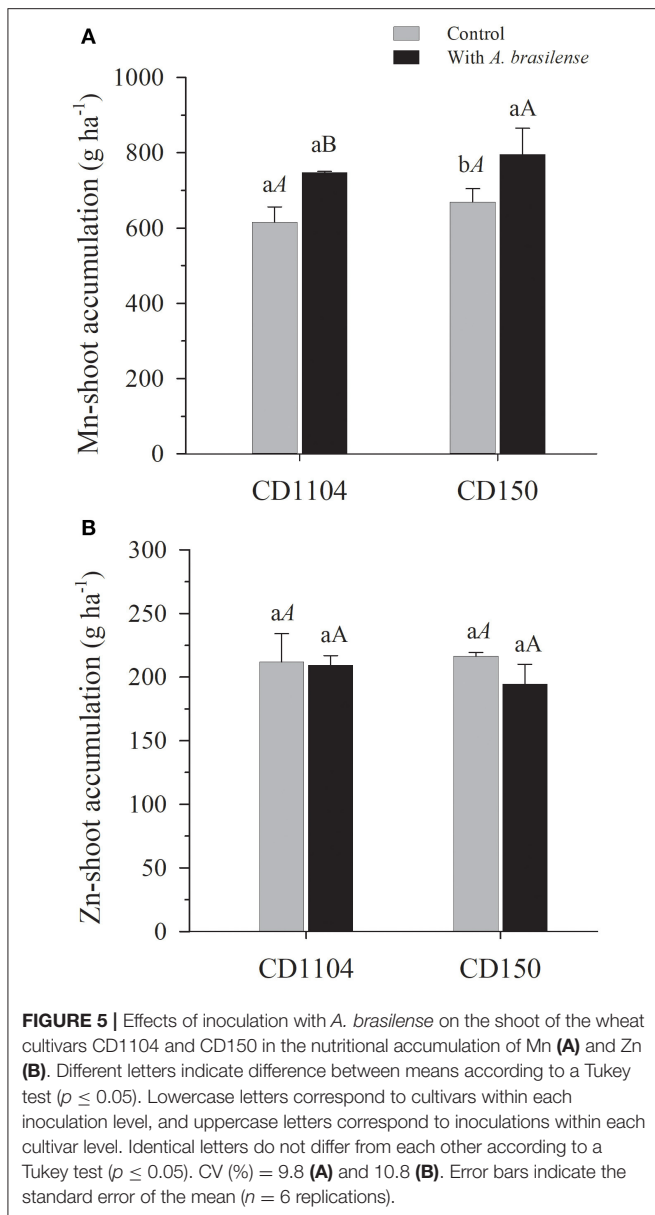
Bacteria of the genus *Azospirillum* are free-living gram-negative species (Galindo et al., 2016), native to the soil, and its association with plants has a series of beneficial responses such as increasing the synthesis of photosynthetic pigments (chlorophyll) (Bulegon et al., 2017), increasing the content of ascorbic acid (Fasciglione et al., 2015), providing greater tolerance to stress (Forni et al., 2017) and greater accumulation and availability of nutrients (Hungria et al., 2010; Galindo et al., 2016), and finally, helping to achieve a higher grain yield (Munareto, 2016; Galindo et al., 2019a).

The accumulation of S followed a similar trend as that of K under cultivar–inoculation interaction (Figure 3B, Table 2). Involved in structural and metabolic functions, S has a synergistic relationship and a triple bond with N in the synthesis of proteins, in addition to being involved in the metabolism of carbohydrates and lipids and decarboxylation reactions (Astolfi, 2009). Similar results were found by Teixeira Filho et al. (2017), where the wheat inoculated with *A. brasilense* was observed with higher accumulation of S in the straw as compared to the non-inoculated. This is probably due to the greater development of the root system of the inoculated wheat, which allowed greater absorption of S-SO<sub>4</sub><sup>2-</sup> in the depth of 0.20–0.40 m, where normally we find higher sulfur contents in the soil.

Evaluating the performance of wheat cultivars in the accumulation of micronutrients, responses to boron were observed in both cultivars (Figure 4A; Supplementary Table 2). Boron participates in the development of pollen grains and pollen tube, promoting cell wall deposition. Therefore, it participates in cell division, increasing cell size and transporting carbohydrates in plants (Marschner, 2012). Manganese is an important enzyme activator, especially in redox reactions of the Krebs cycle, and acts

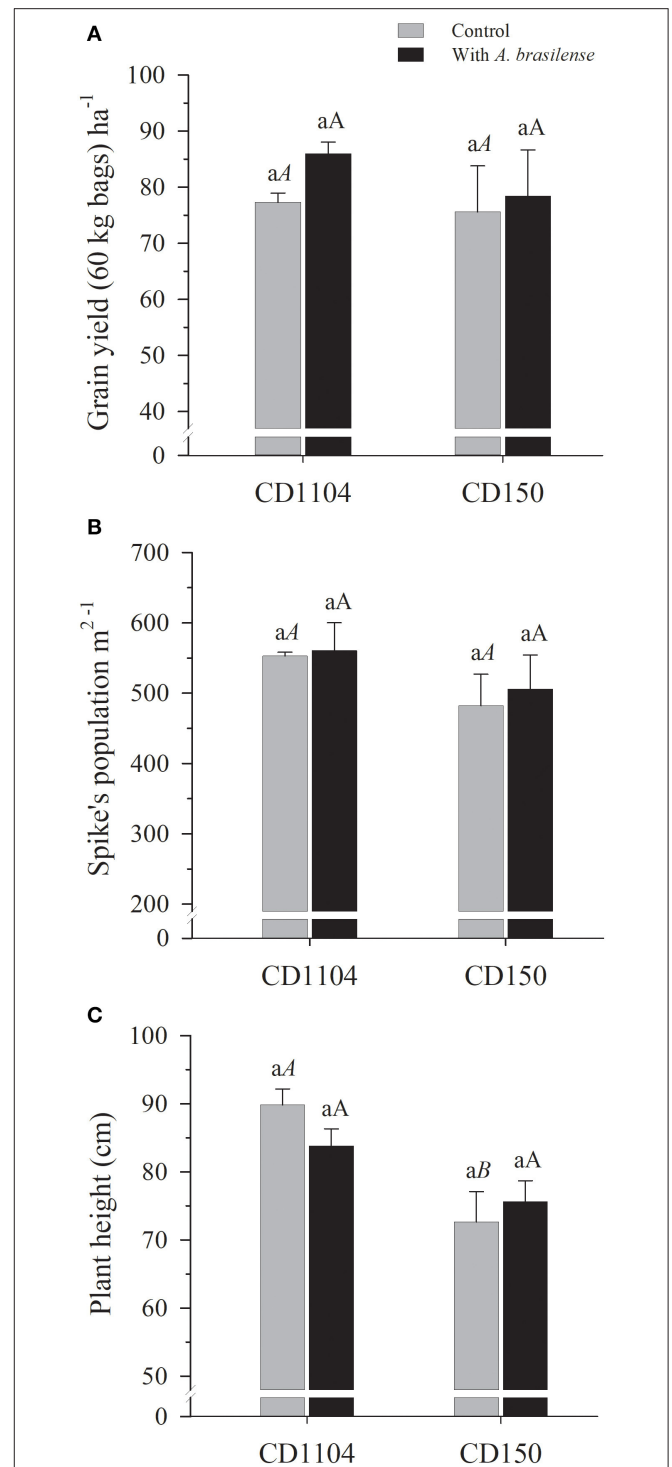






as a co-factor in the the process of photosystem II (Hawkesford et al., 2012).

Regarding the greater absorption of micronutrients due to inoculation with *A. brasilense* (Figures 4, 5A), Teixeira Filho et al. (2017) also reported positive responses of *A. brasilense* in the nutrition of irrigated wheat in the Brazilian Cerrado. These findings demonstrated the importance and response of PGPBs not only in fixing atmospheric N<sub>2</sub>, but it is also involved in several other mechanisms such as production of siderophores, low molecular organic compounds that boost iron uptake from the soil, solubilizing and transporting it to plant (Banik et al., 2016), synthesis of abscisic acid (ABA) and zeatin, tolerance to pathogens



(Tortora et al., 2011; Sahoo et al., 2013), production of kinetin (Patel and Saraf, 2017), and cytokinin that promotes growth.

According to Galindo et al. (2017), *A. brasilense* is a bacterium with high mobility in plants and assists in several metabolic processes of plant tissues to better use available water in the soil (Shakir et al., 2012); to increase soluble sugar, amino acid, and proline contents (Bano et al., 2013); and to absorb and assimilate nutrients (Galindo et al., 2017). It has also improved the assimilation of P (Rosa et al., 2020) by solubilization of non-available P in the soil rhizosphere (Rodriguez et al., 2004) to increase its availability.

Regarding the absorption and accumulation of copper builders in different cultivars (Figure 4B, Table 2), this behavior of *A. brasilense* in CD150 may be related to greater absorption of other cationic micronutrients such as B, Fe, and Mn, which were promoted with the inoculation with *A. brasilense* in the same cultivar, by 44, 49.9, and 21.9% (Figures 4A–C, 5A). The cationic micronutrients compete for the same sites of absorption in the cell of the plant root (Marschner, 2012), which may justify the accumulation in CD150. Furthermore, when analyzing the contents of these nutrients in the soil (Table 1), all micronutrients were at a high level for the cultivation of wheat and other cereals (Raij, 2011). These findings support the hypothesis of multiple researches triggered by *A. brasilense* in cereal which go far beyond the capacity of biological nitrogen fixation (Pankievicz et al., 2015) and synthesis of phytohormones (Bashan and De-Bashan, 2010). The inoculation with *A. brasilense* signals intrinsic absorption processes (Fukami et al., 2017) and solubilization (Rodriguez et al., 2004; Arzanesh et al., 2011; Banik et al., 2016), promoting better use of micronutrients (Galindo et al., 2019b), which, therefore, highlights the need for more specific studies to understand these mechanisms and metabolic pathways of plants.

Regarding the influence of *A. brasilense*, wheat genotypes and their effect on biometric evaluations (height and spike population) were studied at harvest time and resulted in taller plants (Figures 6B,C). This characteristic was clearly observed in the current experimental stand, which can be observed in Supplementary Figure 1. The attributes regarding plant height and size were consistently high in the technical data of each genetic material (COODETEC, 2017). However, regions with a high incidence of wind blow can damage the cultivation of CD1104, as taller wheat plants are more prone to lodging which may lead to more losses in the harvest (Nascimento et al., 2016).

In contrast to plant height, grain yield in the present study was not influenced by wheat genotypes or inoculation with *A. brasilense* during the harvest (Figure 6A). It was studied by Tabassum et al. (2017) and Ferreira et al. (2019) in an extensive review on PGPB-induced capacity of plant tolerance to stress, and they highlighted that inoculation with *A. brasilense* does not always increase productivity. The authors postulated that inoculation responses are most strongly expressed in plants in adverse situations such as water deficit (Shakir et al., 2012), pathogen attack, and plant–weed competition, and light, water, and nutrient deficiencies are among the other abiotic factors (Forni et al., 2017).

In the present study, the climatic situations were normal, and no adverse situations occurred, coinciding with the explanations proposed by Tabassum et al. (2017) and Ferreira et al. (2019) for the none increasing productivity (Figure 6). Climatic values can be consulted from the temperature and exclusion graphs during the experimental period (Figure 1). In addition, it is important to note that the experimental area has a central pivot irrigation system, grown with cereals (cereals and legumes for over 30 years), the last 12 years being under the no-tillage system with straw conservation and minimal soil turnover aiming at the conservation of the soil–plant–microorganism system.

## CONCLUSIONS

The inoculation with *A. brasilense* increases the accumulation of B and Cu in the aerial part of wheat, by 27.7 and 57.4%, respectively, for the cultivar CD150. *A. brasilense* increased the accumulation of B, Fe, and Mn by 43.8, 49.9, and 22%, respectively, and reduced Cu by 21.9% in the cultivar CD1104.

The wheat cultivar CD150 has greater efficiency of N shoot accumulation of +35.5 kg N ha<sup>-1</sup> in comparison with cultivar CD1104. However, the grain yield of the wheat cultivars was similar.

Interactions between *A. brasilense* inoculation and cultivars resulted in greater accumulation of S and K in the wheat shoot for cultivar CD150, as well as Cu shoot accumulation in the CD1104 genotype. *A. brasilense* has more sustainable contribution to wheat nutrition with no significant increase in crop productivity.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

EB wrote the manuscript, with contributions from AJ and MT. FS, OF, and SB corrected and improved the manuscript. EB, WR, JS, and FS conducted the samplings and data collection. EB and BL did the analysis, with the support of SB, WR, and MS. All authors contributed to the article and approved the submitted version.

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# Enhanced Crop Productivity and Sustainability by Using Native Phosphate Solubilizing Rhizobacteria in the Agriculture of Arid Zones

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The importance of phosphate solubilizing rhizobacteria (PSB) has been well-documented as an option for enhancing sustainable agriculture. As a particular group of plant growth promoting rhizobacteria (PGPR), PSB play an important role in the soil phosphorus cycle, increasing the bioavailability for growth and plant development. This study analyses the plant growth promoting effects of 5 strains (BN0009, BN0013, BN0015, BN0024, and BN0035) out of 180 isolated from *Jarava frigida* (Phil.) F.Rojas (*Poaceae*), a native grass from the Andean Atacama desert from North of Chile. The five bacterial isolates (BN strains) were identified as non-pathogenic *Erwinia* sp. and show a high phosphate solubilization capacity for  $\text{Ca}(\text{PO}_4)$  ranging from 608.9 to 781.4 mg/L. Strains IAA production varies between 23.5 and 35.9 mg/L, siderophores, phosphatase (alkaline and acid) production was also observed, but none of the five isolated presented antagonism against plant pathogens *Botrytis* sp. and *Sclerotinia* sp. All isolates enhanced seed germination in *Lactuca sativa* and *Solanum lycopersicum* (excepting BN0009). Additionally, all strains stimulated the early root elongation and seedling development in lettuce and tomato. Pot experiments displayed that BN0015, BN0024, and BN0035 significantly promote plant growth regarding root and leaf area, root and leaf weight, as well as leaf number compared with non-treated plants. In a field experiment with lettuce and two fertilization treatments (50 and 100% of the recommended crop fertilization), BN0024 application improved crop productivity compared to respective control. P content in plants with bacterial inoculations increased significantly compared to control in either fertilization treatment, suggesting an improved nutrient uptake. Also, lettuce with 50% fertilization and inoculation with BN0024 equate productivity with the control 100% fertilization. Finally, we discuss these results in the context of applicability to enhance the agroecosystem productivity in arid and semiarid zones.

**Keywords:** PGPR - plant growth-promoting rhizobacteria, nutrient uptake, *Erwinia*, biostimulant, sustainable agriculture, horticulture crops

## INTRODUCTION

The rhizosphere involves dynamic interactions between plant roots and soil microorganisms, where the composition of root exudates influences the nature and structure of the microbial community (Shi et al., 2011; Eisenhauer et al., 2017; López-Angulo et al., 2020). Particularly, beneficial soil microorganisms like mycorrhiza and Plant Growth Promotion Rhizobacteria (PGPR) enhance the adaptive responses and plant development by direct and indirect mechanisms (Backer et al., 2018). PGPR facilitate the bioavailability of nutrients in the soil, e.g., phosphate, considered a major macronutrient for growth and physiological plant performance (Bechtaoui et al., 2020). However, it is well-known that the bioavailable P in the soil is very limited reaching the level of 1.0 mg kg<sup>-1</sup> soil (Goldstein, 1994). To raise assimilable P, conventional agriculture has been using chemical fertilizers. Nonetheless, the efficiency of P fertilizer throughout the world is around 10–25%, as a large proportion of the applied P is quickly transformed into insoluble forms, limiting the crop productivity and increasing environmental pollution (Kanter and Brownlie, 2019; Mallin and Cahoon, 2020). Phosphate-solubilizing bacteria (PSB) mobilize insoluble P forms from the mineral matrix to the bulk soil where they can be absorbed by plant roots (Sashidhar and Podile, 2010). In turn, plants liberate several sources of carbohydrates, which can be metabolized for bacterial activity (Vives-Peris et al., 2020). PSB can produce various organic acids with low molecular weight, which alter the soil pH, solubilizing the phosphate from acid or alkaline soils (Kim et al., 1998; Gyaneshwar et al., 2002; Liu et al., 2020). Another possible mechanism is proton-excretion accompanying ammonium ion assimilation, which is assumed to be the most probable explanation for microbial solubilization without acid production (Illmer and Schinner, 1995). Species of the bacterial genera *Aerobacter*, *Acetobacter*, *Azospirillum*, *Bacillus*, *Burkholderia*, *Erwinia*, *Pseudomonas*, and *Rhizobium*, have been widely reported to be able to solubilize various forms of insoluble phosphates (Ranjan et al., 2013; Goswami et al., 2016). Additionally, the use of PSB alone or in combined bioformulations with mycorrhizal fungi has been proven to be highly effective in increasing soil phosphate availability and to improve crop productivity (Singh and Kapoor, 1999; Nacoon et al., 2020). Different authors demonstrated these effects for several crops, like cotton (Qureschi et al., 2012), maize (Yazdani et al., 2009), sunflower Ekin (2010), rice (Rasul et al., 2019), or faba bean (Bechtaoui et al., 2020). Due to the activity that PSB expose by solubilizing the phosphate ion trapped in the soil constituents, the seed bacterization, and the preparation of specific biofertilizer with PSB strains are emerging as a potential strategy to promote the transition to sustainable agriculture (Babalola, 2010; Parani and Saha, 2012).

In Chile, soils of the Atacama Desert and adjacent regions are predominantly calcareous, poor in available phosphate, organic matter, and carbon content (Gómez-Silva et al., 2008). However, the presence of a high diversity of native plants adapted to arid environments and the high percentage of endemic organisms justified the consideration as one of the 35 hotspots of biodiversity around the world (Mittermeier et al., 2005). This fact

suggests the presence of ecological networks that harbor bacterial strains with high biotechnical potential and beneficial effects on the productivity and growth of crops, which are grown on arid or semiarid agroecosystem. In fact, some PSB strains, isolated from arid environment, demonstrated promising applicability in agriculture (Goswami et al., 2014; Emami et al., 2020; Wahid et al., 2020).

Despite the benefits that PSB represent to the plants, the information about biotechnological applications of native microorganism, especially from Chile, still is very scarce. Therefore, the present work aims to contribute to our knowledge about PSB, their functional activity as well as potential uses in modern agriculture. We characterize PSB associated to the roots of *Jarava frigida* (Phil.) F.Rojas (*Poaceae*), a native grass that dominates the high Andean desert grasslands (Gajardo, 1994). We analyse their P solubilizing capacity and their ability to promote growth of lettuce and tomato plants; and select the best strain for a field experiment with different levels of fertilization. Finally, we discuss their applicability as biofertilizers for agriculture in arid and semi-arid zones.

## MATERIALS AND METHODS

### Rhizospheric Soil Sampling

For this study, we sampled a *Jarava frigida* population (23°02'50"S–67°39'16"W) at an altitude of 4,650 m above sea level, located in the commune of San Pedro de Atacama (Antofagasta, Chile). The sampling site is a little terrain depression with an average vegetation cover of 5%, the mean annual temperature is 7°C, and annual precipitation is ~120–150 mm. Plants of *J. frigida* were harvested from the study site with a spade. Their root-sediment ball was kept intact, and they were transferred to the laboratory in sterile plastic bags. Besides the rhizoplane-root sample, soil samples were taken. Soil chemistry was analyzed by a commercial laboratory (CTSyc, University of Talca).

Soil chemistry: The pH of Atacama Desert soil samples was moderately acidic (Table 1). The total N and P level was 103 ppm v/s 0.027%, respectively. The soil samples exposed a low level of organic matter (0.63%). However, the organic N level was 0.6 times higher compared to organic P. (16 ppm v/s 10 ppm, respectively). The electric conductivity (EC), expressed as a ds/m measurement was low, but a high concentration of minerals was detected particularly for iron and copper (Table 1). In addition, a high concentration of boron was also measured, a common feature of desert regions.

### Isolation of Phosphate Solubilizing Bacteria

Approximately 5 g of roots and rhizosphere (soil attached to the roots) were placed in an Erlenmeyer flask with 50 ml distilled water and shook at 120 rpm at room temperature for 2 h. Serial diluted (103–105) samples were dispersed with borosilicate glass balls on PVK plates, containing glucose 10 g/l, yeast extract 0.5 g/l, ammonium sulfate 0.5 g/l, magnesium sulfate heptahydrate 0.1 g/l, calcium phosphate 5 g/l, sodium chloride 0.2 g/l, potassium chloride 0.2 g/l, manganese sulfate 0.002 g/l,

**TABLE 1** | Soil characteristics from the isolate collection site in San Pedro de Atacama (as indicated by CTSyC laboratory).

Soil characteristics	Pasto ID 01-05-09	Comments
P Total	103.95 ppm	
N Total	0.027%	
P available	10 ppm	Medium
N available	16 ppm	Low
K	315 ppm	Medium—high
Organic matter	0.63%	
pH	6.12	Moderately acid
CE	0.183 dS/m	No risk
<b>Trace minerals</b>		
Mn	11.02 ppm	High
Zn	0.75 ppm	Medium
Cu	2.97 ppm	High
Fe	9.99 ppm	High
B	2.65 ppm	High
<b>Cation exchange</b>		
Ca	2.10 cmol(+)/kg	Low
Mg	0.84 cmol(+)/kg	High

ferrous sulfate 0.002 g/l, agar 15 g/l, pH adjusted to 7 (Vazquez et al., 2000). Plates were incubated for 3 days at 25°C. Afterward, solubilizing strains were picked and purified. Their solubilizing efficiency on PVK plates (triplicates) was expressed as  $E = \text{halo diameter/colony diameter} \times 100$  (Nguyen et al., 1992). All isolates with ratio  $>200$  were stored at  $-80^{\circ}\text{C}$ .

## Biochemical Characterization of PGPR Traits

### Quantification of Solubilized Phosphate

The isolates were inoculated into 20 ml liquid PVK medium (as mentioned above but without agar), where  $\text{Ca}_3(\text{PO}_4)_2$  was replaced by  $\text{KH}_2\text{PO}_4$  and incubated at  $28^{\circ}\text{C}$  for 24 h. Bacterial concentration of this liquid culture was adjusted to  $1 \times 10^5$  CFU in 500  $\mu\text{l}$  and bacterial cells were cleaned. Finally, respectively, 50 ml of liquid PVK per isolate were inoculated and incubated for 72 h with continuous agitation (125 r/min). Every 12 h 1 ml culture was sampled aseptically for the determination of pH and soluble phosphorus. The pH was measured on a pH meter; the phosphorus availability was determined with the Mo blue method (Watanabe and Olsen, 1965). The solubilizing isolates were compared against a negative control and a not-solubilizing isolate. All measurements were taken in triplicates and the procedure was repeated 3 times independently.

### Alkaline and Acid Phosphatase

To detect alkaline and acid phosphatase activity isolates were cultivated on PVK plates (composition mentioned above) for 4 days at  $25^{\circ}\text{C}$ . Afterwards, a 6 mM 4-nitrophenyl phosphate solution was added to the plates containing either acetic acid (100 mM, pH 5.4) for acid phosphatase or Tris (100 mM, pH 9) for alkaline phosphatase. In both cases, plates were incubated for 90 min., inverted and exposed to ammoniac vapor for 2–3 min.

under an extractor hood. The formation of a yellow halo around bacterial colonies indicates phosphatase activity by reduction of 4-nitrophenyl phosphate (colorless) to p-nitrophenol (yellow).

### Siderophores

To determine the excretion of siderophores by the strains, CAS (chrome azurol sulfur) plates were used as described by Alexander and Zuberer (1991). Bacterial strains were inoculated on the plates and incubated for 72 h. The observation of an orange halo indicates the liberation of iron from the colored Fe-CAS complex due to the presence of siderophores produced by the bacteria. Afterwards CAS-positive bacteria were grown in MM medium and incubated for 24 h (at  $25^{\circ}\text{C}$ , 120 rpm). One milliliter of the culture was centrifuged at 8,000 rpm for 5 min, the cell-free supernatant was mixed with 0.5 ml CAS solution and measured at 630 nm (spectrophotometer), as reference a 0.5 ml uninoculated MM medium and 0.5 ml CAS solution was used (Schwyn and Neilands, 1987). The percentage of siderophore units was estimated as the proportion of CAS color shifted using the formula: % Siderophore units =  $[(\text{absorbance reference} - \text{absorbance sample}) / \text{absorbance reference}] \times 100$  (Bholay et al., 2012).

### IAA Production

For each strain, 10 ml liquid PY medium containing Tryptophan (0.1% p/v) were inoculated and incubated for 48 h at  $25^{\circ}\text{C}$  and 175 rpm. The production of Indole-3-acetic acid (IAA) was determined after 24 and 48 h with or without tryptophan. For each measurement, 1 ml bacterial culture was centrifuged to 0.5 ml of the supernatant 1 ml of Salkowsky reagent (1 ml  $\text{FeCl}_3$  0.5 M mixed with 50 ml 35% (p/p)  $\text{HClO}_4$ ; Gordon and Weber, 1951) was added and incubated in darkness for 30 min (Glickmann and Dessaeux, 1995). Absorbance was measured at 530 nm against a blank [PY medium + Tryptophan (0.1% p/v)]. The IAA concentration was calculated based on a standard curve calibrated with dilutions (0–100  $\mu\text{g/ml}$ ) of a commercial IAA reagent (Merck).

### ACC-Desaminase Activity

The capacity to produce ACC deaminase was evaluated qualitatively by placing the isolates on Dworking-Foster plates with ACC as sole nitrogen source (Penrose and Glick, 2003). Bacterial growth on the plate would indicate the presence of the enzyme.

### N-Fixation Ability

The capacity to fix aerial nitrogen was determined using NFB semi-solid medium according to Baldani et al. (2014).

### Antagonism Capacity

To evaluate the antagonistic capacity of the strains against *Sclerotinia* sp. and *Botrytis* sp., we employed the methodology of Salvatierra-Martinez et al. (2018). Briefly, PDA medium Petri dishes were used, placing in the center of each plate, a disc of fungal hyphae (7-d-old, 0.5 cm diameter) surrounded at a distance of 4 cm by five 3  $\mu\text{l}$  liquid culture aliquots of each strain. The strain liquid culture was prepared in LB, grown for 24 h, and diluted 1:10. As control, plates with only a disc of *Sclerotinia* sp.



or *Botrytis* sp. in the center were used. All plates were incubated at 25°C for 5 days. The inhibition of the radial growth of the fungi was calculated using the equation:  $ICM = [(C-T)/C] \times 100$ ; where C = the growth area of the fungus in the control plate and T = the fungal growth area in the treatment (Fiume and Fiume, 2006). The experiment was repeated three times with 3 plates per replication for each strain.

### Strain Identity

To determine the taxonomical identity of our strains we carried out several tests. For the Gram reaction, we used the commercial stain kit (Merck, Company) and KOH test. Additionally, photographs and observations of cell size and morphology were made for each isolate, using a light microscope and the 100 × objective lens.

Finally, we extracted genomic DNA for each pure strain, following the protocol of Yeates et al. (1998). We amplified 16S region (63F (5'-CAG GCC TAA CAC ATG CAA GTC-3') and 1387R (5'-GGG CGG WGT GTA CAA GGC-3'; ~1,000 bp) with the primer pair and PCR protocol as reported by Marchesi et al. (1998). Purified fragments were sent to Macrogen for sequencing, and the resulting 16S rRNA gene sequences were compared with the NCBI database using the basic local alignment search tool (Zhang et al., 2000). The phylogenetic analysis was performed using MEGA 7 (Kumar et al., 2016). The obtained sequences are available in the NCBI database (NCBI accession numbers: MW092897, MW092898, MW092899, MW092900, and MW092901; not released yet).

### Promotion of Plant Growth

The effects of the bacterial isolates on plant growth were evaluated using *Lactuca sativa* L. (lettuce) and *Solanum lycopersicum* L. (tomato).

### Seed Germination Assays

Seeds [*L. sativa* (lettuce) and *S. lycopersicum* (tomato)] were sterilized with sodium hypochlorite and vernalized for 48 h at 4°C. Afterwards, they were submerged in liquid bacterial culture [LB medium, OD = 0.8 ( $\approx 10^7$  CFU)] for 5 h at room temperature on a shaker (180 rpm). Twenty-five prepared lettuce or 15 prepared tomato seeds were displaced in a Petri dish on humid filter paper (each isolate in quadruplicate, negative control without bacteria). Petri dishes were incubated horizontally at 20°C for 3.5 days (lettuce) and 14 days (tomato), respectively. Observations on germination progress were made every 4 h for lettuce and every 8 h for tomato. Root length was determined at the end of the respective incubation period, using the scale: no root, radicle emerged, radicle <1 cm and root > 1 cm.

### Pot Experiment With Lettuce

For this experiment, the five selected bacterial strains (BN0009, BN0013, BN0015, BN0024, and BN0035) were grown on nutrient agar initially. A single colony was transferred into 250 ml flasks containing nutrient broth and grown aerobically on a shaker (150 pm) for 24 h at 28°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of  $10^8$  CFU/ml. Sterile seeds were submerged for 5 h in this bacterial solution.

Inoculated seeds were placed in a pot with a 1:1 sterile mixture of agricultural soil and peat. For each isolate, 10 pots were prepared (replicates) and located randomly. Pots were maintained in a climate chamber at 20°C for 28 days (photoperiod: 16 h light, 8 h darkness). The pots were irrigated daily 5 ml with distillate water. As negative control, 20 pots containing seeds without bacteria were cultured under the same conditions. Photographs were taken every 5 days. At the end of the incubation period, root and leaf area (using WinRhizo), number of leaves, as well as root and leaf fresh and dry weight were recorded. Root and shoot portions of lettuce plants were dried separately for 5 days at 65°C (constant weight).

### Lettuce Field Experiment

Based on the previous results, one strain was selected for the lettuce field experiment. Just before transplantation, lettuce seedlings (28 days old) were soaked for 1 h in a bacterial suspension. Therefore, the strain was grown in liquid LB medium in a flask on a shaker (150 pm) for 24 h at 28°C and this bacterial culture was diluted afterwards to the final concentration of  $10^8$  CFU/ml. The strain was re-inoculated 4 and 8 weeks after the transplant, via drip irrigation adjusting the concentration at the dripper to  $10^8$  CFU/ml for 10 min (a separate irrigation system was installed for these applications). We considered two fertilization treatments with respect to the literature indication for crop management of lettuce (INIA, 2012): 100% (NPK ratio 100-100-80) and 50% (NPK ratio 50-50-40), using KNO<sub>3</sub>, NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, and NH<sub>4</sub>NO<sub>3</sub> diluted in water for applications during drip irrigation. In total, we established four treatments: control 100% fertilization, control 50% fertilization, BN0024 100% fertilization and BN0024 50% fertilization. After 3 months lettuce was cropped and 10 individuals per treatment (randomly picked) were used for analyses. Growth parameters (root and aerial weight, root area) and biochemical parameters (leaf P content (Hylander et al., 1996), protein content (Bradford, 1976), and chlorophyll content (Porra et al., 1989) were measured.

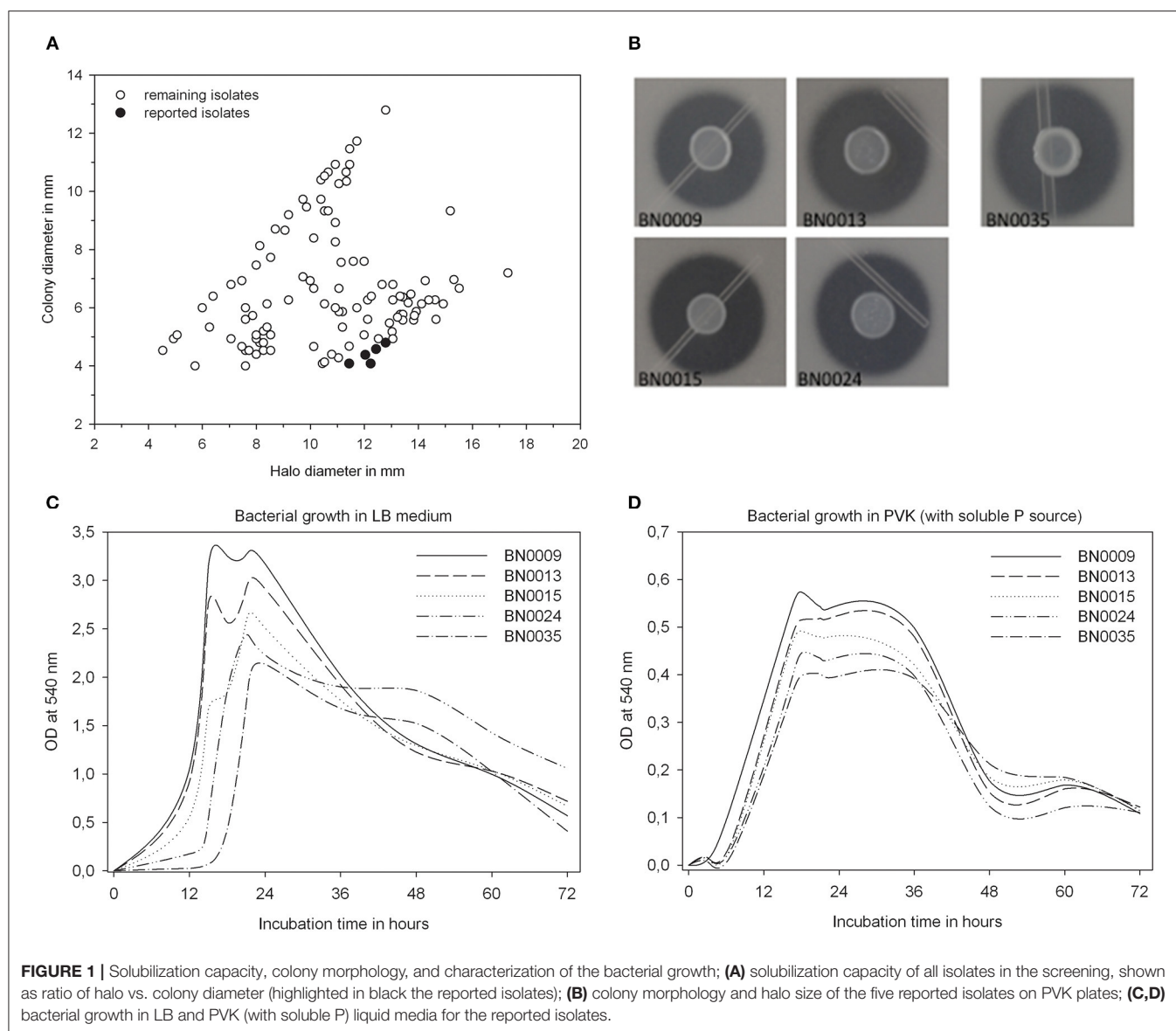
### Statistical Analyses

The experimental data were analyzed regarding homogeneity (Pettitt test), variances (Barlett test), descriptive statistics (standard variation and error, median, mean, asymmetric coefficient, range, maximum, and minimum value). Afterwards, Tukey's range test and ANOVA were applied to determine means (from the bacterial treatments) that are significantly different from the control and/or other treatments ( $P < 0.05$ ). As statistical and graphical software, XLSTAT, and SigmaPlot were used.

## RESULTS

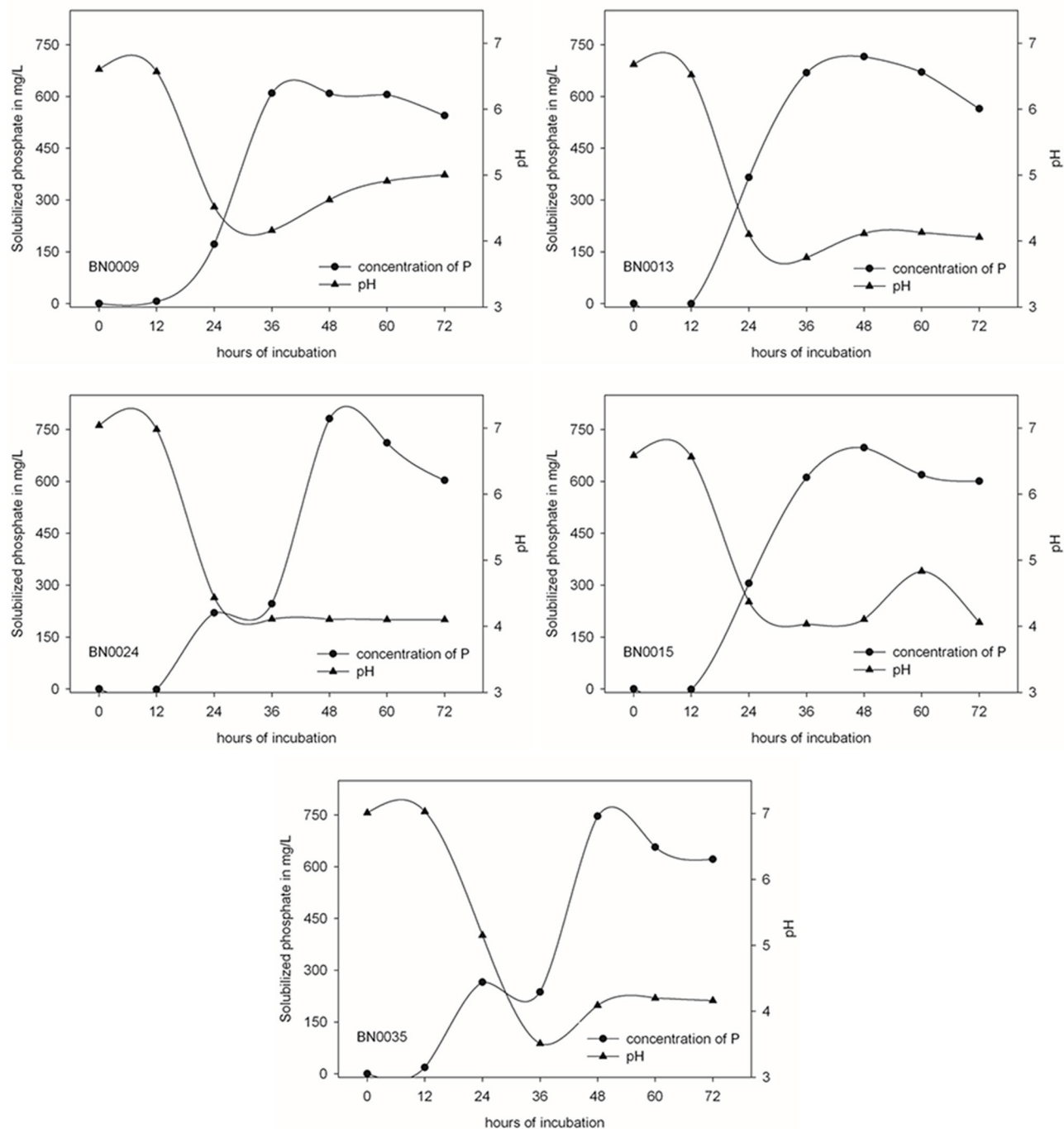
### Screening for Phosphate Solubilizing Bacteria and Quantification of Solubilized Phosphate

From *Jarava frigida* rhizosphere samples, 180 isolates were obtained on PVK plates. The isolates showed different colony morphology features such as color, density and colony elevation (Supplementary Table 1). A qualitative determination of the



phosphate solubilization capacity was carried with the whole collection using PVK agar plates. Measurements of solubilization efficiency *E* ranged between 110 and 300, with an average of 172. Twenty-seven isolates demonstrated a solubilization efficiency of over 200 and were further characterized regarding their capacity to solubilize P in liquid medium (**Supplementary Table 1**). The five most efficient solubilizing isolates were selected for further analyses due to their outstanding *E*-values of 260 to 300 (**Figure 1A**). These isolates were labeled as BN0009, BN0013, BN0015, BN0024, and BN0035. Their colonies were described as round and creamy in color (**Figure 1B**). All BN isolates exposed a high growth capacity on LB medium, with a density peak after 16 h of incubation (**Figure 1C**). At the same time (16 h), the growth of these five isolates in PVK liquid medium containing a P soluble source is noticeable reduced (**Figure 1D**). For these selected isolates a quantification of solubilized P in a

liquid medium was carried out, which demonstrated different levels in their capacity dissolving insoluble phosphate (**Figure 2**). The highest solubilization activity was observed between 36 and 48 h when 608.9–781.4 mg/L P were liberated from BN strains. A longer incubation time did not further increase the solubilized phosphate by each isolated. On the contrary, by the end of the experiment (72 h), the concentration of soluble P decreased slightly in all strains. At the same time pH descends from the initial 7.0 to values ranging from 4.3 to 4.0, apparently in negative correlation to the amount of dissolved P. BN0024 and BN0035 solubilized 781.4 and 746.5 mg/L, respectively, the highest amount of P as bacterial culture (**Figure 2**). Nonetheless, when the capacity of single cells to solubilize phosphate was calculated, the most efficient strain is BN0015 with 10 pg/CFU after 36 h of growth (**Figure 3**).



**FIGURE 2 |** Kinetics of phosphate solubilization and pH changes of the five BN isolates in time, measurements every 12 h during 3 days of incubation.

## Evaluation of PGPR Traits

With respect to the taxonomy and identity, all five BN strains were determined as Gram-negative. Based on the 16S sequences, the strains belong to the genus *Erwinia* sp, with the highest identity scores (> 98.5%) for *Erwinia pyrifoliae* strain 8557, *Erwinia billingiae* strain HMF7415 and strain P3\_3, a bacterial

endosymbiont of *Curculio koreanus* (Supplementary Figure 1). Our strains are closely related to one another but are not clones. The phylogenetic analyses did not allow us to determine the species.

Furthermore, several PGPR characteristics were analyzed (Table 2). First, the ability to produce IAA, an important

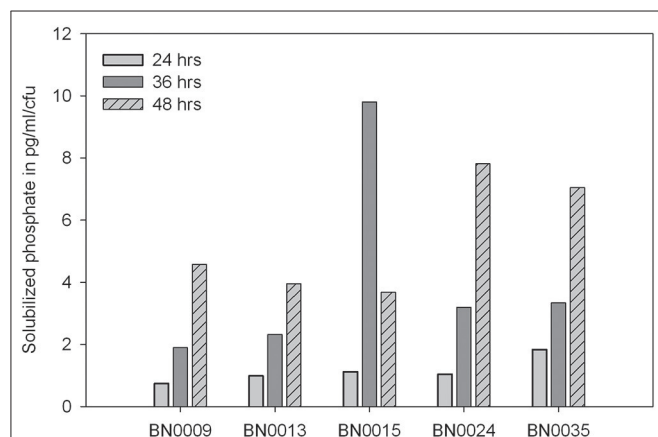
phytohormone for plant growth promotion was assessed and detected for all strains. The IAA production with tryptophan enriched medium ranged from 23 to 54 mg/L (Table 2). Among all BN isolates, BN0009 and BN0035 produced the highest IAA amounts (31 and 54 mg/L, respectively). BN0015, the most efficient P-solubilizer was found to produce an average IAA value (23.5 mg/L). Without tryptophan the IAA production was noticeable lower in all strains, reaching concentrations between 1.4 and 10.3 mg/L (Table 2).

All isolated strains demonstrated activity of alkaline and acid phosphatases and were capable of nitrogen fixation and siderophores production, in the latter values ranged between 0.5 and 10% siderophore units (Table 2). Additionally, only BN0009 and BN0013 were able to grow on ACC medium suggesting that both isolates possess ACC Deaminase activity (Table 2).

None of the five strains presented antagonism against *Sclerotinia* spp and *Botrytis* spp. (Table 2).

## Seed Germination Assays

Seeds of *L. sativa* (lettuce) and *S. lycopersicum* (tomato) seeds were inoculated with the selected strains to evaluate their effect on germination. All BN isolates accelerated the germination of *L. sativa* seed compared to the non-treated control, but the rate of enhancement varied among the bacterial strains (Figure 4A).



**FIGURE 3 |** Phosphate solubilization capacity per cell of the five selected phosphate-solubilizing bacteria at three different times of incubation.

Nonetheless, BN0013 accelerated the germination most (4.6x), reaching up to 93% after 24 h of incubation vs. the control with 20% at the same time (Figure 4A). Additionally, early root development and extension were analyzed 32 h after inoculation (Figure 4B). In *L. sativa* seeds, all isolates fostered early root development compared to control. In seeds treated with BN0009 and BN0013 a 78% (51% of seed with radicle <1 cm, 27% with root >1 cm) and 88% (45% of seed with radicle <1 cm, 43% with root >1 cm), respectively, demonstrated an enhanced root development compared to the non-treated control seeds (14% of seed with radicle <1 cm, 0% root >1 cm) (Figure 4B).

In the germination assay with *S. lycopersicum* seeds, again all isolates accelerated the germination in 2.5 and 2 times in 6 days with respect to the non-treated control (Figure 4C). Meanwhile, BN0024 anticipated germination, reaching the first peak after only 4 days of incubation (45% of the seeds germinated), BN0009 significantly increased the germination rate compared to the non-treated control. After 7 days of incubation, in the non-treated control 50% of the seeds were germinated, whereas the germination rate in inoculated seeds varied between 72 and 92% (Figure 4D). Similar to lettuce, all BN isolates enhanced early root development in *S. lycopersicum* seeds. 45% (BN0024) to 60% (BN0009) of seeds with bacterial treatments already presented roots >1 cm, compared to the control with only 8% of the seeds (Figure 4D).

Finally, the root length of lettuce and tomato seedlings was measured after the respective incubation period of 3.5 and 14 days, respectively (Figure 5). In both crops, the BN isolates induced a significant root elongation compared to the non-treated control. For lettuce, BN0013 caused the longest root extension with 2.3 times compared with the control (Figures 5A,B), followed by BN009 and BN0015. In tomato, the bacterial effect is more equal. However, isolates BN0013 and BN0024 rise with an average root length of 2.0 and 2.2 times compared to the control (Figures 5C,D).

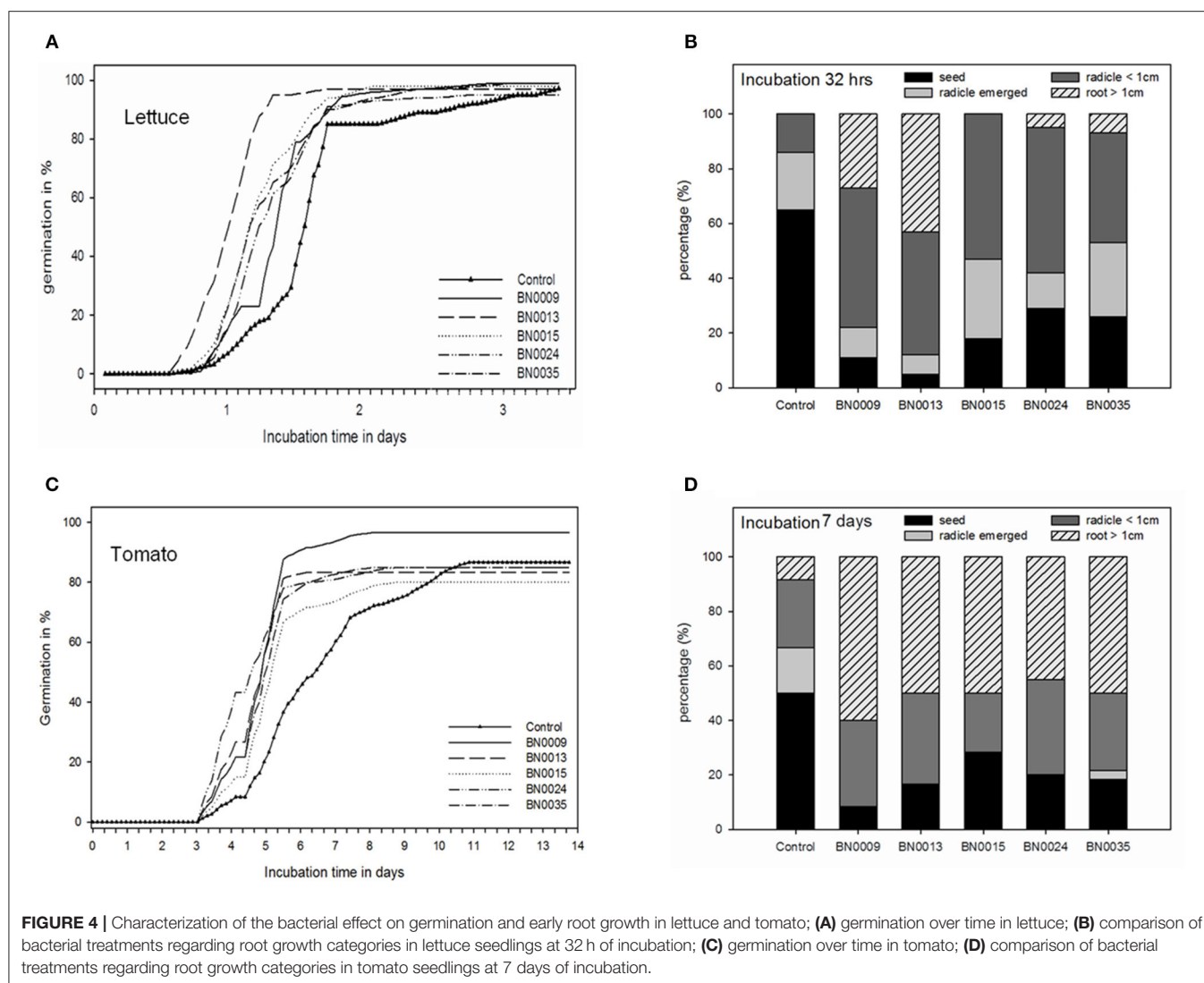
## Pot Experiment With Lettuce

Further insights about growth promotion beyond germination produced by the five strains were evaluated in a lettuce pot experiment. Inoculated seeds were planted and maintained for 35 days under controlled conditions. At harvest, root area, leaf area, number of leaves, fresh, and dry weight were measured. All isolates significantly increased root area (1.4–1.8x), leaf area (1.5–1.7x), and the number of developed leaves (1–2 leaves)

**TABLE 2 |** Different PGPR traits and molecular identity of the 5 reported isolates (\*Antagonism against *Sclerotinia* sp. and *Botrytis* sp.).

ID isolate	Antagonism*	Gram reaction	N fixation	ACC deaminase	IAA mg/L		Phosphatase		% Units of siderophores	Molecular identity
					With tryptophan	Without tryptophan	alkaline	acid		
BN0009	–	–	+	+	31,1	1,4	+	+	0,5	<i>Erwinia</i> sp.
BN0013	–	–	+	+	36,1	10,3	+	+	8,7	<i>Erwinia</i> sp.
BN0015	–	–	+	–	23,5	9,3	+	+	7,1	<i>Erwinia</i> sp.
BN0024	–	–	+	–	34,4	7,6	+	+	10,0	<i>Erwinia</i> sp.
BN0035	–	–	+	–	54,1	8,1	+	+	4,7	<i>Erwinia</i> sp.





compared to the control (Figure 6A, Table 3). Also, the effects were similar when comparing the strains. Seeds treated with BN0024 developed on average one leave more than control, which implies an advance in seedling development of ~1 week. The results for fresh and dry weight after inoculation with BN isolates were more differential (Figure 6B, Table 3). All isolates presented a significantly higher root fresh weight (1.9–2.3x), but the root dry weight was similar to the control. Three strains (BN0015, BN0024, and BN0035) stimulated significantly the leaf development in both fresh and dry weight compared with the control (1.8, and 2x, respectively) (Figure 6B, Table 3). Overall, the inoculation with BN0015, BN0024, and BN0035 produced the strongest promotion of plant growth and development on *Lactuca sativa* during the pot experiment.

### Lettuce Field Experiment

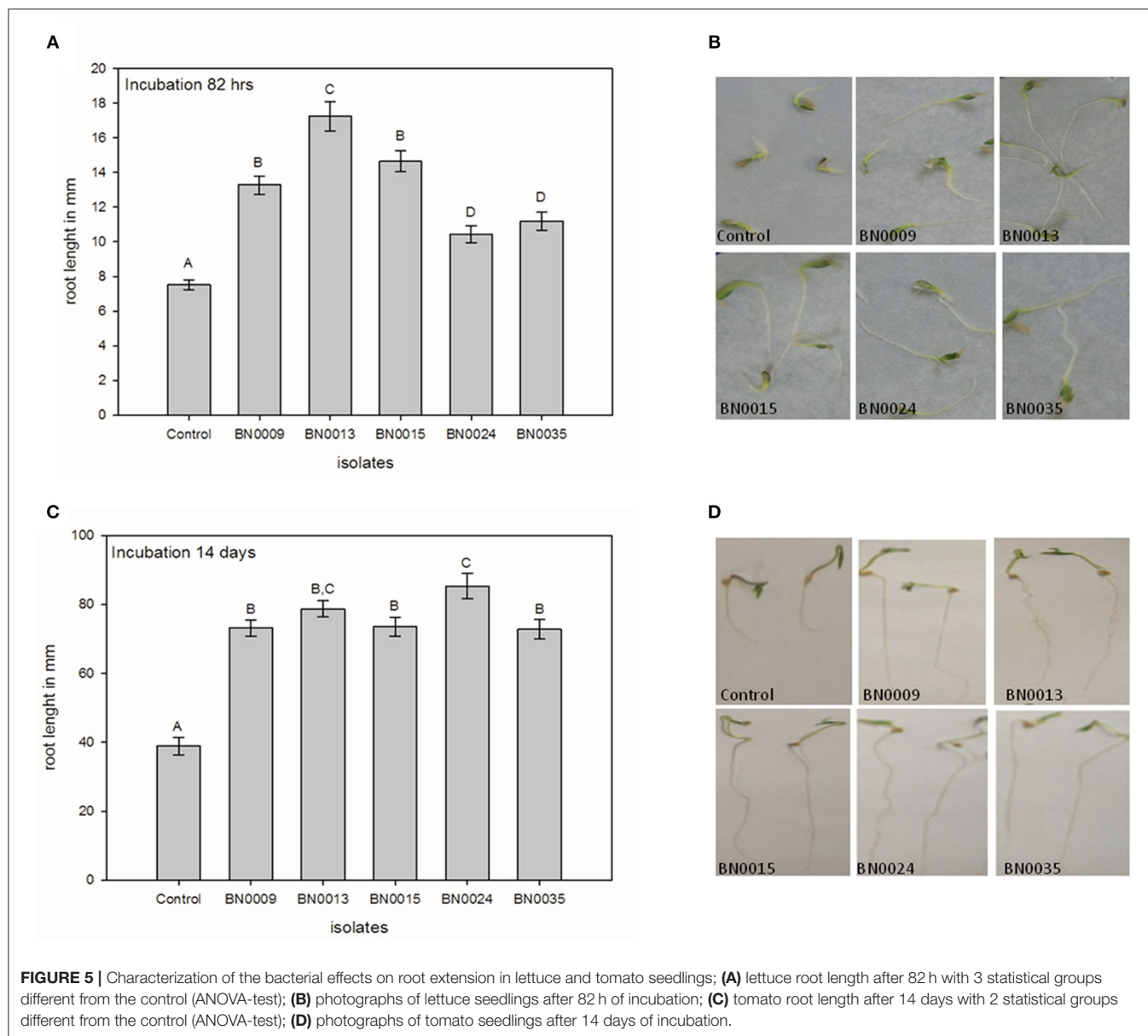
Based on the previous data, BN0024 was selected for a lettuce field experiment with root inoculation during transplant from nursery to field and two fertilization levels. The lettuce plants

responded to both factors (inoculation/fertilization) of the field experiment (Figure 7, Table 4). The effect of bacterial inoculation on plant growth was higher at 50% fertilization, where all evaluated parameters increased significantly (Table 4). With 100% fertilization the evaluated parameters also improved in plants with application of BN0024, nonetheless only root area, P content and protein content were significantly higher compared to control. The P content in plants with bacterial inoculations was significantly elevated compared to control in either fertilization treatment.

Finally, the application of the strain BN0024 with 50% fertilization reached a crop productivity and nutrient content similar to the control with 100% fertilization (Table 4).

### DISCUSSION

In this work, rhizosphere soil samples from *Jarava frigida* (Phil.) F.Rojas (*Poaceae*) were screened for phosphate solubilizing rhizobacteria, which exhibit additional PGPR traits. The



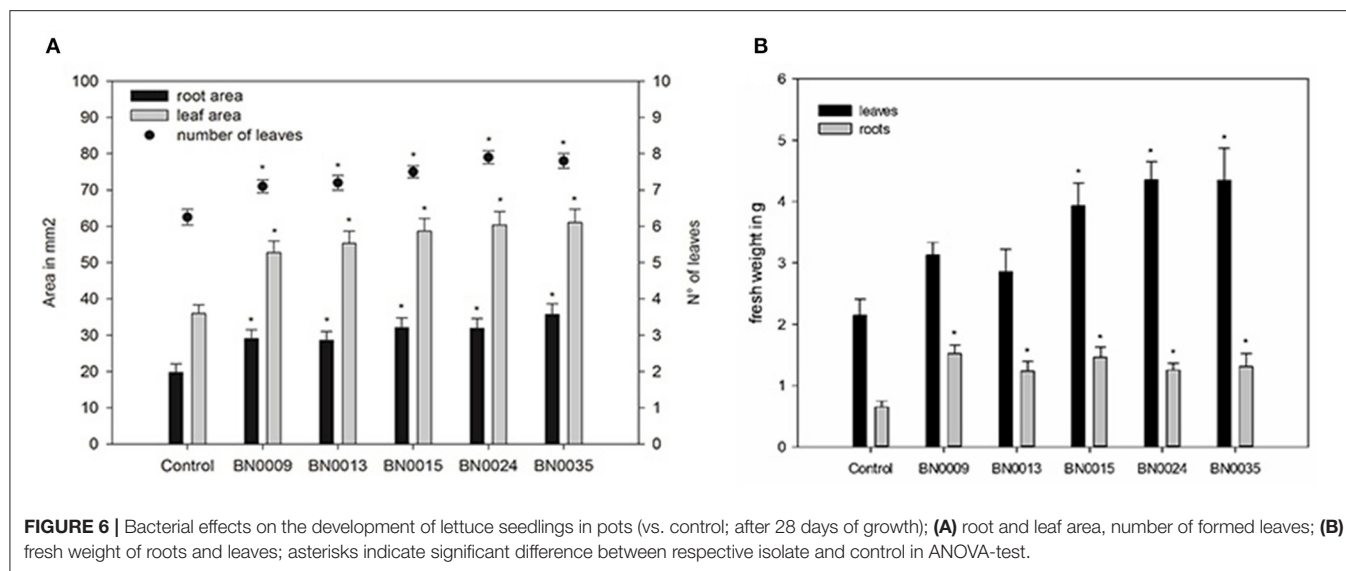
rhizospheric soil showed a low content of organic matter (0.63%), lower electric conductivity and high content of iron and copper, as described for Andean desert soils (Mandakovic et al., 2018).

Among the 180 phosphate solubilizing isolates, five efficient BN isolates were selected for further studies. Each of these five isolates demonstrated a high P-solubilization capacity, ability to N fixation, as well as the production of siderophores and IAA, which are used as indicators for a positive growth promotion aptitude (Backer et al., 2018; Wahid et al., 2020). However, but none of them showed antagonism response against fungal pathogens. The five strains, denominated as BN0009, BN0013, BN0015, BN0024, and BN0035 were identified as *Erwinia* sp., and did not demonstrate pathogenic characteristics. In fact, non-pathogenic *Erwinia* sp. strains were previously described as phosphate solubilizes by

different authors, like Goswami et al. (2016) and Sagar et al. (2018).

In liquid medium each of five BN strains, induced an inverse relationship between pH and the solubilized P concentration, suggesting that the acidification facilitates the inorganic phosphate solubilization (Khan et al., 2007). This capacity is probably complemented with the enzymatic activity of alkaline and acid phosphatases that use organic phosphate as a substrate to convert it into inorganic form (Walpolo and Yoon, 2012; Zhao et al., 2013). Additionally, the increased plant elongation (plant height and root length) can be associated with higher nutrient absorption, particularly phosphorous, which can induce cell elongation and multiplication Richardson and Simpson (2011).

Another important feature in the rhizobacteria—plant interaction, is their ability to produce phytohormones. We



**TABLE 3 |** Growth parameters from the lettuce pot experiment with seed inoculation of the five selected strains BN0009, BN0013, BN0015, BN0024, and BN0035.

	Control	BN0009	BN0013	BN0015	BN0024	BN0035
<b>Roots</b>						
R Area (cm <sup>2</sup> )	19.72 ± 7.48	29.01 ± 7.70	28.57 ± 7.58	32.06 ± 8.50	31.90 ± 8.46	35.69 ± 9.46
R fresh weight (g)	0.65 ± 0.28	1.52 ± 0.45	1.23 ± 0.50	1.46 ± 0.54	1.26 ± 0.34	1.31 ± 0.66
R dry weight (g)	0.07 ± 0.03	0.05 ± 0.02	0.09 ± 0.05	0.09 ± 0.03	0.09 ± 0.02	0.08 ± 0.04
<b>Leaves</b>						
N° leaves	6.25 ± 0.42	7.10 ± 0.57	7.20 ± 0.63	7.50 ± 0.53	7.90 ± 0.57	7.80 ± 0.63
L area (cm <sup>2</sup> )	36.02 ± 7.43	52.79 ± 10.07	55.31 ± 10.55	58.65 ± 11.18	60.41 ± 11.52	61.02 ± 11.64
L fresh weight (g)	2.15 ± 0.34	3.12 ± 0.67	2.86 ± 0.75	3.93 ± 0.65	4.35 ± 0.92	4.35 ± 0.83
L dry weight (g)	0.08 ± 0.03	0.11 ± 0.03	0.11 ± 0.04	0.15 ± 0.03	0.16 ± 0.03	0.15 ± 0.03

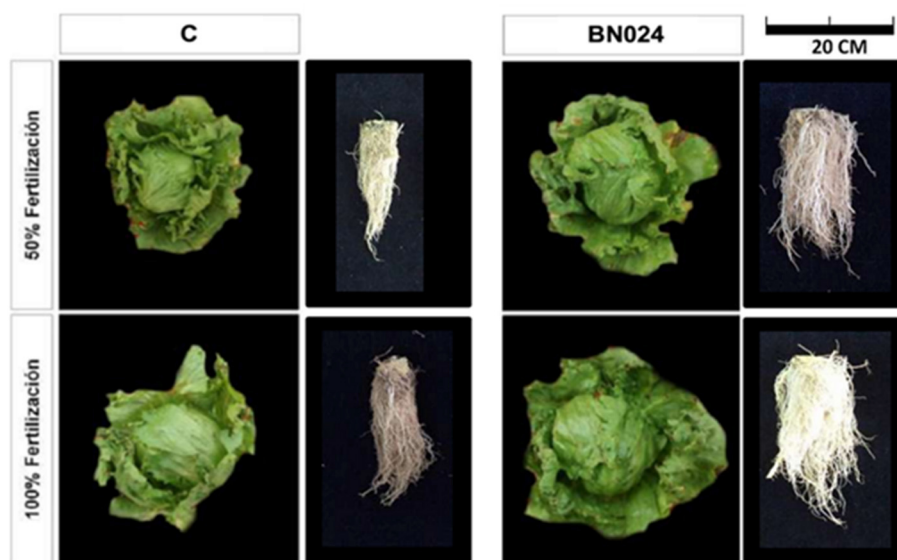
looked especially at IAA, which is involved in the formation of lateral roots and root hair, enhancing the nutrient absorption from soil (Qin and Huang, 2018). At least four pathways have been described for the biosynthesis of IAA in PGPR strains, being one of them tryptophan Trp-independent and two of them (Trp)-dependent (Gusain and Bhandari, 2019). Our five BN isolates produced between 23 and 54 mg IAA /L under the presence of tryptophan, concentrations which could cause important effects on the growth of the host plant. Actually, up to 80% of IAA-producing bacteria colonize root surfaces and stimulate the root system as a response to higher IAA concentrations (plant exogenous and endogenous synthesis) (Patten and Glick, 2002). The five isolated strains also were able to produce IAA in absence of tryptophan, which suggests the presence of a tryptophan-independent pathway (Ribeiro and Cardoso, 2012).

Compared to other studies (e.g., Sayyed et al., 2005), the siderophore production of our strains is relatively low, values are between 0.5 and 10 % siderophore units (Table 2). However, the concentration of available iron in their natural environment is high regarding the soil analysis (Table 1), which could result in lower selective pressure for this bacterial capacity.

All selected isolates demonstrated stimulation on lettuce and tomato germination (Figure 4). All strains accelerated the germination significantly compared to the control and generated a fast root extension in their early growth phase. These results coincide with other PSB studies, using strains alone or in combination, where a strong stimulation in radicle emergence from the seed is observed in plants with economical interest, e.g., rice, asparagus bean, and mung bean (Duarah et al., 2011; Arun et al., 2012).

In the lettuce greenhouse experiments, all five strains enhanced significantly the plant growth regarding root and leaf area, fresh, and dry weight as well as leaf numbers. These results are concordant with other authors who studied the effects of PSB on different crops: canola (Pandey et al., 2006), peanuts Goswami et al. (2014), cotton (Qureschi et al., 2012), maize (Yazdani et al., 2009; Shirinbayan et al., 2019), and rice (Rasul et al., 2019).

Despite the variety of *in vitro* and pot experiments with PSB and PGPR, few studies are available, where their effects were evaluated under productive conditions in field. Here we challenged *Erwinia* sp. strain BN0024, selected due to its constant growth promotion results, in a field experiment with lettuce, comparing two fertilization treatments (50 and



**FIGURE 7 |** Photographs from the lettuce field trail; left: Control (C) aerial and root lettuce development at harvest with 50 and 100% fertilization; right: BN0024 (BN0024) aerial and root lettuce development at harvest with 50 and 100% fertilization.

**TABLE 4 |** Growth parameters from the lettuce field experiment, carried out with two levels of fertilization (50 and 100%) and root application of strain BN0024 at transplant.

Parameters	Fertilization			
	50%		100%	
	Control	BN0024	Control	BN0024
<b>Growth parameters (N = 10)</b>				
Root weight (g)	15.62 ± 1.66	17.37 ± 1.25*	17.38 ± 0.82	17.81 ± 1.39
Root area (cm <sup>2</sup> )	55.61 ± 2.10	65.66 ± 3.65*	58.28 ± 2.84	69.65 ± 2.91*
Aerial weight (g)	619.57 ± 83.77	747.47 ± 55.19*	772.94 ± 74.53	827.26 ± 75.20
<b>Biochemical parameters (N = 6)</b>				
P content (μg/g fresh weight)	75.17 ± 2.32	106.46 ± 3.65*	92.07 ± 2.77	125.69 ± 6.71*
Protein content (mg/g fresh weight)	2.16 ± 0.09	2.61 ± 0.09*	3.07 ± 0.09	3.50 ± 0.19*
Chlorophyll content (μg/g fresh weight)	1.28 ± 0.16	2.43 ± 0.17*	2.45 ± 0.11	2.51 ± 0.28

Asterisks indicate significant difference to respective control (ANOVA).

100% of the recommended crop fertilization). The application of BN0024 improved crop productivity in both fertilization treatments. Particularly, the P content in plants with bacterial inoculations was significantly elevated compared to control in either fertilization treatment, suggesting an improved nutrient uptake (Rezakhani et al., 2019; Bechtaoui et al., 2020). In addition, lettuce with 50% fertilization and inoculation with BN0024 equate the productivity of the control plants with 100% fertilization. Recently, similar experiences reducing the phosphate fertilizer requirements of crops between 50 and 75% have been reported for other soils, e.g., Brazilian Latosol with sugarcane (*Saccharum* sp.) production integrating PSB (Rosa et al., 2020), Chinese Troporthents and Rhodustuts with tea (*Camellia sinensis* (L.) O. Kuntze) production integrating PGPR (Tennakoon et al., 2019). Our results gain special importance in

the context of P availability in agricultural soil of semiarid and arid zones, which could be mediated integrating PSB application in the crop management (Bechtaoui et al., 2020; Wahid et al., 2020) reducing environmental pollution (Mallin and Cahoon, 2020) and production costs for agricultures.

Beyond aridity, soils of arid zones possess other complex characteristics (pH, low soil organic matter, low availability of macronutrients, bound by soil chemistry), which need to be addressed in agriculture management. While aridity is compensated by the use of irrigation systems, the low availability of macronutrients like phosphorus, quickly bound to the soil matrix and not accessible for plant growth, still is a major issue in agriculture and the use of P solubilizing PGPR can offer an alternative to improve fertilization efficiency—as demonstrated by our results. Our results encourage the use of PGPR for these



purposes, improving not only the efficiency of P-fertilization, but also allowing to improve crop quality and productivity as well as reduce costs.

Future studies will focus on two aspects: (1) further biochemical, physiological, and molecular characterization of the five *Erwinia* sp. isolates to understand more entirely their metabolism and interaction with plants; (2) development of application strategies for these strains as part of integrated crop management. This information will enable the design of eco-friendly alternatives to stimulate the productivity of agriculture carried out under the limiting conditions of semi-arid and arid zones.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI accession numbers: MW092897, MW092898, MW092899, MW092900, and MW092901.

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## AUTHOR CONTRIBUTIONS

AS, JB, and VO designed the research. SM, AR, BA, and PM sampled and performed laboratory work under the guidance of AS and CJ. JA, MG, and CS carried out data analysis. All authors wrote the manuscript, designed Tables and Figures, revised the manuscript, and approved the final version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# PGPR Mediated Alterations in Root Traits: Way Toward Sustainable Crop Production

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The above ground growth of the plant is highly dependent on the belowground root system. Rhizosphere is the zone of continuous interplay between plant roots and soil microbial communities. Plants, through root exudates, attract rhizosphere microorganisms to colonize the root surface and internal tissues. Many of these microorganisms known as plant growth promoting rhizobacteria (PGPR) improve plant growth through several direct and indirect mechanisms including biological nitrogen fixation, nutrient solubilization, and disease-control. Many PGPR, by producing phytohormones, volatile organic compounds, and secondary metabolites play important role in influencing the root architecture and growth, resulting in increased surface area for nutrient exchange and other rhizosphere effects. PGPR also improve resource use efficiency of the root system by improving the root system functioning at physiological levels. PGPR mediated root trait alterations can contribute to agroecosystem through improving crop stand, resource use efficiency, stress tolerance, soil structure etc. Thus, PGPR capable of modulating root traits can play important role in agricultural sustainability and root traits can be used as a primary criterion for the selection of potential PGPR strains. Available PGPR studies emphasize root morphological and physiological traits to assess the effect of PGPR. However, these traits can be influenced by various external factors and may give varying results. Therefore, it is important to understand the pathways and genes involved in plant root traits and the microbial signals/metabolites that can intercept and/or intersect these pathways for modulating root traits. The use of advanced tools and technologies can help to decipher the mechanisms involved in PGPR mediated determinants affecting the root traits. Further identification of PGPR based determinants/signaling molecules capable of regulating root trait genes and pathways can open up new avenues in PGPR research. The present review updates recent knowledge on the PGPR influence on root architecture and root functional traits and its benefits to the agro-ecosystem. Efforts have been made to understand the bacterial signals/determinants that can play regulatory role in the expression of root traits and their prospects in sustainable agriculture. The review will be helpful in providing future directions to the researchers working on PGPR and root system functioning.

**Keywords:** root architecture, microbial determinants, root system functioning, gene regulation, hormonal regulation



## INTRODUCTION

Rhizosphere is the ecological niche comprising the soil surrounding the plant roots which is under the influence of root exudates (Hiltner, 1904) and plays a major role in supporting the growth and activity of a large but variable community of microorganisms. Plant growth promoting rhizobacteria (PGPR) are a group of bacteria that colonize the rhizosphere and are capable of augmenting plant growth by a variety of direct and indirect mechanisms. The beneficial microorganisms colonizing the microhabitats, rhizoplane, and root endosphere (Hartman and Tringe, 2019) function as the root microbiome and exhibit plant growth promoting activities. In turn, the secretion of carbon compounds from plants into the surrounding soil results in greater microbial populations (100–1,000 times higher) in the rhizosphere relative to the bulk soil (Lynch, 1987; Bending, 2003; Goswami et al., 2016). Due to the influence of root exudates, the microbial populations present in the rhizosphere are relatively different from those present in the bulk soil (Burdman et al., 2000). The most important and well-documented group of plant beneficial microbes comprises bacteria that form endosymbiotic associations with leguminous plants by inhabiting the root nodules and benefit the host plant mainly by fixing atmospheric nitrogen. PGPR do not need to form endosymbiotic association and exert positive effect on plant through one or more direct and/or indirect mechanisms. A rhizobacteria qualifies as PGPR only if it is able to produce a positive effect on the host plant upon inoculation, hence demonstrating good competitive skills over the native rhizosphere communities. It has been suggested that ~2–5% of rhizosphere bacteria act as PGPR (Antoun and Prévost, 2005; Barriuso et al., 2008). PGPR may affect plant performance through direct and indirect mechanisms (Figure 1). Direct mechanisms operate through the production of plant growth promoting substances (i.e., phytohormones), and enhanced availability and uptake of nutrients in soil through biological nitrogen fixation, solubilization of fixed form of nutrients to plant available form (P, K, Zn), chelation of nutrients (Fe) through siderophore production etc. (Glick, 2014; Goswami et al., 2016). Indirect mechanisms include suppression of plant pathogens (Beneduzi et al., 2012; Ribeiro and Cardoso, 2012) and abiotic stress tolerance (Grover et al., 2011; van Oosten et al., 2017). Credible group of PGPR include bacteria belonging to genera *Acinetobacter*, *Agrobacterium*, *Arthobacter*, *Azotobacter*, *Azospirillum*, *Burkholderia*, *Bradyrhizobium*, *Rhizobium*, *Frankia*, *Serratia*, *Thiobacillus*, *Pseudomonads*, and *Bacillus* (Vessey, 2003; Goswami et al., 2016).

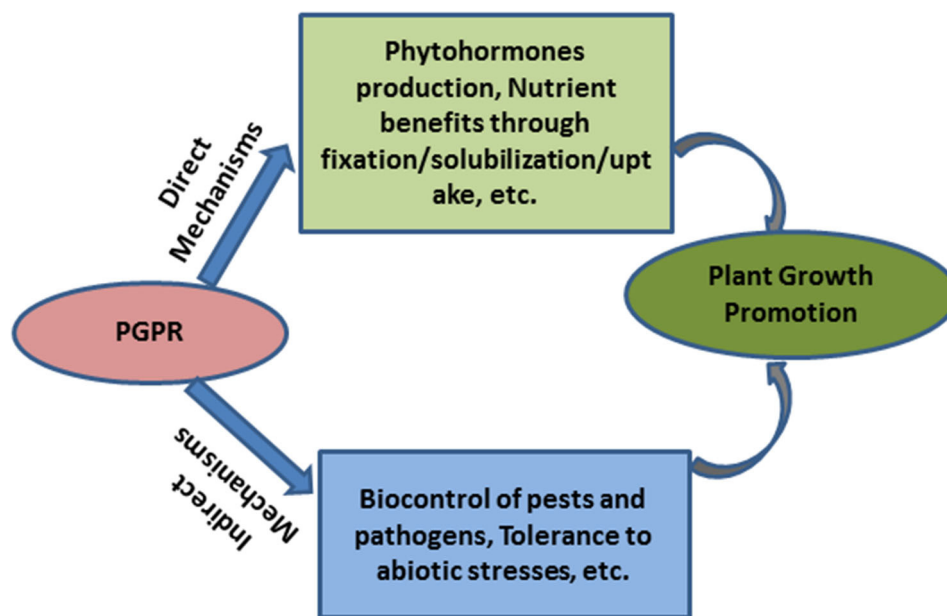
Positive effect of PGPR inoculation has been reported on aboveground as well as belowground plant part, although, more attention has been given to the above ground part due to economic importance of the aerial parts as food and fodder and, ease of sampling and recording observations. However, accumulating evidences on the role of root traits in ecosystem functioning have attracted the attention of the researchers to understand and explore the root trait based interventions in driving ecosystem processes (Bardgett et al., 2014). The primary function of the roots is to provide anchorage as well as support to the aboveground biomass and uptake water and

nutrients (macro and micro) from the soil for plant growth. In addition, roots also play important role in nutrient cycling by providing organic matter and by influencing the activity of soil microbial communities, in soil structure formation through soil aggregation and in carbon sequestration through recalcitrant components etc. Root traits are highly plastic in response to nutrient and water gradient, environmental gradients and to biotic interactions with other species. Hence, the root traits show high variation both between and within the species (Bardgett et al., 2014). Thus, any of these external stimuli may influence the roots traits. In this respect, PGPRs have been reported for altering the root architecture of plants (Mantelin et al., 2006). Similarly, root acquisition of water and nutrients is essential for plant growth and crop productivity. As a primary target, root is the organ that shows the first stimulatory effects of bacterial interactions (Nosheen et al., 2011). This is particularly remarkable in plants inoculated with PGPR belonging to genus *Azospirillum* (Okon, 1985). Inoculation with *Azospirillum* has been found to cause remarkable positive alterations in the growth and morphology of the roots accompanied by better water and mineral uptake and marked increase in plant yield (Creus et al., 2005). One of the most striking effects of PGPR inoculation on roots is stimulation of lateral root development (Mantelin et al., 2006). This increase in development of lateral roots causes an increase in root surface which improves plant nutrient uptake and therefore, can be considered as an important factor for promotion of plant growth by PGPR.

An improved understanding of root system development and functioning, to identify root traits contributing to crop yields in various scenarios, as well as mechanisms by which they are enhanced is a research frontier that might enable a second Green Revolution needed to sustain world food security (Lynch, 2007). PGPR mediated modulations of root traits can be advantageously explored for improving the efficiency of agroecosystems. PGPR able to induce desired root traits for harnessing the soil resources can be a way toward sustainable agricultural production. The role of PGPR in improving plant productivity has been reviewed by many workers (Beneduzi et al., 2012; Backer et al., 2018). However, there is limited knowledge on how PGPR modify the root traits. The present review focuses on the PGPR mediated changes in root traits and key determinants responsible for root-microbe cross talk. Contributions of PGPR mediated root traits to ecosystem service and crop productivity are also discussed.

## IMPORTANCE OF ROOT TRAITS

Root act as interface between plant and soil besides providing anchorage and support to the growing plant. Also, roots act as storage organ in many plants (Smith and De Smet, 2012). Majority of the terrestrial plants have well-developed system of roots for exploration of the soil and for nutrient acquisition to support the growth and development of the growing plant. Root is a complex organ that comprises different regions including root tip, meristem, differentiation and elongation zones, and emerging lateral roots (Scheres et al., 2002). All these regions have discrete roles. For example, the terminal portion of a root or



**FIGURE 1** | Mechanisms of plant growth promotion by PGPR.

root branch usually including the root cap and the meristematic region behind it is often the region of differentiation, elongation, and root hair formation. The purpose of the root cap is to enable downward growth of the root tip while, root hairs are the differentiated epidermal cells vital for uptake of minerals (Ahn et al., 2004). Lateral roots help in branching of root systems and play a significant role in the uptake of water, ion, and nutrients and also facilitate the diffusion of oxygen from the base to the apex thus contributing significantly in plant development (Colmer, 2003; Kotula et al., 2009a,b). The functional specificity of roots is also known by the extent of interactions between plant and the microbes. For example, in Fabaceae, root tip is the main region where the process of rhizobial colonization is initiated leading to root nodule formation (Desbrosses and Stougaard, 2011). While, PGPR preferentially colonize root hairs and lateral roots, where they might exhibit beneficial properties for the plant (Pothier et al., 2007; Combes-Meynet et al., 2011).

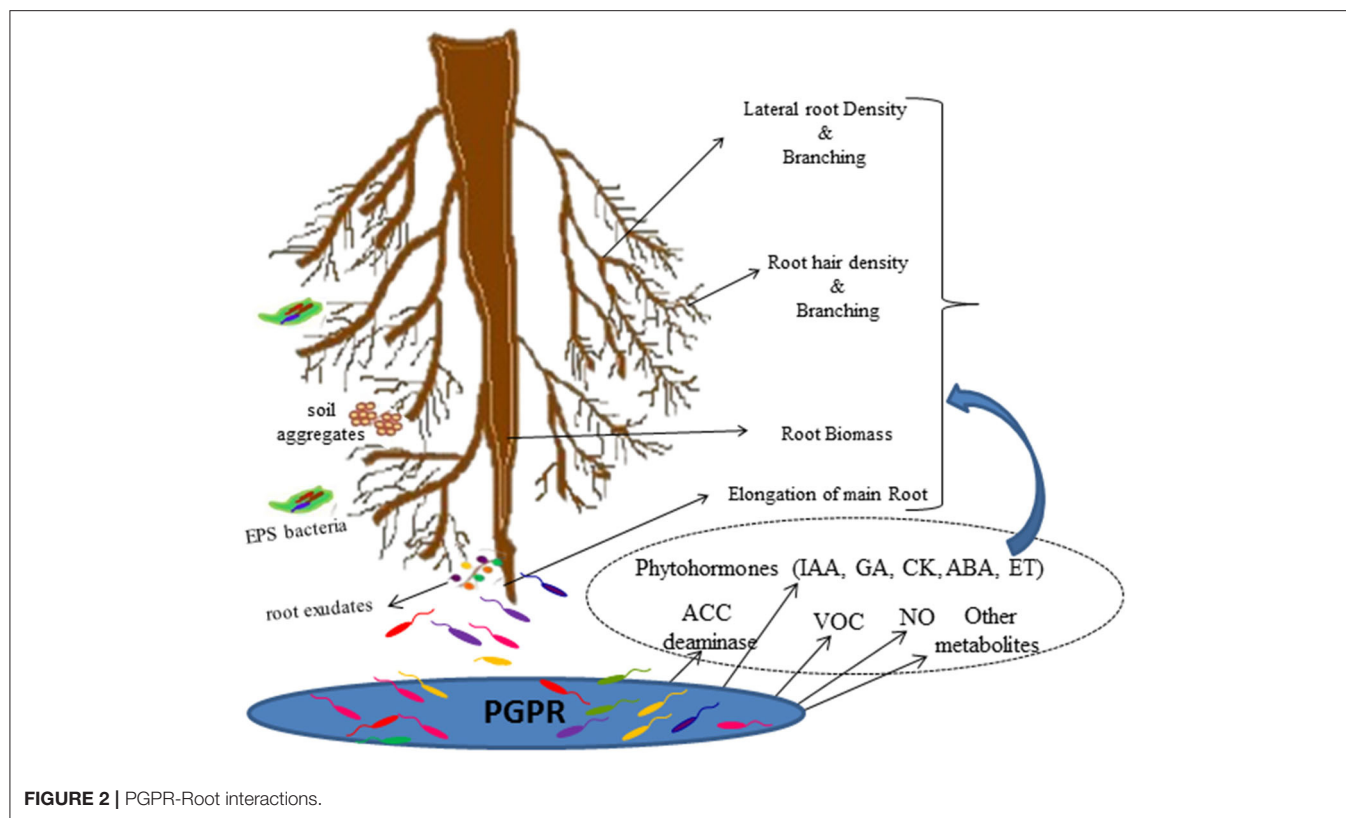
According to Bardgett et al. (2014), kinds of root traits which can potentially affect the rhizosphere functioning, include: (1) architectural root traits, that decide the topology and spatial configuration of the entire root system of a plant, like root number, depth of root, and root length density; (2) morphological root traits that refer to features of individual roots, such as diameter of the roots, specific length of roots, density of root tissues, and dry matter content of the roots; (3) physiological root traits like nutrient uptake kinetics, release of root exudates, and root respiration; and (4) biotic traits which include direct interactions between roots and soil microbiota that affect nutrient capture, such as associations with mycorrhizal fungi and rhizobia (in legumes). However, the biotic interaction of roots with rhizospheric microorganisms also influence the architectural, morphological, and physiological traits of roots

thus, influencing the root system functioning as indicated by the recently accumulating evidences (Nosheen et al., 2011; Droque et al., 2013; Vacheron et al., 2013; Mesa-Marín et al., 2018), although majority of earlier studies on PGPR effect on root traits have reported only effects in terms of root length, biomass and volume.

Root architecture refers to the spatial configuration of the root system that includes topology and distribution of an entire root system or a large subset of the root system of an individual plant. The spatial deployment of the root system helps the plant to explore the unevenly distributed or spatially localized resources in the soil and thus play important role in plant productivity (Lynch, 1995). The architecture of a root system also responds dynamically to the localized availability of soil resources through meristematic activity and transport functions. Indeed, root architecture has been linked with plant acquisition of water and nutrients that move with water, as well as immobile nutrients such as P (Lynch, 1995). The root architecture also determines the role of roots in providing mechanical support to the aboveground parts of the plant (Ennos and Fitter, 1992). The morphological and physiological traits of roots play essential roles in ecological processes (Dong et al., 2016). Morphological changes in roots are often caused due to internal physiological changes, induced in response to external stimuli (Makita et al., 2011; Gong and Zhao, 2019).

## PGPR AND ROOT TRAITS

Understanding and quantifying the impact of PGPR on roots and the whole plant remain challenging (Vacheron et al., 2013). Several *in vitro* studies conducted to study the effect of PGPR inoculation on plant growth and root traits, reveal that many



PGPR reduce the growth of main root, increase the number, and/or length of lateral roots and stimulate root hair elongation thus enhancing the uptake of water and nutrients and resulting in increased plant growth and development (**Figure 2**; Vacheron et al., 2013; Cassán et al., 2020). Positive effects of PGPR inoculation on root and shoot biomass have also been reported from soil based pot and field studies (El-Zemrany et al., 2006; Minorsky, 2008; Veresoglou and Meneses, 2010; Walker et al., 2011). **Table 1** summarizes the reports on PGPR mediated effects on root traits and other plant growth parameters. As evident from the **Table 1**, majority of the studies have reported the effect in terms of root length and biomass. Members of *Azospirillum* and *Rhizobium* are most studied PGPR for their effect on root traits. *Azospirillum*-root association has been extensively studied all over the world in different crops including cereals, forage, vegetables, and the stimulatory effects of inoculation on root elongation, root biomass, development of lateral and adventitious roots, root hairs and branching of root hairs have been well-documented (Hadas and Okon, 1987; Bashan and de-Bashan, 2010). These cumulative effects on root traits results in significant improvement in the root system functioning. However, these effects have been found to vary with inoculum concentration and environmental conditions (Okon and Kapulnik, 1986).

Among the initial studies on *Azospirillum*-root interaction, Tien et al. (1979) observed that the morphology of pearl millet (*Pennisetum glaucum* L.) roots with respect to increased number of lateral roots and increased density of root hair when inoculated with *A. brasilense*. Umali-Garcia et al. (1980)

described the adsorption and colonization of *A. brasilense* Sp7 on the root surfaces of pearl millet and guinea grass (*Megathyrus maximus*) under controlled conditions. Inoculated roots of both the plants produced more mucilaginous sheath, root hairs, and lateral roots as compared to the uninoculated controls. The presence of bacteria was observed within the mucilaginous sheath accumulated on the root cap and along the root axes. Pectolytic activities detected in pure cultures of *A. brasilense* provided a possible explanation for colonization of intercellular spaces of the outer root cortex. It was also observed that inoculated bacteria showed firm attachment to root hairs when no nitrogen source was added to the culture medium, whereas, no firm attachment of the inoculated bacteria was observed in the culture medium supplemented with potassium nitrate. Okon and Kapulnik (1986) summarized many such studies that documented the presence of *Azospirillum* in the cortical tissues, in the regions of lateral root emergence, along the inner cortex, inside the xylem vessels and between the pith cells. Inoculation of various crops including wheat (*Triticum aestivum* L.), corn (*Zea mays* L.), sorghum (*Sorghum bicolor* L.), and setaria (*Setaria viridis* L.) with different strains of *Azospirillum* resulted in root morphological changes that started immediately after germination. The effect on root length and surface area varied with age and cell level of inoculated bacteria. Inoculation increased the number and branching of root hairs (Jain and Patriquin, 1985) and number of lateral roots during the initial 3 weeks post-germination, but root biomass increased at later stages (Okon and Kapulnik, 1986). Inoculation also induced irregularity in the arrangement of cortical cells.

**TABLE 1** | Influence of PGPR on root traits and other growth parameters of inoculated plant.

Microorganism	PGP traits/mechanism	Plant	Effect on root traits	Effect on other plant parameters	References
<i>Bacillus aquimaris</i> strain 3.13, <i>Micrococcus luteus</i> strain 4.43	IAA, EPS, ACC deaminase	Jerusalem artichoke ( <i>Helianthus tuberosus</i> L.)	Increase in weight, length, diameter, volume, area and surface	Increase in shoot length, shoot weight, leaf area and chlorophyll levels, photosynthesis and harvest index	Namwongsa et al., 2019
<i>Azospirillum brasilense</i> , <i>Azotobacter vinelandii</i> KhSr1, <i>Pseudomonas stutzeri</i> , KhSr3	Phytohormone (IAA, cytokinin) production	Safflower ( <i>Carthamus tinctorius</i> L.)	Altered morphology and distribution pattern with increased length, diameter and area	–	Nosheen et al., 2011
<i>Pseudomonas putida</i> REN <sub>5</sub> , <i>Pseudomonas fluorescens</i> REN <sub>1</sub>	IAA, ACC deaminase, siderophore production	Rice ( <i>Oryza sativa</i> L.)	Increased length, fresh weight and dry weight, branching	Increased shoot length, fresh weight, dry weight	Etesami and Alikhani, 2016
<i>Serratia proteamaculans</i> strain J119	ACC-deaminase	Chickpea ( <i>Cicer arietinum</i> L.)	Increased length of lateral roots, number, total root biomass	Improved shoot length, weight, Increased number of nodules, total nodule weight, number of pods, and grain yield	Shahzad et al., 2010
<i>Bacillus megaterium</i> TV-91C, <i>Pantoea agglomerans</i> RK-92, <i>Bacillus subtilis</i> TV-17C	Gibberellic acid, Salicylic acid, Absciscic acid, Indole acetic acid	Cabbage ( <i>Brassica oleracea</i> )	Enhanced root fresh weight, dry weight	Enhanced shoot fresh and dry weight, stem diameter, chlorophyll content, leaf area and leaf number	Turan et al., 2014
<i>Bacillus amyloliquefaciens</i> subsp. <i>plantarum</i> UCMB5113	Enhanced expression of plant hormones cytokinins, gibberellin, brassinolide	Arabidopsis ( <i>Arabidopsis thaliana</i> )	Increased lateral root outgrowth and elongation and root-hair formation.	Increased fresh weight of shoot	Asari et al., 2016
<i>Bacillus</i> sp. 12D6, <i>Enterobacter</i> sp. 16i	Indole-3-acetic acid (IAA), salicylic acid (SA)	Wheat ( <i>Triticum aestivum</i> ) and maize ( <i>Zea mays</i> )	Significantly increased root branching, root length, surface area of root, number of root tips	Alleviated drought stress symptoms in both wheat and maize	Jochum et al., 2019
<i>Bacillus</i> RC23, <i>B. subtilis</i> OSU142, <i>Bacillus</i> RC03, <i>B. megaterium</i> RC01, <i>B. simplex</i> RC19, <i>Paenibacillus polymyxa</i> RC05, <i>Comamonas acidovorans</i> RC41	IAA production	Kiwifruit ( <i>Actinidia deliciosa</i> )	Higher numbers of main roots, root length, root diameter, root dry weight, root quality and rooting percentage	–	Erturk et al., 2010
<i>Enterobacter</i> sp., <i>Serratia</i> sp.	IAA production	Chickpea ( <i>Cicer arietinum</i> L.)	Primary root growth, lateral root density, lateral root length, root dry weight	–	Fierro-Coronado et al., 2014
<i>Burkholderia pyrrocinia</i> (R-46), <i>Pseudomonas fluorescens</i> (R-55), coinoculation with <i>Trichoderma asperellum</i>	Auxins, siderophore production	Rice ( <i>Oryza sativa</i> L.)	Improved root length and diameter, expansion of cortex and aerenchyma, Increase in no. of protoxylem poles, metaxylem vessel elements, and total phenol, flavonoid and lignin contents	–	Ferreira Rêgo et al., 2014
<i>Bacillus simplex</i> 30N-5, <i>B. subtilis</i> 30VD-1, <i>Rhizobium leguminosarum</i> bv. <i>viciae</i>	Siderophore production, phosphate solubilization, antifungal activity	Pea ( <i>Pisum sativum</i> )	Emergence of more lateral roots, highly branched vascular bundles, improved root dry weight	Larger nodules, improved shoot length	Schwartz et al., 2013
<i>Bacillus megaterium</i> BOFC15	Secretion of polyamine, spermidine	<i>Arabidopsis thaliana</i>	More lateral roots, longer primary roots, leaf area, improved fresh, and dry weight of root	Improved plant growth and photosynthesis rate, Induced high absciscic acid, improved drought tolerance	Zhou et al., 2016
<i>Bacillus megaterium</i> , <i>B. subtilis</i> , and <i>Pseudomonas</i> sp., co-inoculated with <i>Bradyrhizobium</i> sp.	IAA production, antagonistic against root rot disease, biofilm formation, lytic enzyme production	Peanut ( <i>Arachis hypogaea</i> L.)	Improved root length, root dry weight	Shoot length and shoot dry weight	Yuttavanichakul et al., 2012

(Continued)



TABLE 1 | Continued

Microorganism	PGP traits/mechanism	Plant	Effect on root traits	Effect on other plant parameters	References
<i>Bacillus megaterium</i> strain (RmBm31)	Production of IAA, VOC	<i>Arabidopsis</i> ( <i>Arabidopsis thaliana</i> )	Root fresh weight, number and length of lateral roots, root hair length	Increased plant biomass, induced expression of genes involved in the signaling pathways of ethylene, jasmonic acid, and salicylic acid	Dahmani et al., 2020
<i>Bradyrhizobium japonicum</i> USDA 110, <i>Pseudomonas putida</i> TSAU1	IAA production, nitrogen and phosphorus accumulation	Soybean ( <i>Glycine max</i> )	Improved root length, surface area, root volume, number of nodules per plant root	Improved shoot length, shoot fresh weight	Egamberdieva et al., 2017
<i>Azospirillum brasilense</i> , <i>Azotobacter vinelandii</i> , <i>Pseudomonas stutzeri</i>	Phytohormone (IAA, cytokinin) production	Safflower ( <i>Carthamus tinctorius</i> L.)	Increased root area, width, root length	–	Nosheen et al., 2011
<i>Azospirillum brasilense</i> strain Cd	Malate dehydrogenase (MDH) activity	Tomato ( <i>Lycopersicon esculentum</i> L. cv Marmande and cv M-82)	Increased root length, root dry weight, root hairs, asymmetrical growth of the root tip, root respiration	Increased leaf surface area	Hadas and Okon, 1987
<i>Azospirillum lipoferum</i> CRT1	IAA production	Maize ( <i>Zea mays</i> L.)	Increased root biomass, root length, diameter, larger numbers of tips	–	El-Zemrany et al., 2007
<i>Azospirillum brasilense</i> Sp245	Nitric oxide production	Tomato ( <i>Lycopersicon esculentum</i> L.)	Enhanced lateral root and root hair development	–	Creus et al., 2005
<i>Pseudomonas fluorescens</i> F113, <i>Azospirillum lipoferum</i> CRT1, coinoculated with <i>Glomus intraradices</i> JJ291	Synthesis of Benzoxazinoids	Maize ( <i>Zea mays</i> L.)	Enhanced total root surface, total root volume and/or root number	–	Walker et al., 2011
<i>Azospirillum brasilense</i> SM	IAA production	Sorghum ( <i>Sorghum bicolor</i> )	Significantly increased root length, number of lateral roots	Increased shoot length and plant dry weight	Malhotra and Srivastava, 2009
<i>Azospirillum brasilense</i> Sp245	IAA production	Bean ( <i>Phaseolus vulgaris</i> L.)	Enhanced root dry weight, nodule number	Increased shoot dry weight	Remans et al., 2007
<i>Phyllobacterium brassicacearum</i> STM196	IAA production	<i>Arabidopsis</i> ( <i>Arabidopsis thaliana</i> )	Increased lateral root length	–	Contesto et al., 2010
<i>Pseudomonas putida</i> WCS417, <i>Pseudomonas fluorescens</i> WCS374	Auxin regulated gene expression	<i>Arabidopsis</i> ( <i>Arabidopsis thaliana</i> )	Increased root length, lateral roots, root hair, increased the number of cortical cells	Stimulated shoot fresh weight	Zamioudis et al., 2013
<i>Bacillus</i> spp.	Synthesis of cytokinin	Lettuce plants ( <i>Lactuca sativa</i> L., cv Lolla Rossa)	Increased root fresh weight	Improved shoots biomass, increased chlorophyll and carotenoids in leaves	Arkhipova et al., 2005
<i>Azospirillum brasilense</i> strain Az39, <i>Bradyrhizobium japonicum</i> strain E109	IAA, gibberellic acid (GA <sub>3</sub> ), Zeatin (Z) production	Corn ( <i>Zea mays</i> L.) and Soybean ( <i>Glycine max</i> L.)	Improved radicle length, root dry weight, number of nodules per plant.	Promoted seed germination, improved shoot length and dry weight	Cassán et al., 2009
<i>Azospirillum lipoferum</i> (Al op 33, Al iaa 320, and ATCC 29708)	Auxin production	Maize ( <i>Zea mays</i> L., hybrid Cargill 147)	Improved root growth, enhanced root hair growth and density	Increased shoot weight	Fulchieri et al., 1993
<i>Pseudomonas fluorescens</i> 63-28R	Production of antifungal metabolites	Pea ( <i>Pisum sativum</i> L.)	Induced accumulation of lignin in root cells and inhibited colonization by the oomycete <i>Pythium ultimum</i>	Enhanced defense against <i>Pythium ultimum</i>	Benhamou et al., 1996
<i>Pseudomonas putida</i> Corvallis	–	Bean ( <i>Phaseolus vulgaris</i> )	Accumulation of lignin in root cells	Increased plant weight, protection against <i>F. solani</i> through delayed wilting and lesion formation	Anderson and Guerra, 1985
<i>Azospirillum brasilense</i> CA-10	IAA production	Rice ( <i>Oryza sativa</i> )	Enhanced PG (polygalacturonase) activity in roots, better root morphogenesis	–	Sekar et al., 2000

(Continued)

TABLE 1 | Continued

Microorganism	PGP traits/mechanism	Plant	Effect on root traits	Effect on other plant parameters	References
<i>Frankia</i> UGL010708	BNF	Common alder ( <i>Alnus glutinosa</i> )	Increased root branching and total number nodules and root hairs	Enhanced seedling growth	Orfanoudakis et al., 2010
<i>Bacillus megaterium</i> UMCV1	Influences auxin and ethylene signaling pathways	Common bean ( <i>Phaseolus vulgaris</i> )	Increased lateral root number and growth, and root hair length	Increased the fresh and dry weights of plants	López-Bucio et al., 2007
<i>Bacillus subtilis</i> GB03	Production of VOC	Arabidopsis	Increased lateral root density and length	Regulation of auxin homeostasis. Enhanced seedling growth	Zhang et al., 2007
<i>Bacillus</i> sp., LZR216	Strengthening of Auxin responses	Arabidopsis	Inhibition of primary root elongation, Enhanced number and density lateral roots	Enhanced seedlings growth, Increase in the transcriptional levels of auxin biosynthesis genes	Wang et al., 2015
<i>Azospirillum Brasilense</i> Sp245, <i>Azospirillum Brasilense</i> Sp7	IAA production	Wheat ( <i>Triticum aestivum</i> )	Inhibited root length while enhanced root hair formation	–	Dobbelaere et al., 1999
<i>Rhizobium leguminosarum</i> 899	BNF	Common bean ( <i>Phaseolus vulgaris</i> )	Increased root dry weight	Increased shoot dry weight	Franzini et al., 2010
<i>Rhizobium</i> sp., Coinoculated with AM fungi	BNF	Black-eyed pea ( <i>Vigna unguiculata</i> )	Increased root length root dry weight	Improved shoot length and biomass, chlorophyll content, nodule no. and dry weight	Arumugam et al., 2010
<i>Rhizobium leguminosarum</i> PR-1, <i>Pseudomonas</i> sp. strain PGERs17	Increase uptake of N,P,K, Zn, Fe	Lentil ( <i>Lens culinaris</i> )	Increased root length, nodule number, nodule dry weight, root dry biomass	Increased shoot dry weight, chlorophyll content.	Mishra et al., 2011
<i>Rhizobium phaseoli</i> 123, <i>Pseudomonas</i> sp. LG, <i>Bacillus</i> sp. Bx	IAA, phosphate solubilization	Common bean ( <i>Phaseolus vulgaris</i> )	Improved root length, root dry weight, nodule dry weight	Increased shoot dry weight	Stajković et al., 2011
<i>Rhizobium</i> sp. CR2, CR3	BNF	Chickpea ( <i>Cicer arietinum</i> )	Increased root length, dry weight, nodule number, nodule dry weight	Improved plant height, shoot dry weight.	Solaiman et al., 1970
<i>Rhizobium</i> sp. NSFBR-1, NSFBR-15	IAA, phosphate solubilization and ACC utilization	Faba bean ( <i>Vicia faba</i> L.)	Increased root length, number of nodules, nodule dry weight	Improved numbers of leaves, branches, and pods, pod length, seed weight	Argaw, 2012
<i>Rhizobium japonicum</i>	BNF	Black gram ( <i>Vigna mungo</i> ) and Green gram ( <i>Vigna radiata</i> )	Increase in root numbers, improved nodulation	Increased plant height, and no. of leaves and branches	Ravikumar, 2012
<i>Rhizobium</i> sp strain JUR4, <i>Trichoderma Hamatum</i> JUF1	P solubilization, Antagonistic activity	Black gram ( <i>Vigna mungo</i> )	Increased root length, fresh weight	Increased shoot length, chlorophyll and protein content	Badar and Qureshi, 2012
<i>Rhizobium</i> spp., <i>Rhizobium tropici</i> CIAT899	BNF	<i>Phaseolus vulgaris</i>	Increased root length, number of roots, number of nodules, root dry weight	Increased in shoot dry weight	Karaca and Uyanoz, 2012
<i>Rhizobium leguminosarum</i> EAL-110	BNF	Faba bean ( <i>Vicia faba</i> )	Increased root length, root fresh weight, number of nodule per plant, nodule dry weight and volume	Increased uptake of P, Zn	Desta et al., 2015
<i>Rhizobium</i> sp. R4, R6, R9	Production of phytohormones, salt tolerant	Chickpea ( <i>Cicer arietinum</i> L.)	Increased root dry weight, nodule number, root length,	Improved shoot dry weight, shoot length	Khaitov et al., 2016

Morphological changes also influenced the physiological traits of inoculated roots as evident from the reduced activities of oxidative enzymes, and low lipid and suberin content. These observations suggested presence of larger proportion of younger roots in inoculated roots than in uninoculated roots. The inoculated seedlings also exhibited higher rate of  $\text{NO}_3^-$ ,  $\text{K}^+$ , and  $\text{H}_2\text{PO}_4^-$  uptake in inoculated seedlings. Field studies revealed

faster rates of dry matter, N, P, and K accumulation with high water content in *Azospirillum* inoculated plants (Okon and Kapulnik, 1986). Inoculation with wild strains of *A. brasilense* Sp245 and Sp7 in wheat caused significant reduction in root length, but increased the number of root hairs (Dobbelaere et al., 1999). Inoculation of tomato (*Lycopersicon esculentum* L.) seedlings with *A. brasilense* Sp245 could promote both lateral

and adventitious roots formation. Application of cell free culture supernatants of *A. brasilense* ATCC 29710 to rice (*Oryza sativa* L.) roots increased root elongation, root surface area, root dry matter, and development of lateral roots and root hairs (El-Khawass and Adachi, 1999). In a similar study, Molla et al. (2001) reported increased number of roots and root length in soybean (*Glycine max* L.) plants treated with cell-free supernatant of *A. brasilense* Cd., indicating the release of root growth promoting substances by the cells into the medium. El-Zemrany et al. (2007) assessed the result of inoculation of maize (*Zea mays*) seeds grown in a luvisol soil with *Azospirillum lipoferum* CRT1. The observations recorded after 35 days of plant growth revealed significant increase in the number of root tips and total root length in the inoculated plants. The authors reported that due to changes in root traits, the inoculated roots had a larger surface area to interact with soil particles, soil water and microbes than the uninoculated plants. Further, the mechanical and biochemical (lignin content) properties of the root system of inoculated plants showed less mature characteristics as compared to uninoculated control plants. This observation indicated that the growth of new root tips in the early stages of inoculation resulted in the dominance of primary root tissue. Moreover, roots of inoculated plants showed more stiffness that may contribute to anchorage thus increasing the resistance of the plants to lodging. However, such types of studies need to be conducted at regular intervals during the crop growth period, to evaluate if the differences between inoculated and uninoculated plants endure or vanish with time until the harvest.

Increased formation of lateral roots in barrel medic (*Medicago truncatula*) by the Nod factor of *Sinorhizobium meliloti* was observed by Oláh et al. (2005). Inoculation with *Bacillus megaterium* UMCV1 in *Arabidopsis thaliana* resulted in inhibition of primary-root growth (due to decreased cell elongation and proliferation in the root meristem,) and an increase in number and length of lateral roots and root-hair (López-Bucio et al., 2007). Similar results were reported by Zhang et al. (2007) and Gutiérrez-Luna et al. (2010) with volatile organic compounds (VOCs) producing *Bacillus* strains. Co-culturing of *Serratia marscescens* with *Arabidopsis* resulted in inhibition of elongation of primary roots, while inducing lateral roots (Shi et al., 2010). On contrary, inoculation with *Phyllobacterium brassicacearum* STM196 in *Arabidopsis* exerted a positive effect on the total length of lateral roots but no effect on the primary root length or lateral root density (Mantelin et al., 2006; Contesto et al., 2010). An increased root branching was seen in alder (*Alnus glutinosa* L.) due to root colonization by *Frankia* UGL010708 (Orfanoudakis et al., 2010). The root architecture in terms of increased primary length of roots, number of lateral roots and root fresh weight in *A. thaliana* was modified by the rhizobacterium *Pseudomonas aeruginosa* PAO1 (Ortiz-Castro et al., 2011). Interestingly, Nosheen et al. (2011) observed inoculation effects of *Azotobacter vinelandii* Khsr1, *Azospirillum brasilense* and *Pseudomonas stutzeri* Khsr3 on physiological root traits in safflower (*Carthamus tinctorius* L.). The authors observed that the inoculation caused significant increase in root diameter (upto 76% increase), resulting in greater biomass. Ferreira Rêgo et al. (2014) observed significant

changes in architectural, morphological and physiological traits in the roots of rice plant, treated individually or as combination with rhizobacterial strains *Burkholderia pyrrocinia* R-46 and *Pseudomonas fluorescens* R-55 as individual or combined treatments. Rhizobacterial treatment caused increase in length and diameter of roots along with expansion of cortex and aerenchyma space as compared to uninoculated control treatment. Rhizobacterial inoculation also caused increase in number of protoxylem poles and metaxylem vessel elements and also improved total phenol content and flavonoid content in the roots. Also, treatment with *Pseudomonas fluorescens* R-55 improved lignin content. The modification caused by rhizobacterial treatment resulted in root plasticity in rice plants. Interestingly, a thicker pericycle was presented in plants inoculated with *B. pyrrocinia*, which is linked to the development of lateral roots. The process by which lateral roots are formed includes two major stages: pericycle cell cycle reactivation and establishing a new meristem. The PGPR mediated stimulation of pericycle and protoxylem may result in increased lateral root formation and consequently more vigorous establishment of rice plants (Ferreira Rêgo et al., 2014). Co-inoculation of common bean (*Phaseolus vulgaris* L.) with two rhizobial strains, i.e., IITA-PAU 983 and IITA-PAU 987 significantly increased root dry weights and nodule weight of plants when compared to uninoculated control (Korir et al., 2017). Several PGPR, not classified as mutualistic partners, have also been shown to affect the architecture of plant roots.

## MICROBIAL METABOLITES/MOLECULES THAT INFLUENCE ROOT TRAIT PATHWAYS IN PLANTS

### Phytohormones and Root Traits

Root traits components like branch number, branching pattern, length, orientation, angle, and diameter are controlled by intricate genetic pathways that are also involved in plant's response to environmental stimuli (Jung and McCouch, 2013). These pathways belong to either "intrinsic pathways" or "extrinsic pathways" (Malamy and Ryan, 2001). Intrinsic pathways involve hormones, their receptors, signaling components, and transcription factors (TFs) whereas extrinsic pathways involve receptors for environmental stimuli, their downstream transduction and TFs. Many components of extrinsic pathways are shared with or interregulated by intrinsic pathway and are mediated by hormonal regulation (Jung and McCouch, 2013).

Phytohormones coordinate metabolic activities associated with growth of microorganisms in different plants tissues. Modifications at organ, cellular and molecular level of root systems and plant tissues get triggered by beneficial and detrimental rhizosphere microorganisms, through modifications of phytohormonal balances (Boivin et al., 2016). Many of the PGPR present in rhizosphere are well-known to secrete hormones for uptake by the plant roots or for manipulation of plant hormonal balance, which finally regulate root/shoot

growth as well as stress response of the plant (Tien et al., 1979; Gupta et al., 2015). In this respect, the hormones involved majorly include auxins, cytokinins, ethylene, and to a lesser extent gibberellins and abscisic acid (ABA) has also been reported (Vacheron et al., 2013). The auxin-cytokinin balance is definitely the main regulating mechanism for organogenesis in plants that defines the architecture of roots (Aloni et al., 2006). In this respect, particular changes that occur in the structure of roots are: total root growth, length of primary and lateral roots, number of lateral roots, and the manner in which lateral roots are positioned (Contesto et al., 2010).

### Role of Microbial Auxins on Root Traits of Plants

Auxins are responsible for division, extension, and differentiation of plant cells and tissues and are known to increase the rate of xylem and root formation (Bashan and de-Bashan, 2010). A brief and simple explanation of the function of auxin in development of roots is the promotion of lateral root development, while inhibiting elongation of roots. The development of lateral roots involves a cycle of cell divisions in the activated endodermal/pericycle cells which form the root primordia that eventually differentiate and emerge as the new lateral roots (Péret et al., 2012). All these processes i.e., initiation, development, emergence, and elongation of lateral roots are regulated by the biosynthesis, transport, and signaling of auxins (Péret et al., 2009; De Smet, 2012). Increase in the amount of auxins can cause increased formation of roots in an amount dependent manner, as evidenced by genetic engineering studies which augment levels of auxin (Malamy, 2005).

PGPR stimulate the root growth mainly due to their potential to secrete indole acetic acid (IAA). Many PGPR are known to synthesize auxins that impart a considerable effect on root growth and architecture (Vacheron et al., 2013). For example, auxin producing PGPRs *Pseudomonas extremaustralis* IB-k13-1A and *Paenibacillus illinoisensis* IB 1087, when inoculated in wheat plants, resulted in increased root biomass and root auxin levels (Kudoyarova et al., 2017). Seed inoculation of wheat plant with *P. extremaustralis* IB-k13-1A increased the crop yield under field conditions (Arkhipova et al., 2019). In several studies (as reviewed by Bashan and de-Bashan, 2010; Vacheron et al., 2013), morphological changes induced by *Azospirillum* in roots were mimicked by applying a combination of plant growth substances, indicating the involvement of bacterial auxin in root proliferation and consequent plant growth promotion. Jain and Patriquin (1984, 1985) identified IAA as the “branching substance” in the culture filtrate of *A. brasilense* Sp245. Fallik et al. (1989) reported that the levels of IAA and IBA (indole-3-butyric acid) were increased in maize plants inoculated with *Azospirillum*, thereby justifying the role of auxins in promotion of plant growth. Dobbelaere et al. (1999) reported that wheat plants when inoculated with the wild strains of *A. brasilense* Sp245 and Sp7 caused a significant reduction of root length and an increase in the formation of root hairs. Addition of tryptophan further enhanced the effect of inoculation on the root morphology. Also, the replacement of *Azospirillum* cells with exogenous IAA resulted in a similar response in the wheat roots (Dobbelaere et al., 1999). On these lines, when IAA was applied exogenously

to the roots of bean plants, the results were similar to that obtained with *A. brasilense* Sp245 inoculation (Remans et al., 2008).

Use of IAA-attenuated mutants could provide strong evidence for the role of IAA in PGPR-induced root growth promotion (de-Bashan et al., 2008). Inoculation of canola (*Brassica napus* L.) plants with auxin deficient *Pseudomonas putida* GR12-2 strain resulted in reduced root growth (Patten and Glick, 2002a). Likewise, inoculation with IAA-deficient mutants of *Pseudomonas moraviensis* in wheat caused reduction in root surface area by 13–38% as compared to that with the control strain (Hassan and Bano, 2019). Barbieri and Galli (1993) inoculated wheat seedlings with wild-type strain *Azospirillum brasilense* Sp6 and a very low-IAA producing mutant *Azospirillum brasilense* SPM7918 and observed reduced ability of the mutant strain to stimulate root system in terms of number and length of lateral roots and the distribution of root hairs. They also observed that by increasing the inoculum concentration of SpM7918, no increase in the number and length of lateral roots or in the density of root hair was observed, thus, indicating the involvement of additional factors in root growth. In another study, Kundu et al. (1997) observed that inoculating pearl millet seedlings with *A. brasilense* mutant strain with high N<sub>2</sub>-fixation activity but low phytohormone production ability could not cause any increase in root growth, whereas, the mutant strain able to produce higher levels of phytohormones had a considerable positive effect on the morphology of roots when compared with the uninoculated control plants (Kundu et al., 1997). The wheat seedlings inoculated with *A. brasilense* Sp245 strain with a mutated IAA synthesis gene, could not cause reduced root length or enhance the formation of root hairs when compared with the control (Dobbelaere et al., 1999). However, the insertion of the heterologous genes for indole acetamide pathway (*iaaM* and *iaaH*) into the strain *A. brasilense* SM significantly enhanced the levels of IAA and inoculation of sorghum with engineered strain resulted in marked increase on the lateral branching of sorghum roots and dry weight of roots as compared to inoculation with wild-type strain (Malhotra and Srivastava, 2006). Hence, it can be inferred that the analysis of mutations for studying the role of auxins in various plant-microbe interactions provide constant results in a widespread bacterial genera and plant species. Sukumar et al. (2013) reviewed the role of auxin pathways in regulating architecture of roots during plant-microbe interactions. Inoculation with auxin producing PGPR strain induced transcriptional changes in hormone- and defense-related genes, as well as in plant cell wall-related genes in the host plant (Spaepen et al., 2014), resulting in induction of long roots (Hong et al., 1991), increased root biomass and decreased size and density of stomata (Llorente et al., 2016) as well as induction of auxin responsive genes that stimulate plant growth (Ruzzi and Aroca, 2015).

Auxins are implicated in the process of formation of nodules by rhizobia in leguminous plants, like founder cell specification (auxin transport inhibition mainly by flavonoids), initiation and differentiation of nodules (auxin accumulation), formation of vascular bundles, and the number of nodules (long distance auxin transport). Auxin produced by bacteria may change the balance



of auxins in the plant and hence, indirectly affect the nodulation in legumes (Mathesius, 2008). Generally, rhizobia can produce auxins to enhance cell division, differentiation, root growth and increase nodule formation. IAA is most widely produced and studied among different rhizobial species including *R. japonicum*, *R. lupine*, *R. leguminosarum*, *R. phaseoli*, *R. meliloti*, *R. trifolii*, *Bradyrhizobium japonicum*, *B. elkanii*, and *Sinorhizobium* sp. (Gopalakrishnan et al., 2015). The co-inoculation of IAA producing strains *B. japonicum* SB-1 and *Bacillus thuringiensis* KR1 resulted in enhanced plant growth and nodulation in pea (*Pisum sativum* L.) (Mishra et al., 2010). Inoculation with IAA overproducing *R. leguminosarum* bv. *viciae* strain LPR1105 caused 60-fold increase in IAA content in the root nodules of the vetch plant (Camerini et al., 2008). The co-inoculation of *Azospirillum* and *Rhizobium* resulted in early and faster nodulation with higher crop yields. Additionally, the number of nodules and biological nitrogen fixation also increased. While, when *ipdC* mutants of *Azospirillum* were used for inoculating the plants, they synthesized only 10% of the IAA as compared to wild type strain and exhibited reduced ability to promote root development, without any increase in nodulation as well as nitrogen fixation, thus representing that the IAA biosynthesis by bacteria play significant role in plant-microbe symbiosis (Barbieri and Galli, 1993; Dobbelaere et al., 1999; Remans et al., 2008).

*Azospirillum* spp. are well-known for their potential to synthesize plant hormones and among them, indoles, specifically IAA and gibberellins (GAs) may play a larger role (Spaepen et al., 2007; Bashan and de-Bashan, 2010). Although auxins are known to cause stimulatory effect, their inhibitory effects on the elongation of roots has also been reported. The inhibitory effects could be dose-dependent (increases with auxin concentration) (Arteca and Arteca, 2008) as the increasing concentration of auxins may stimulate production of ethylene (Glick, 2014). Ethylene is a major phytohormone that causes inhibition of root elongation and transport of auxins, while promoting senescence and organ abscission, leading to ripening of fruits (Glick et al., 2007; Splivallo et al., 2009). Since, the direct precursor of ethylene is 1-aminocyclopropane-1-carboxylate (ACC) and hence bacterial ACC deaminase activity can control excessive ethylene production. The ACC deaminase can alleviate the repressing effect of ethylene, thus enhancing elongation of roots, in spite of high concentrations of potentially inhibitory auxin (Kudoyarova et al., 2019). *Enterobacter cloacae* UW4 that exhibited IAA and ACC deaminase activity promoted root elongation in canola (*Brassica napus* L.) plants, whereas its mutant lacking ACC deaminase activity had no effect on root growth and thus, highlighting the significance of ACC deaminase enzyme in elongation of roots in the presence of high auxins (Li et al., 2000). Thus, both IAA and ACC deaminase act together for stimulating elongation of roots (Patten and Glick, 2002b). Over expression of ACC deaminase in rhizobia enhanced the competitiveness of the bacterium and increase nodule number in legumes such as pea, *Lotus* sp. (*Lotus japonicus* and *L. tenuis*) (Glick et al., 1998; Conforte et al., 2010). According to Glick (2014), the high level of ACC was observed in IAA producing rhizobia, which promote root and shoot elongation, enhanced mineral uptake, and nodulation. It was also observed that

ACC deaminase enzyme producer rhizobia are potent nitrogen fixers. In *Mesorhizobium* sp., the regulation of ACC deaminase gene (*acds*) is under the control of *nif* promoter which also regulates *nif* gene expression for nitrogen fixation (Nascimento et al., 2012). Even though, auxins produced by microorganisms enhance branching in roots, which in turn stimulate uptake of water and nutrients, their higher concentrations can also be inhibitory for root elongation. Its implication can be harmful during conditions of drought, when longer roots are required for extracting water from deeper layers of soil. However, this can be prevented by PGPR that also have ACC deaminase activity along with high auxin producing capability.

On the other hand, in some studies, application of synthetic IAA could not stimulate the effect on root growth as induced by bacterial inoculation, thus showing that no correlation exists between IAA synthesis and the root growth stimulation (Kapulnik et al., 1985; Harari et al., 1988; Bothe et al., 1992). *A. thaliana* mutants (*aux1-7*, *axr4-1*, *eir1*, *etr1*, *ein2*, and *rhid6*), defective in either auxin or ethylene signaling, were found to increase the number of lateral roots and to develop long root hairs when inoculated with *B. megaterium* UMCV1, indicating that plant-growth promotion and root-architectural alterations by *B. megaterium* may involve auxin- and-ethylene independent mechanisms (López-Bucio et al., 2007). In addition, many studies indicated that the total positive effect on the organogenesis of roots and plant growth cannot be governed by IAA biosynthesis alone (Spaepen et al., 2007). According to Cassán et al. (2001), bacterial phytostimulation is critical in the stages of early development (germination and early seedling growth) and leads to other processes that occur later on during interaction between *Azospirillum* and plants. Although, IAA production from *Azospirillum* and other PGPR is well-documented, it is not the only mechanism in bacteria that regulates plant growth.

### Microbial Cytokinins and Root Traits of Plants/Nodulation in Legumes

Cytokinins through regulating cell division and differentiation play important role in development of plant and regularly act in conjunction with other phytohormone. Mostly, the equilibrium between the levels of auxin and cytokinins is considered a major regulator of plant organogenesis and root architecture (Vacheron et al., 2013; Liu et al., 2017; Kudoyarova et al., 2019). In the shoot, cytokinins stimulate proliferation of cells, including the apical and axillary meristem activities. Increased levels of cytokinins in shoots have been linked to yield gain (Kieber and Schaller, 2018). In contrast to their stimulatory effect on shoot, cytokinins have inhibitory effect on root growth, which is due to promotion of cell differentiation in root apical meristem and regulation of root branching. By inhibiting initiation of lateral roots and elongation of primary roots, cytokinins control root architecture, and can also regulate functions of roots wherein, they regulate transport of nutrients and uptake of proteins (Argueso et al., 2009; Werner et al., 2010). Further, in concert with auxins, cytokinins are also responsible for the regulation of vascular development, as cytokinins promote phloem and auxins develop xylem through couple of mutually inhibitory interactions.

Cytokinin production (particularly zeatin) has been reported in many PGPR like *Arthrobacter*, *Azospirillum*, *Bradyrhizobium*, *Bacillus*, *Pseudomonas*, and *Paenibacillus* (Cacciari et al., 1989; Timmusk et al., 1999; de-García Salamone et al., 2001; Perrig et al., 2007; Cassán et al., 2009; Hussain and Hasnain, 2009; Vacheron et al., 2013). Plants that are inoculated with cytokinin producing bacteria show enhanced growth of shoot, with reduced root to shoot ratio (Arkhipova et al., 2007). Additionally, various PGPRs synthesize cytokinins which cause increased production of root exudates by the plant (Ruzzi and Aroca, 2015) and thus in turn increase the PGPR interactions with the plant. The role of auxin and cytokinin in the regulation of *Rhizobium* nitrogen-fixing symbiotic interaction has been documented. In this regard, Gonzalez-Rizzo et al. (2006) used RNA interference approach for specifically targeting different putative cytokinin receptors that affect lateral root development in barrel medic roots and also its symbiotic interaction with *Sinorhizobium meliloti*. The authors observed that RNAi of the cytokinin receptor homolog Cytokinin Response1 (Mt CRE1) led to cytokinin-insensitive roots, which showed an increased number of lateral roots and a strong reduction in nodulation. Further, the development of *S. meliloti* infection and the formation of nodule primordium were also affected. The authors also identified two cytokinin signaling response regulator genes, Mt RR1 and Mt RR4, which were induced in the beginning of the symbiotic interaction. However, in the mutants that were affected in the NoD factor signaling pathway, the induction of these genes was altered by *S. meliloti* infection. This indicated that Mt CRE1 is responsible for cytokinin regulation of the early nodulin gene, *Nodule Inception1* (Mt NIN). The authors thus concluded that Mt NIN, Mt RR1, and Mt RR4 are activated at the beginning of *S. meliloti* interaction, and thus permitting interaction between cytokinins of the plant and Nod factor signals of bacteria. Similarly, highly reduced nodulation capacity was observed in the *lhk1/hit1* (*lotus histidine kinase 1/hyperinfected 1*) mutant of lotus (*L. japonicas*), which affected the closest homolog of cytokine receptor MtCRE1 (Murray et al., 2007). During nodule formation in legumes, the cell division is initiated by cytokinin responsible gene in the cortical cells, which leads to nodule development (Frugier et al., 2008; Plet et al., 2011; Held et al., 2014). Cooper and Long (1994) reported that rhizobia was capable to produce cytokinin in sufficient amount to induce cell division in cortical cells of alfalfa (*Medicago sativa* L.). Similarly, rhizobia enhanced the synthesis of cytokinin in legumes by expression of Nod factor cascade, which is helpful in understanding the coordination among cortical and epidermal cells during nodulation (Oldroyd, 2007). Interaction of *Bradyrhizobium* sp. strain ORS285 with legume plants, *Aeschynomene afraspera* and Indian joint vetch (*A. indica* L.) and displayed nodule formation by production of cytokinin through organism and also change size and numbers of nodules but initiation of nodule formation was also observed in cytokinin deficient mutants (Podlesakova et al., 2013).

Cytokinins regulate differentiation of root meristem, cause proliferation of root hairs, but have inhibitory effect on the formation of lateral roots (Silverman et al., 1998) and elongation of primary roots (Riefler et al., 2006). Primary root growth in *A. thaliana* was inhibited by *Bacillus amyloliquefaciens* UCMB5113,

which can be attributed to the production of cytokinin (Asari et al., 2016). However, inoculation of wheat rhizospheres with zeatin producing *B. subtilis* IB 22 could not cause any reduction in the root biomass, but increased rhizodeposition (Kudoyarova et al., 2014). Arkhipova et al. (2005) observed that after inoculation with rhizobacteria *B. subtilis* IB22, zeatin riboside were detected in roots in the beginning followed by accumulation in shoots and corresponding decline in their concentration in roots. As the cytokinins produced by the bacterium are ribosylated, they get transported out of the roots. Hence, their accumulation was not observed in the roots and the growth of the roots was normal. It can thus be inferred that introduction of microbes that produce cytokinins into the rhizosphere may not essentially cause inhibition of root growth, provided they are migrated to the shoots.

### Role of Microbial ABA and Gibberellins on Root Traits of Plants

Various studies have reported the ability of PGPR to synthesize ABA or gibberellic acid, or to regulate the level of these hormones in plants (Richardson et al., 2009; Dodd et al., 2010). ABA is a well-known phytohormone for its role during conditions of drought stress. The increased ABA levels during water limiting conditions cause closure of stomata and thus help in reducing loss of water (Bauer et al., 2013). Nevertheless, the hormone is also known to act differently during development of lateral roots (De Smet et al., 2006; Dodd et al., 2010). An increase in ABA levels in *A. thaliana* was observed when inoculated with *Azospirillum brasilense* Sp245, particularly under osmotic stress conditions (Cohen et al., 2008).

Gibberellins act in combination with other phytohormones and additional regulatory factors, and stimulate primary root elongation along with lateral root extension (Yaxley et al., 2001). Many PGPR including, *Achromobacter xylosoxidans*, *Acinetobacter calcoaceticus*, *Azospirillum* spp., *Azotobacter* spp., *Bacillus* spp., *Herbaspirillum seropedicae*, *Gluconobacter diazotrophicus*, and rhizobia are known to produce gibberellins (Gutiérrez-Mañero et al., 2001; Bottini et al., 2004; Dodd et al., 2010). In a study, when gibberellic acid at similar levels as those produced by *Azospirillum* was applied on maize was found to promote root growth. Further, the amount of gibberellins also increased in maize roots inoculated with *Azospirillum* strains (Fulchieri et al., 1993). Yanni et al. (2001) noted that indigenous *Rhizobium leguminosarum* bv. *trifoli* C6 could colonize rice roots in the Egyptian Nile delta where *Trifolium alexandrinum* L. is grown in rotation with rice. These IAA and gibberellin producing *Rhizobium* strains promoted root and shoot growth in rice, thereby improving seedling vigor hence resulting in higher grain yield.

The production of phytohormones by PGPR has been well-documented. Inoculation with phytohormone producing PGPR may influence the phytohormone levels in plants and thus resulting in altered growth. However, the role of individual hormone in influencing plant growth is difficult to measure as different phytohormones are co-produced by bacterial strains and they work in combination with other hormones and regulatory factors to produce an effect on plant

system. Development of bacterial mutants lacking the ability to produce individual phytohormone from a strain producing multiple phytohormones can help in deducing the role of each phytohormone. However, the effect may vary with the dose and host plant. In future, transcriptomics can help in deciphering pathways up-regulated or down-regulated due to PGPR inoculation.

## Role of Microbial VOCs on Root Traits of Plants

A number of VOCs and secondary metabolites produced by PGPRs helps in improving tolerance of plants to stress conditions and also aid in stimulation of plant growth by specifically altering root traits. VOCs are thus considered to be effective mediators of chemical crosstalk be it, attracting, repelling or warning signals. Microbes secrete VOCs for a variety of reasons like, crosstalk and protection (Kai and Piechulla, 2009). Plant root system can quickly and efficiently sense the volatiles released by its allied microbes. Ortíz-Castro et al. (2008) demonstrated that plant roots can perceive quorum sensing signals to modulate the root system architecture. Gutiérrez-Luna et al. (2010) using a divided Petri plate assay, observed the positive effect of certain rhizobacteria isolated from lemon (*Citrus limon* L.) on root morphogenesis and biomass production in *A. thaliana* seedlings, indicating the role of VOCs in plant growth modulation. The response of the plant to VOCs via alteration of root architecture may be of ecological significance for increasing colonization of roots and strengthening of beneficial mutual interactions between plants and their coupled bacteria (Gutiérrez-Luna et al., 2010). These chemical cross talks/communications might regulate how plant will perform in various agricultural soils (Camarena-Pozos et al., 2018). The effect is achieved by affecting root growth and improving availability of nutrients in the rhizosphere along with other beneficial contributions w.r.t. plant immunity by the microorganisms (Ortíz-Castro et al., 2009). Inoculation of *A. thaliana* with *Bacillus megaterium* UMCV1 resulted in altered root-system architecture with inhibition of primary-root growth and an increase in number and length of lateral roots. The solid-phase microextraction coupled to a gas chromatography—mass spectrometry (SPME-GC-MS) based analysis identified acetoin in the volatiles produced by UMCV1 (López-Bucio et al., 2007). VOCs (aldehydes, ketones and 1-butanol) released by some *Bacillus* isolates (L254, L263, L265a, L265b, L266, L270, L271, and L272a) resulted in modulation of root-system architecture in *A. thaliana* plants, consequently resulting in enhanced biomass (Gutiérrez-Luna et al., 2010). Farag et al. (2006) also reported 1-butanol among the VOCs produced by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a by using SPME-GC-MS technique. Ryu et al. (2003) reported the stimulatory effect of acetoin or 2,3-butanediol producing *Bacillus* strains on *A. thaliana* growth. The volatiles of *B. subtilis* GB03 stimulated plant growth via signaling of cytokinin and ethylene, whereas, VOCs of *B. amyloliquefaciens* IN937a exerted their effect independently without the involvement of cytokinin and ethylene (Ryu et al., 2003; Farag et al., 2006), indicating the role of different VOCs in phytostimulation. Gutiérrez-Luna et al.

(2010) detected butyrolactone in the VOCs produced by *Bacillus* sp. strain L265a. Butyrolactone as an autoinducer of bacterial quorum-sensing (QS) signaling has also been reported (Polkade et al., 2016).

Tobacco (*Nicotiana attenuata* L.) plants cultivated under sulfur-limiting conditions showed enhanced growth under the influence of dimethyl disulfide produced by *Bacillus* sp. strain BG55. The observed effect was partially attributed to absorption and assimilation of dimethyl sulphoxide (Meldau et al., 2013). Cordovez et al. (2018) demonstrated that VOCs from *Microbacterium* sp. strain EC8 caused an increase in root biomass and lateral root density in *A. thaliana* plants. The authors also reported similar effects in other crop plants like lettuce (*Lactuca sativa* L.) and tomato. Further, enrichment of sulfur-containing compounds like dimethyl disulfide and dimethyl trisulfide, compounds found usually from bacteria, and also rarer compounds, such as S-methyl 2-methylpropanethioate and S-methyl pentanethioate was also observed in the headspace of EC8. Genome-wide transcriptome analyses further identified 1,361 (698 upregulated and 663 downregulated) differentially expressed genes (DEGs) in root tissues induced by microbial VOCs. In root tissues, DEGs involved in sulfur metabolism were mostly downregulated, DEGs involved in nitrate assimilation were upregulated, whereas DEGs involved in nitrate reduction were downregulated. While, upregulated expression of genes encoding three nitrate transporters (NRT2.1, NRT2.6, and NRT2.7) and a chlorine channel (CLC-A) proteins was observed. Other genes involved in nitrate-related processes, like *NIA1* and *NIA2*, encoding nitrate reductases, were downregulated. It has been reported that nitrate is not a mere nutrient for plants but also acts as a signal for regulating metabolism of carbon and nitrogen (Scheible et al., 1997). The transporter gene of nitrogen, *NTR2.1*, displayed an upregulation of 14-folds in *A. thaliana* when exposed to VOCs from EC8, is reported to be regulated by nitrate and act as a negative regulator of lateral root initiation under high-sucrose and low-nitrate conditions, whereas *NRT2.6* is reported to be a part of growth stimulation of *A. thaliana* by the rhizobacterium *Phyllobacterium brassicacearum* STM196 (Little et al., 2005; Kechid et al., 2013). The transporter gene *NRT2.6*, along with *NRT2.5*, was upregulated in the leaves of *A. thaliana* that were inoculated with the bacteria, thereby inferring that these genes may be a part of the regulation of nitrogen control for root development (Kechid et al., 2013). The identification of the bioactive volatiles produced by PGPR and characterization of their ecological functions can open up new avenues for improving crop productivity in a sustainable way.

## Microbial Nitric Oxide (NO) in Plant Signaling

Nitric oxide is a small, diffusible, lipophilic, volatile free radical and a ubiquitous bioactive molecule, which participates in a broad spectrum of metabolic, signaling, defense, and developmental pathways (Lamattina et al., 2003). The NO has the potential to operate simultaneously on a number of discrete biochemical nodes via modulation of cell redox status and  $\text{Ca}^{2+}$  cytosolic concentrations. This unique property makes NO



a molecule with remarkable signaling as well as homeostatic characteristics for the synchronization and organization of cellular metabolism (Lamattina et al., 2003). Many studies suggest extensive linkage between NO and plant hormones (Molina-Favero et al., 2008).

Rhizobacteria can produce NO by different pathways (Molina-Favero et al., 2008; Arasimowicz-Jelonek and Floryszak-Wieczorek, 2014). Bacterial nitric oxide synthase (bNOS) can synthesize NO by oxidation of L-arginine to L-citrulline, in presence of oxygen (Stuehr, 1997). The other major pathway for NO synthesis in free bacteria is through the process of anaerobic denitrification. The established denitrification pathway is a multistep process that reduces nitrate ( $\text{NO}_3^-$ ) to nitrogen  $\text{N}_2$  via activities of nitrate reductase (NR), nitrite reductase (NiR), NO reductase (NoR), and  $\text{N}_2\text{O}$  reductase (Meilhoc et al., 2011). Nitric oxide is synthesized as a free intermediate by the enzyme nitrite reductase. In addition, denitrification can also occur under aerobic conditions. The process involves a periplasmic nitrate reductase (Nap) rather than the classical membrane bound respiratory nitrate reductase (Nar) found in anaerobic denitrification. Steenhoudt et al. (2001) identified a Nap in *Azospirillum brasilense* Sp245 which is not sensitive to oxygen. Another pathway for bacterial NO production is heterotrophic nitrification which causes sequential oxidation of ammonia to hydroxylamine ( $\text{NH}_2\text{OH}$ ),  $\text{NO}_2^-$ , and  $\text{NO}_3^-$ , with NO being produced as an intermediate during the reduction of  $\text{NO}_2^-$  to  $\text{N}_2$  (Wrage et al., 2001). Pothier et al. (2007) reported that *nirK* gene in *A. brasilense* Sp245 encodes a NO-producing nitrite reductase copper-containing enzyme. Further, the expression of *nirK* was induced by wheat seed or root extracts (Pothier et al., 2007).

Bloom et al. (2003) have very well-reviewed various molecules and signals which are involved in development of roots. Amongst them, nitrogen species as NO, ammonium and nitrate are evidently occupied in growth and development of roots. NO production by the microbial partner in the legume–*Rhizobium* symbiosis has been reported by Horchani et al. (2011). Various approaches have also shown that denitrification is responsible for generation of NO in bacteroids, especially during hypoxic conditions (Meakin et al., 2007; Sanchez et al., 2010). In addition, Horchani et al. (2011) also demonstrated that around one-third of the NO generated by *Medicago truncatula*–*Si. meliloti* nodules is synthesized by the rhizobial denitrification pathway. Gouvêa et al. (1997) indicated that application of NO donors like sodium nitroprusside (SNP) and nitrosoglutathione stimulated growth and elongation in maize roots, thus postulating the intermediary role of NO in IAA-induced root elongation. Pagnussat et al. (2002, 2003) showed that NO acts as a signal molecule in the IAA-induced signaling pathway, and thus causing development of adventitious roots in cucumber (*Cucumis sativus* L.). The authors also noted that application of two NO donors, SNP and S-nitroso, N-acetyl penicillamine (SNAP) to hypocotyl cuttings of cucumber could induce *de novo* root-organogenesis, whereas application of NO scavengers, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) with SNP or SNAP blocked the effect. Microscopic observation revealed similar anatomical structure in NO- and IAA- induced roots.

Moreover, the hypocotyls treated with SNP or SNAP along with IAA showed enhanced response and displayed better root organogenesis as compared to the hypocotyls treated with NO donors or IAA alone (Pagnussat et al., 2002). Pagnussat et al. (2003) strongly indicated the role of endogenous NO as a downstream component in the IAA-mediated root organogenesis. Correa-Aragunde et al. (2004) reported that NO has a major role in lateral root formation (LRF) in tomato. Molina-Favero et al. (2008) analyzed the aerobic NO production in *A. brasilense* Sp245 and its mutants Faj009 (IAA attenuated) and Faj164 (lacking periplasmic nitrate reductase activity) and correlated it with root-organogenesis in tomato. The strains Sp245 and Faj009 could produce 120 nmols of NO per g bacteria, whereas, strain Faj164 could produce only 5.6 nmols of NO per g bacteria, indicating aerobic denitrification as major source of NO in bacteria. Inoculating tomato seedlings with Sp245 and Faj009 could promote both lateral and adventitious roots formation, whereas no such effect was observed with Faj164 inoculation, indicating the role of NO in *Azospirillum*-induced root branching, without any regards to the potential of bacteria to synthesize IAA. Creus et al. (2005) investigated the ability of aerobically grown *Azospirillum brasilense* Sp245 to synthesize NO, using NO specific fluorescent probe 4, 5-diaminofluorescein diacetate (DAF-2 DA), followed by epifluorescence microscopy. Quantification of NO was done by electron paramagnetic resonance (EPR). The production of green fluorescence indicated the production of NO which was drastically reduced in presence of specific NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO). They further studied the role of NO as the inducer molecule in the *Azospirillum*-mediated stimulation of the tomato roots by incubating *Azospirillum*-inoculated and uninoculated tomato roots with DAF-2 DA followed by fluorescence microscopy. The *Azospirillum* inoculated roots exhibiting induction of lateral root formation also showed high fluorescence intensity majorly in the vascular tissues and sub-epidermal cells of roots. Treatment with cPTIO completely blocked the *Azospirillum*-mediated induction of lateral root formation (LRF), while addition of NO donor sodium nitroprusside partly reversed the inhibitory effect of cPTIO. These results strongly indicated the role of NO in the *Azospirillum*-mediated root growth promotion in tomato seedlings. Other studies have attributed the ion nitrite produced by *Azospirillum* metabolism to be accountable for the observed effects on the plant growth and other developmental processes on inoculation of roots (Didonet and Magalhaes, 1993).

The auxin competitive inhibitor, p-chlorophenoxyisobutyric acid (PCIB) was found to reduce the stimulating effect of *Azospirillum brasilense* Sp245 on LRF, indicating the involvement of auxins in the *Azospirillum*-induced effects (Creus et al., 2005), possibly causing an increase in the levels of NO as reported previously also (Pagnussat et al., 2002). In all together, these findings very well-support the hypothesis that NO is necessary for the *Azospirillum*-promoted LRF and both auxins and NO, act as cellular messengers that are involved in the close communication between *Azospirillum* and plant root leading to LRF. The increased build-up of NO in the inoculated plants can also be a result of some events rather than production of NO by



*Azospirillum* itself, viz. (i) induced reduction of nitrite into NO in inoculated plant roots (Stohr and Ullrich, 2002); (ii) acidification of the root apoplast by *Azospirillum* which eventually induce a non-enzymatic production of NO in plant roots (Bethke et al., 2004). As majority of the studies indicate, effect on the root traits of plants can also result from the indirect stimulation of the plant auxin pathway by microbially produced NO in the rhizosphere.

## CONTRIBUTIONS OF PGPR TO AGROECOSYSTEM FUNCTIONING

Colonization of plant roots with bacteria is mutually beneficial for plant and bacteria. Plants secrete fixed carbon by root exudation and feed the rhizosphere bacteria and in turn bacteria help the plant through exhibiting an array of growth promoting traits. Apart from direct and indirect PGP traits, PGPR mediated altered root traits also contribute to agroecosystem functioning and plant productivity as discussed in the following sub-sections.

### PGPR and Soil Structure

Root adhering soil play important role in uptake of nutrients and water for plant growth. Root traits play key role in shaping the physical structure of soils (Leifheit et al., 2013). Morphological root traits, such as root length density and root diameter, strongly influence soil stability because denser, finer root systems bind soil more effectively than do coarse root systems (Loades et al., 2010). As the diameter of larger root increases with growth, the soil particles adjacent to the root get pushed aside thus increasing the soil density. Finer roots on the other hand increase the soil porosity by improving the soil aggregation (Gyssels et al., 2005). Architectural root traits such as branching of roots helps in increasing the anchorage by fixing the soil, hence increasing soil stability and protection against erosion (Bardgett et al., 2014). PGPR strains able to enhance root morphological, physiological, and architectural characteristics thus can help in improving soil structure and stability. However, focussed studies need to be conducted to have direct evidence on PGPR mediated improvement of soil structure mediated through root traits.

Root exudation is another root trait that has strong influence on soil aggregation and stability, as the polysaccharides (mucilaginous substances) and proteins present in the root exudates act like glue, that bind clay and mineral particles together to form soil aggregates (Czarnes et al., 2000; Whalley et al., 2005). PGPR inoculation has been found to enhance rhizodeposition by the host plant (Kudoyarova et al., 2014), that provide nutritional niche for the rhizosphere microbial communities. However, not much information is available on how the PGPR mediated alteration in quality and quantity of rhizodeposits influences soil structure. Nonetheless, many of the rhizosphere microorganisms produce exopolysaccharides (EPS) for attachment to other cells, soil particles and root surfaces. EPS bind with the soil particle to form aggregates and stabilize the soil structure. The improved structure increases cation exchange and water holding capacity of the soil (Upadhyay et al., 2011). EPS producing *Rhizobium* spp. strain YAS34 significantly increased the root adhering soil (RAS) aggregation

and soil macropore volume. The inoculation also improved root diameter and overall plant growth of sunflower (*Helianthus annuus* L.) (Alami et al., 2000). This significant increase in RAS mass around the roots of inoculated plants could be the result of either an increase in soil adhesion to roots or a higher soil aggregate stability around roots, or both (Alami et al., 2000). The improvement in soil aggregation due to microbial inoculation may be attributed to microbial EPS. Seed inoculation of sunflower with an EPS producing *Pseudomonas putida* strain GAP-P45 resulted in increased root adhering soil/root tissue ratio, increased the stable soil aggregates percentage and also improved plant biomass under drought stress (Sandhya et al., 2009). Two salt-tolerant strains *Halomonas variabilis* HT1 and *Planococcus rifietoensis* RT4 able to form biofilm and accumulate EPS, improved chickpea (*Cicer arietinum* Var. CM-98) plant growth and soil aggregation under salinity stress (Qurashi and Sabri, 2012). Similar effects on RAS improvement have been observed in wheat under *P. polymyxa* CF43 inoculation (Gouzou et al., 1993). Mutation in *sacB* gene (encoding levansucrase for synthesis of levan) of *P. polymyxa* impaired its ability to cause soil aggregation, thus implicating the role of levan produced by *P. polymyxa* in the aggregation of root-adhering soil in wheat. EPS producing *Bacillus amyloliquefaciens* p16 improved soil aggregation and soil water retention (Deka et al., 2018). Inoculation of EPS producing *Pantoea agglomerans* strain NAS206 in wheat increased aggregation and stabilization of root-adhering soil, as indicated by significant increase in aggregate mean weight diameter, aggregate macro-porosity, RAS/RT ratio, and water stable aggregates (Amellal et al., 1998). Similarly, inoculation with EPS producing rhizobacteria (*Planomicrobium chinense* P1, *Bacillus cereus* P2, and *Pseudomonas fluorescens* P3) in wheat significantly enhanced the water holding capacity of sandy soil and improved soil aggregation around the plant roots (Khan et al., 2016). Under field conditions, inoculation with EPS producing *Pseudomonas mendocina* Palleroni along with *Glomus intraradices*, an arbuscular mycorrhizal fungus onto lettuce showed stabilization of soil aggregation (Kohler et al., 2006).

PGPR can contribute toward soil structure and stability through EPS production, influencing root exudation and other root traits. However, intricacies of the mechanisms need to be explored through focussed studies on these aspects.

### PGPR and Carbon Sequestration

Soil is incorporating 6,000 billion tons of carbon approximately and acts as natural reservoirs of biogeochemical carbon cycle. More carbon is getting emitted into the atmosphere due to industrial development (Cristea et al., 2020). Microorganisms can sequester carbon into the soils and is sustainable method to lowering atmospheric carbon. Selection of suitable microbial inoculant is important to improve agriculture land capability to sequester and store carbon (Ahmed et al., 2019). Microbial consortia of (*Pseudomonas protegens*, *Bacillus paramycoides* and *Bacillus paramycoides*) when applied in tall perennial aromatic grass species including vetiver (*Vetiveria zizanioides*), lemongrass (*Cymbopogon citratus*), palmarosa (*Cymbopogon martinii*), and citronella (*Cymbopogon winterianus*) improved the carbon

sequestration (Maddhesiya et al., 2020). High C: N ratio of soil treated with PGPR highlight the significant role of inoculants in enhancing carbon sequestration. Co-inoculation of rhizobacteria and cyanobacteria significantly increased the grain yield of rice and showed positive influence on carbon sequestration in soil (Prasanna et al., 2011). Under elevated CO<sub>2</sub> soil microbe *Pseudomonas fluorescens* increased the capacity to store carbon and nitrogen in plant (Nie et al., 2015). Combined application of rice husk biochar (RHB) along with PGPR (*Pseudomonas* species, *Azotobacter chroococcum*, and *Azospirillum brasilense*) resulted in significantly higher rice yield, nutrient uptake, and also increased organic content of soil (Singh et al., 2017). Glomalin-Related Soil Protein (GRSP) improves the soil quality, C and N storage. Cell walls of hyphal Arbuscular mycorrhizal fungi (AMF) produce glomalin and, death of hyphae deposited in the soil, further incorporated into soil organic matter (SOM) pool. Interaction of AMF and PGPR improved the glomalin production in rhizosphere of pea (*Pisum sativum* L.) and further enhanced C and N storage in rhizosphere (Walley et al., 2014). In boreal forest ecosystems root-associated fungi acts as organic decomposers and belowground mediators for C transport and respiration (Clemmensen et al., 2013).

## PGPR in Water and Nutrient Acquisition

For efficient nutrition in plants, the acquisition of nutrients by the roots plays the most important role (Gutschick, 1993). Efficiency in acquisition depends on root size and morphology, physiology and biochemistry. Root exudation influence the soil nutrient cycling by exuding compounds rich in organic acids that increase availability of nutrients by desorption and solubilization from mineral surfaces and by priming the rhizospheric microbial communities for enhanced mineralization of organic matter resulting in increased nutrient availability in the soil (Bardgett et al., 2014). However, priming of microbial growth by root exudates can also increase immobilization of nutrients, thereby reducing their availability to the plants (Bengtson et al., 2012). Thus a fine balance between mineralization and immobilization dynamics is probably controlled by variations in the quality of root exudates, the extent of nutrient limitation for microbial growth (Dijkstra et al., 2013; Drake et al., 2013), and the response of specific groups of microbes involved in nutrient transformations (Bremer et al., 2007). The relationships between root traits and nutrient cycling have been studied in the field and mixed results have been reported.

PGPR may improve the plant nutrition by affecting the plant nutrient uptake and/or the plant growth rate (Mantelin and Touraine, 2004). It is generally considered that increase in root surface area triggered by PGPR, results in enhanced nutrient uptake (Vacheron et al., 2013). However, the activity of root ion transporters can be regulated according to the nutritional demand of the plant (Lappartient et al., 1999). Hence, PGPR must interfere with the development and nutrition pathways of the host plant to modulate both nutrient acquisition and growth (Vacheron et al., 2013). Maize cultivar Seiddi inoculated with *A. lipoferum* CRT1 exhibited increased lateral

root growth and enhanced photosynthetic potential unlike non-responsive cultivar FuturiXX (Rozier, 2016). GC-MS based profiling of small organic substances in the ascending xylem sap of plantlets revealed reduced content of 17 substances, including primary metabolites, such as glucose, maltose, sucrose, TCA cycle intermediates, GABA, amino acids and shikimate pathway metabolites in both the cultivars after *A. lipoferum* CRT1 inoculation. Whereas, the content of 28 substances, including glucose, lactic acid, acidic intermediates of the pentose phosphate and ascorbate/aldarate pathways and defense-related hydroxycinnamic acids, decreased in the xylem sap of the *A. lipoferum*-phytostimulated plantlets of responsive cultivar (Seiddi) only, suggesting the role of xylem-transported metabolic signaling in *A. lipoferum* induced phytostimulation of maize. Glucose or other metabolites that are retrograde transported through the xylem to the shoot by transpirational pull may act as feedback signals of the root status. Such signals may stimulate leaves to enhance photosynthesis-mediated C-assimilation that is needed to sustain *A. lipoferum*-triggered root growth (Rozier, 2016).

Li et al. (2020) reported improved nutrient acquisition of N, P, Ca<sup>2+</sup> and K<sup>+</sup> in maize by novel PGPR strain *Kocuria rhizophila* Y1. Inoculation of consortium of *Bacillus megaterium* BHU1, *Arthrobacter chlorophenolicus* BHU3, and *Enterobacter* sp. BHU5 significantly improved Cu, Zn, Mn, and Fe nutrients in wheat (Kumar et al., 2014). According to Sabir et al. (2012) root inoculation with PGPR strains *Azospirillum brasilense* Sp245 and *Bacillus subtilis* OSU-142 enhanced growth and nutrient acquisition (N, P, K, Ca, and Mg) in grapevine rootstocks (*Vitis* spp.). Inoculation of soybean with a consortium of *Pseudomonas aeruginosa* LSE-2 and *Bradyrhizobium* sp. LSBR-3 exhibiting multiple PGP traits (IAA, P solubilization, ACC deaminase, biofilm formation) enhanced root dry weight, nodule number, dry weight, leghaemoglobin content, and nutrient (N, P, K) acquisition (Kumawat et al., 2019) adding toward overall plant growth and yield. Safflower (*Carthamus tinctorius* L.) inoculation with *Bacillus* strains (*B. subtilis* OSU-142 and *B. megaterium* M3) enhanced nutrient acquisition and improved seed and oil yield under semi-arid condition (Ekin, 2020). Treatment with rhizobacteria (*Pseudomonas* sp. R185) and endophytic bacteria (*Pseudomonas mosselii* E240) had a positive effect on P, Zn, and Fe contents in wheat grains in phosphorous deficient soils (Emami et al., 2018). Rana et al. (2012) reported 2-fold enhancement in P and 66.7% increase in the N content in leaves of wheat treated with a combination of *Bacillus* sp. AW1 and *Providentia* sp. AW5. They also observed significant correlation between plant biomass, grain weight, N, P, and Fe contents with acetylene reduction activity of rhizosphere soil, indicating the role of N fixation in crop productivity.

PGPR can directly increase bioavailability of nutrients through nitrogen fixation and nutrient mobilization of key nutrients (iron, phosphorous, and potassium) to crop plants (Rashid et al., 2016). Phosphate solubilization is an important effect of PGPR on plant nutrition. Although, the soils generally have high levels of phosphorus, most of the soil P exists in fixed forms and only a small proportion is available for plants.

Soil microorganisms play important role in the solubilization and mineralization of organic or insoluble forms of P into plant available forms (Richardson et al., 2009; Ramaekers et al., 2010) through the release of organic acids and hydrolytic enzymes (phosphatases and phytases) (Richardson and Simpson, 2011).

Plant phosphate use efficiency (PUE) is generally correlated with P acquisition efficiency and with root length and architectural traits (Ruiz et al., 2019). Root hairs can increase the effective surface area of plant roots and help the roots to exploit non-accessible stocks of P through accessing the finer pores than the main root axis can enter, thus length and density of root hairs play important role in nutrient acquisition (Ma et al., 2001). PGPR able to influence the root traits including root length, lateral branching, and root hair length and density, thus can help in improving plant nutrition acquisition capacity.

Biological nitrogen fixation is another important trait of many PGPR that contributes to fulfill the nutritional demands of the plant. Nitrogen fixing bacteria possess the nitrogenase enzyme that converts atmospheric elemental dinitrogen into ammonia. Nitrogen fixing PGPR can be symbiotic, associative or free living. Symbiotic nitrogen fixers like rhizobia and *Fankia* form nodules on the plants root by modifying the root architecture and can benefit the plant by directly supplying the fixed form of N in the root nodules (Brock, 2000). *Rhizobium*-legume symbiosis is most studied for its nitrogen contributions. Rhizobia can fix  $180 \times 10^6$  tons of nitrogen annually through BNF process at global level (Sahgal and Johri, 2003) which causes efficient increment in the productivity and quality of crops (Herridge et al., 2008; Krapp et al., 2011).

Associative nitrogen fixers like *Azospirillum* are known for their effect on root architecture and associative nitrogen fixation in many crops (Bashan et al., 2004). The important free living and associative nitrogen fixing genera include *Azospirillum*, *Azotobacter*, *Gluconacetobacter*, *Bacillus*, *Burkholderia*, *Clostridia*, *Kelbsiella*, *Mycobacterium*, and *Pseudomonas*.

With increasing attention on the genetic, molecular and physiological regulation for root architecture as related to plant nutrient efficiency, a number of genes and regulators have been identified and demonstrated to participate in regulatory networks linking root architecture to nutrient efficiency in different crops including *Arabidopsis*, maize, rice, soybean (Li X. et al., 2016). The expression studies of these genes and regulators under the influence of PGPR inoculation can help in understanding the regulatory role of PGPR in nutrient acquisition.

## PGPR and Abiotic Stress Tolerance in Plants

Abiotic stresses include excessive or deficient water, high or low temperature, high salinity, heavy metals, and ultraviolet radiation, are malicious for growth and development of plant, lead to crop yield losses (He et al., 2018). PGPR plays defensive role against abiotic stresses (Kaushal and Wani, 2016). While it is difficult to elucidate the explicit mechanism by which PGPR act to enhance drought tolerance by affecting root traits of the plant, in certain cases it has been demonstrated. For example, Sandhya et al. (2009) reported production of

extracellular matrices by PGPR for the maintenance of a hydrated root environment and thus, increasing root-adhering soil and stability. In drought stressed *A. thaliana* plants, inoculation with spermidine producing *Bacillus megaterium* BOFC15 exhibited reduction in reactive oxygen species, upregulation of ABA biosynthesis and response genes, and augmented photosynthesis and root system architecture (Zhou et al., 2016). In a study, thuricin produced by *Bacillus thuringiensis* NEB17 was applied to soybean (*Glycine max*) under drought conditions and it caused modification in root structures and increase in root length and biomass, nodule biomass, ABA content, and total nitrogen content (Prudent et al., 2015). IAA producing strains *Pseudomonas putida*, *Pseudomonas* sp., and *Bacillus megaterium* could stimulate shoot, root biomass and water content in white clover plants (*Trifolium repens*) under drought condition. *Bacillus megaterium* was found to be more effective when applied along with arbuscular mycorrhizal fungi against drought (Marulanda et al., 2009). Limited water availability results in low rate of photosynthesis and insufficient nutrient uptake. In wheat, inoculation of phosphorus-solubilizing and drought-tolerant PGPR *Pseudomonas libanensis* EU-LWNA-33 improved plant growth and phosphorus uptake under water deficient condition (Kour et al., 2019). Inoculation with *Bacillus* sp. 12D6 and *Enterobacter* sp. 16i delayed onset of drought symptoms in wheat and maize seedlings. Inoculation of wheat with *Bacillus* sp. (12D6) increased the root length, whereas, in maize inoculation with both *Bacillus* sp. strain 12D6 and *Enterobacter* sp. strain 16i increased the root length and surface area (Jochum et al., 2019). Seed treatment with endophytic bacteria *Bacillus subtilis* MSEB78 and *Corynebacterium hansenii* MSEB3 improved chlorophyll content, relative water content, shoot fresh and dry biomass under drought stress (Bodhankar et al., 2019).

Among the various PGP traits implicated in conferring drought tolerance, synthesis of enzyme ACC deaminase is most studied. Through production of ACC deaminase, PGPR maintain the level of plant stress hormone ethylene below inhibitory levels, maintaining normal root growth by diverting the excess of auxins and delaying senescence under drought (Marasco et al., 2013). ACC deaminase producing PGPR strains *Ochrobactrum* sp. SB2.ACC2, *Bacillus* sp. SB1.ACC3, and *Alcaligenes* sp. SB1.ACC2 reduced ethylene production and promoted the plant growth by inducing salinity tolerance in rice (Bal et al., 2012). Inoculation of Jerusalem artichoke (*Helianthus tuberosus* L.) with IAA and ACC deaminase producing *Bacillus aquimaris* strain 3.13 and *Micrococcus luteus* strain 4.43 drought conditions increased shoot height and weight, length, diameter, volume, area of root ultimately improving harvest index (Namwongsa et al., 2019). Similarly, Rice (*Oryza sativa*) seedlings inoculated with IAA and ACC deaminase producing *Pseudomonas fluorescens* strain REN, exhibited enhanced root length under flooding (Etesami and Alikhani, 2016). The ability of PGPR to produce both auxins and ACC deaminase becomes particularly important under stress conditions (Glick, 2014). Phytohormones mediated increase in root length, root surface area, and the number of root tips, causing increased uptake of water and nutrients, represents a major contributor for the improvement of survival chances of the plant under



water limiting conditions (Glick, 2011; Marasco et al., 2013). ACC deaminase producing *Pseudomonas lini* DT6 and *Serratia plymuthica* DT8 mitigated drought stress and promoted the growth of jujube plant by regulating ABA and IAA (Zhang et al., 2020). ACC deaminase producing rhizobacteria *Enterobacter cloacae* and *Achromobacter xylosoxidans* amended with biochar improved the maize growth and productivity under drought stress (Danish et al., 2020). Root colonization of wheat plants with IAA and ACC deaminase producing *Stenotrophomonas maltophilia* SBP-9 improved root, shoot length and biomass, chlorophyll content under salinity stress. Inoculation decreased level of proline and malondialdehyde (MDA), and increased activities of antioxidant enzymes, superoxide dismutase (SOD), catalase (CAT), peroxidase (POX) (Singh and Jha, 2017). The PGPR exhibit enzymatic and non-enzymatic antioxidant responses to scavenge the toxic compounds like reactive oxygen species produced under stress conditions. In Okra (*Abelmoschus esculentus*) combined inoculation with *Bacillus megaterium* UPMR2 and *Enterobacter* sp. UPMR18 improved germination percentage, plant growth parameters (shoot fresh weight and root dry weight) and adapted to salinity stress by eliminating reactive oxygen species (ROS) through SOD (superoxide dismutase), CAT (catalase) and APX (Ascorbate peroxidase) activities (Habib et al., 2016). PGPR inoculation increased proline accumulation in soybean, lettuce, wheat, maize plants under abiotic stress conditions (Han and Lee, 2005; Kohler et al., 2009; Zarea et al., 2012; Bodhankar et al., 2019). Salt tolerant PGPR *Bacillus aquimaris* SU8 accumulated total soluble sugars, improved growth and yield of wheat under saline soil (Upadhyay and Singh, 2014). Priming of wheat seeds with *Bacillus safensis* (NCBI JX660689) and *Ochrobactrum pseudogrignonense* (NCBI JX660688) improved temperature stress tolerance of wheat seedlings by lowering ROS levels, increasing chlorophyll content and accumulating osmolytes (Sarkar et al., 2018).

Role of VOCs released by PGPR have been implicated in stress tolerance. VOCs produced by *Bacillus subtilis* GB03 stimulated choline synthesis in *A. thaliana* that helped in maintaining cell turgor through preventing the water loss (Zhang et al., 2010). Moreover, *Pseudomonas chlororaphis* O6 induced stomata closure in the colonized plants, by producing VOC, 2R, 3R-butenediol (Cho et al., 2011). In mungbean [*Vigna radiata* (L.) R. Wilczek] inoculation with *Pseudomonas aeruginosa* GGRJ21 strain elicited drought tolerance as indicated from better plant growth, osmolyte accumulation, antioxidant enzyme status, and up-regulation of drought responsive genes i.e., dehydrin (DHN), catalase (CAT1), and dehydration-responsive element binding protein (DREB2A) (Sarma and Saikia, 2013). Sheibani-Tezerji et al. (2015) reported up-regulation of genes involved in detoxification of reactive oxygen species (ROS) in the drought-exposed potato (*Solanum tuberosum*) plants colonized with root endophytic bacteria *Burkholderia phytofirmans* PsJN. Similarly, *Pseudomonas putida* MTCC5279 inoculation improved drought stress response of chick pea as indicated from gene expression (stress responsive genes), membrane stability, water status, plant growth, antioxidant enzymes (Tiwari et al., 2016). Treatment of wheat seedlings with *Bacillus velezensis* 5113 exposed to cold/freezing, heat or drought stress, improved

the plant survival under stress conditions. Protein profile of wheat leaves showed differential expression of several proteins with cold stress showing strong impact on protein profile than heat and drought (Abd El-Daim et al., 2019). PGPR also help the plant under stress conditions by nutrient cycling through diazotrophy, phosphorus solubilization, and siderophore synthesis, by enhancing photosynthesis, increasing fine root production and overall root surface area and reducing the stress ethylene emission, thus contributing toward better plant performance (Casanovas et al., 2002; Timmusk et al., 2014; Gagné-Bourque et al., 2016). Thus, PGPR through various direct and indirect mechanisms (Figure 3) can enhance plant's tolerance to abiotic stress, hence contributing toward sustainable production.

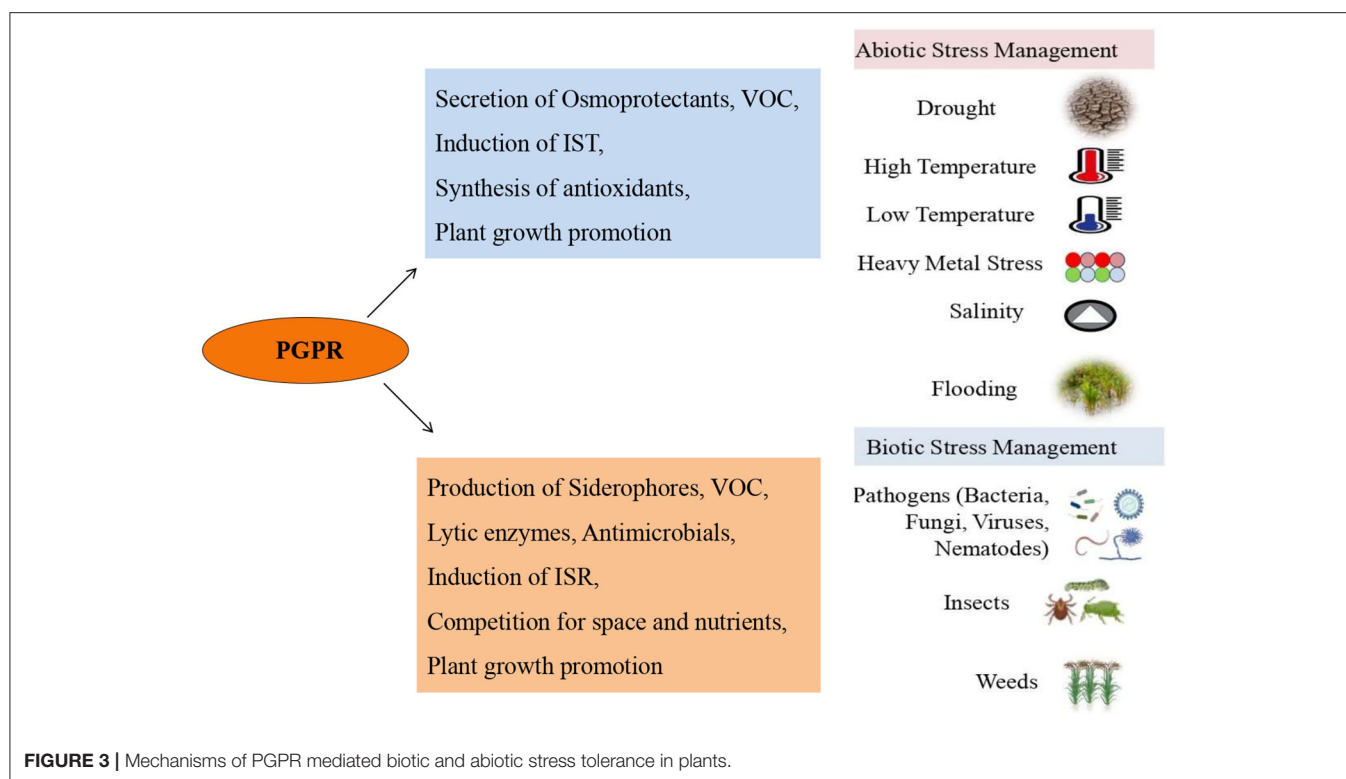
## PGPR and Biotic Stress Tolerance in Plants

Use of chemical inputs to control phytopathogens poses a serious threat to soil fertility, crop productivity, and nutritional value of farm produce. Application of PGPR can help in mitigating biotic stresses and thus improving crop yield in a sustainable way (Sayyed, 2019). The antagonistic relationship between microbes and plant pathogens may be complex. It may involve the competition for nutrient and space, production of antimicrobial compounds or triggering of the host defensive response (Figure 3). PGPR eliciting ISR may modulate defense responses of the plant to their own benefit (Zamioudis and Pieterse, 2012). In the absence of pathogens ISR poses slight fitness costs on the plant, however, the benefits of ISR greatly outweigh the costs when the plant is attacked by pathogens. The cross communication between the plant and the ISR-eliciting PGPR, reduce the susceptibility of the plant to pathogen attack (Choudhary et al., 2007; Bakker et al., 2013).

Many PGPR especially *Pseudomonas* and *Bacillus* species have been well-reported to play important roles in plant growth promotion through the biocontrol of a broad range of plant pathogens, by eliciting ISR, production of antimicrobial compounds (lipopeptides, antibiotics, and enzymes) and acting as competitors for growth factors (space and nutrients) with other pathogenic microorganisms through colonization (Pal et al., 2001; Haas and Defago, 2005; Weller, 2007; Loganathan et al., 2014; Singh et al., 2014; Bodhankar et al., 2017; Shafi et al., 2017; Hashem et al., 2019; Wang et al., 2019). Certain PGPR like *Azospirillum* spp. are reported to reduce the damage caused by plant pathogens through indirect mechanisms like competition and displacement of pathogens, general enhancement of plant health to resist pathogen attack, and possible inhibition of fungal growth via the production of toxic substances (Bashan and de-Bashan, 2010). The role of *Rhizobium* spp. in disease management has been associated with production of lytic enzymes, antimicrobial metabolites and ISR. Also, rhizobial mediated plant growth promotion and/or symbiotic efficiency also contribute toward disease suppression by the host plant (Volpiano et al., 2019).

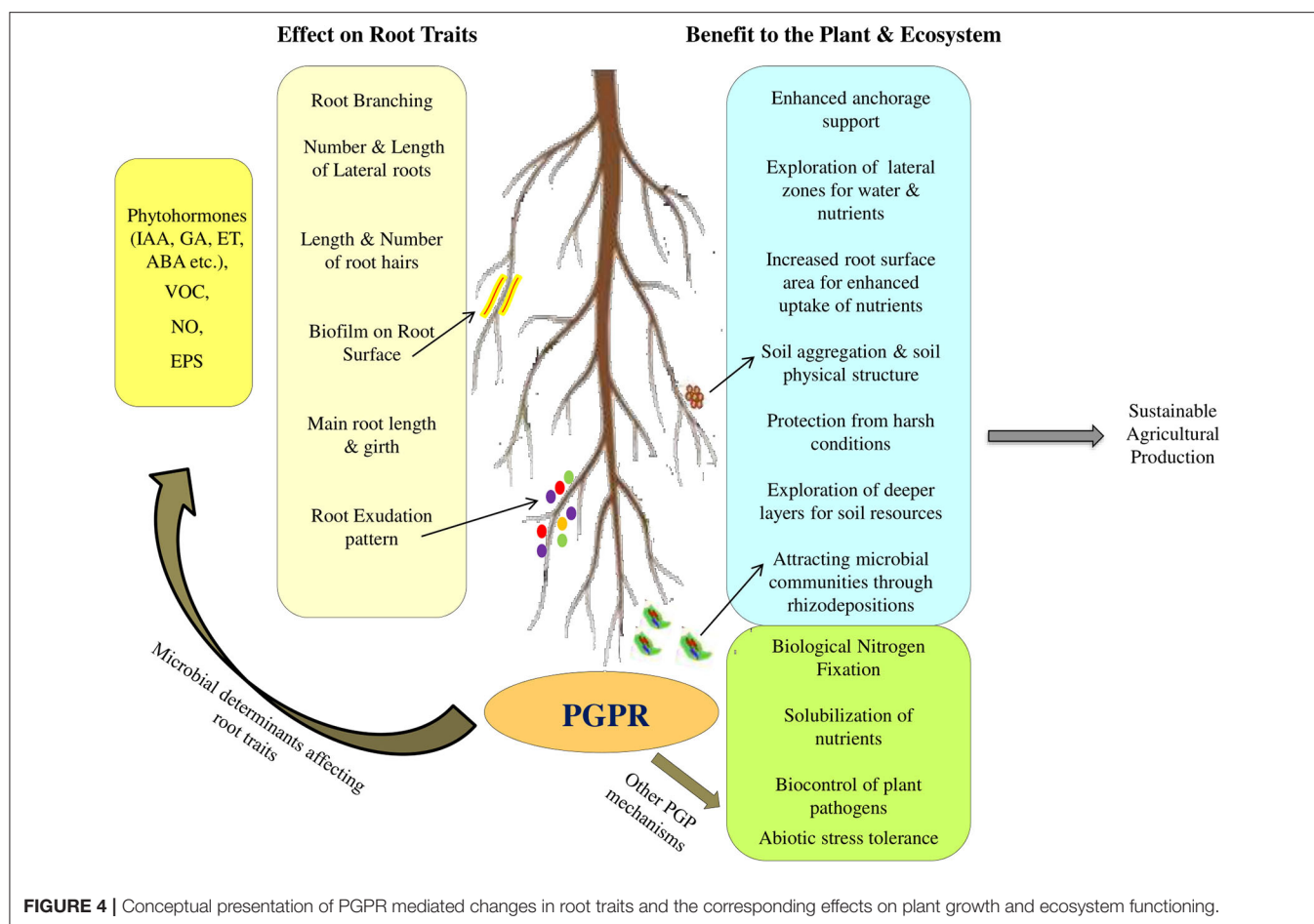
Umpteen literature reports provide evidence for PGPR mediated pathogen control in different plant. For example, a PGPR *Bacillus subtilis* BS2 was found to be effective under field condition against a pathogen *Fusarium oxysporum* f.sp.





*lycopersici* causing tomato wilt. Tomato plants pretreated with *B. subtilis* BS2 has significantly induced defense mechanisms viz., polyphenol oxidase, peroxidase, chitinase, phenolics and phenylalanine ammoniolyase. Inoculation also improved the nutritional quality and fruit yield (Loganathan et al., 2014). *Bacillus amyloliquefaciens* SQR9 a beneficial bacteria showed strong antifungal activity against six tested soil-borne pathogens *Fusarium solani*, *Verticillium dahliae* Kleb, *Rhizoctonia solani* Kahn, *Sclerotinia sclerotiovum*, *Fusarium oxysporum* f. sp. *cucumerinum*, and *Phytophthora parasitica* var. *nicotianae* the bacterium produces a different spectrum of antifungal compounds in response to different fungal species (Li et al., 2014). In rice plants, inoculation with *B. amyloliquefaciens* (SN13) enhanced immune response against *R. solani* by modulating various molecular, metabolic and physiological functions (Srivastava et al., 2016). Treatment with PGPR *Bacillus amyloliquefaciens* improved plant growth and induces resistance in chili (*Capsicum annum* L.) against anthracnose disease (Gowtham et al., 2018). Inoculation with *Peanibacillus lentimorbus* B-30488 in *Nicotiana tabacum* cv. white burley significantly reduced the level of cucumber mosaic virus RNA in the leaves. Inoculation caused an increase in the expression of pathogenesis-related (PR) genes and antioxidant enzymes suggesting induced resistance against the virus. Colonization by B-30488 improved tissue health and physiology of cucumber plants, which produced more flowers and seeds (Kumar et al., 2016). *P. lentimorbus* B-30488 also produced ACC deaminase and could induce tolerance against southern blight disease in tomato caused by *S. rolfii*. The inoculated plants showed modulation of

the ethylene pathway and antioxidant enzyme activities; systemic tolerance was corroborated by PR gene expression analysis (Dixit et al., 2016). PGPR strains fluorescent *Pseudomonas* sp. EM85 and two *Bacillus* spp. MR-11(2) and MRF isolated from maize rhizosphere showed strong antagonistic activity against *Fusarium graminearum*, *Macrophomina phaseolina*, and *Fusarium moniliforme* causal agents of wilting and root rot, stalk rot/collar rot, and charcoal rot of maize, respectively (Pal et al., 2001). Seed treatment of chickpea with combined triple potential rhizosphere microbes, viz., *Trichoderma harzianum* THU0816, fluorescent *Pseudomonas aeruginosa* PHU094, and *Mesorhizobium* sp. RL091 significantly improved plant growth parameters and also triggered the defense in chickpea against the attack of *Sclerotium rolfii* infection (Singh et al., 2014). In french bean (*Phaseolus vulgaris*) combined inoculation of *Pseudomonas fluorescens* along with AMF (arbuscular mycorrhizal fungi) has reduced the root rot incidence caused by *Rhizoctonia solani* apart from promoting plant growth and yield (Neeraj and Singh, 2011). Intercropping of cotton with *Sesbania aculeata* with PGPR (*Azospirillum* AZ204 and *Pseudomonas fluorescens*) inoculation significantly improved cotton yield and reduced the root rot incidence caused by *Rhizoctonia bataticola* (Marimuthu et al., 2013). Inoculation with *Methylobacterium* spp. reduced the disease intensity in tomato plants challenged with *Ralstonia solanacearum* through reduced ethylene levels and ACC accumulation in tomato plants and enhanced accumulation of PR proteins/defense enzymes (Yim et al., 2013). Inoculation with *Enterobacter asburiae* BQ9 induced resistance of tomato plants against yellow leaf curl virus



**FIGURE 4 |** Conceptual presentation of PGPR mediated changes in root traits and the corresponding effects on plant growth and ecosystem functioning.

through enhanced expression of antioxidant enzymes, including catalase, superoxide dismutase, peroxidase and phenylalanine ammonia lyase, and defense-related genes (Li H. et al., 2016). Acyl-homoserine lactones (AHL)-producing *Serratia liquefaciens* MG1 and *P. putida* IsoF elicited induced systemic resistance (ISR) in tomato against *Alternaria alternata* whereas AHL-null mutant strains of both PGPR resulted in reduced ISR (Schuhegger et al., 2006). Root exudates have been found to contain chemicals that mimic AHL signals, stimulating beneficial rhizosphere associations while inhibiting pathogenic bacteria (Teplitski et al., 2000). Treatment of PGPR *Pseudomonas aeruginosa* and *Burkholderia gladioli* in tomato plants controlled root-knot nematode infection through alteration in the synthesis of different secondary metabolites in plants (Khanna et al., 2019).

Studies have reported that plants under pathogen attack recruit beneficial microorganisms in their rhizospheres (Rudrappa et al., 2008; Lee et al., 2012). Weller et al. (2002) reported that in monocultures of wheat, after an initial outbreak of take-all disease, recruitment of 2,4-diacetylphloroglucinol producing pseudomonads occurs that results in increased disease suppressiveness. Mavrodi et al. (2012) also reported evidence in support of cry for help hypothesis in the wheat rhizosphere, in which 2,4-diacetylphloroglucinol producing pseudomonads were recruited under irrigated conditions (*G. graminis* var.

*tritici*, sensitive to 2,4-diacetylphloroglucinol, is the major pathogen under irrigated conditions) and phenazine producing pseudomonads were recruited under dry conditions where *R. solani*, sensitive to phenazines, is the major pathogen.

As indicated by several studies, PGPR have tremendous potential as biocontrol agents and thus can contribute to sustainable agricultural production by reducing the pesticides load and thus saving the soil health.

## CONCLUSIONS AND FUTURE PROSPECTS

Benefits of PGPR to plant growth and yield are well-documented and majority of the studies have emphasized on plant parameters aboveground. However, interaction of PGPR with the plant starts in the rhizosphere through root exudation followed by attachment, colonization on the rhizoplane, and/or in the endorhizosphere. PGPR, by releasing various metabolites including phytohormones, and volatile organic compounds influence the root traits through influencing hormonal balance and/or root trait pathways. The microbial effect on the altered root traits may manifest in terms of root morphological, physiological, architectural, and biotic traits. The altered root

traits may contribute toward plant growth and development and ecosystem functioning through cumulative effects in terms of enhanced anchorage due to root branching, better uptake of soil resources due to increased root surface area as presented in **Figure 4**. PGPR inoculation improves soil aggregation due to increased microbial polysaccharide production. In addition, PGP traits exhibited by the PGPR communities like biological nitrogen fixation, solubilization of nutrients like P and K, biocontrol of plant pathogens and abiotic stress management etc. also contribute significantly to plant growth and yield. The application of fixing/mobilizing/acquisitioning PGPR can save significant amount of chemical fertilizers, besides contributing toward sustainable agriculture. However, the PGPR mediated effect on root traits and subsequent benefits to the plants and ecosystem services need to be estimated/calculated through systematic and focused research/methodologies. Seedling stage screening of presumptive PGPR strains for inducing beneficial

root traits can be advantageously used for selecting promising PGPR strains. It is an interesting field of research with direct applications in sustainable agriculture.

## AUTHOR CONTRIBUTIONS

MG and SB have written the major portions of the review article. AS, PS, and JS have written sections of the article. SB and JS have made the Table. LN has given her expertise in the field with relevant suggestions, and knowledge along with editing and improvising the review article. All authors contributed to the article and approved the submitted version.

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# Rhizobium-Linked Nutritional and Phytochemical Changes Under Multitrophic Functional Contexts in Sustainable Food Systems

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Rhizobia are bacteria that exhibit both endophytic and free-living lifestyles. Endophytic rhizobial strains are widely known to infect leguminous host plants, while some do infect non-legumes. Infection of leguminous roots often results in the formation of root nodules. Associations between rhizobia and host plants may result in beneficial or non-beneficial effects. Such effects are linked to various biochemical changes that have far-reaching implications on relationships between host plants and the dependent multitrophic biodiversity. This paper explores relationships that exist between rhizobia and various plant species. Emphasis is on nutritional and phytochemical changes that occur in rhizobial host plants, and how such changes affect diverse consumers at different trophic levels. The purpose of this paper is to bring into context various aspects of such interactions that could improve knowledge on the application of rhizobia in different fields. The relevance of rhizobia in sustainable food systems is addressed in context.

**Keywords:** multitrophic interactions, phytochemicals, *Rhizobium*, nitrogen, carbon, plant tolerance

## INTRODUCTION

*Rhizobium* is a group of bacteria that were first described in the year 1889 by the German botanist Dr. Albert Bernhard Frank (1839-1900) (Hassen et al., 2020). Such bacteria are part of complex microbiomes that exist endophytically in root nodules of leguminous plants (Martínez-Hidalgo and Hirsch, 2017; Hansen et al., 2020). With the advent of modern molecular identification tools (Young and Haukka, 1996; Deng et al., 2011), root nodulating rhizobia have currently been sub-classified into at least 15 genera including the alphaproteobacteria *Rhizobium*, *Ensifer* (syn. *Sinorhizobium*), *Allorhizobium*, *Pararhizobium*, *Neorhizobium*, *Shinella* (Rhizobiaceae), *Mesorhizobium*, *Aminobacter*, *Phyllobacterium* (Phyllobacteriaceae), *Ochrobactrum* (Brucellaceae), *Methylobacterium*, *Microvirga* (Methylobacteriaceae), *Bradyrhizobium* (Bradyrhizobiaceae), *Azorhizobium* (Xanthobacteraceae), and *Devosia* (Hyphomicrobiaceae), as well as the betaproteobacterial genera *Paraburkholderia*, *Cupriavidus*, and *Trinickia* (Burkholderiaceae).



(De Lajudie et al., 2019; Hassen et al., 2020). The term “*Rhizobium*” has mostly been retained for general reference and as a common name for these genera. However, apart from these root nodulating rhizobia, diverse strains of non-nodulating rhizobia do exist endophytically and in the rhizospheres of various leguminous plants (Wu et al., 2011; Gano-Cohen et al., 2016; Martínez-Hidalgo and Hirsch, 2017).

Symbiotic *Rhizobium* species associated within root nodule symbiosomes of leguminous plants endophytically fix molecular nitrogen ( $N_2$ ) through reactions catalyzed by nitrogenase enzyme (Flores-Tinoco et al., 2020; Jangir et al., 2020). The biologically fixed nitrogen is assimilated by legumes to meet the nutritional demands especially under N-limited conditions (Basu and Kumar, 2020). In return, the autotrophic legumes (macrosymbionts) provide the heterotrophic *Rhizobium* bacteroids (microsymbionts) with organic carbon for respiration derived from photosynthetic reactions (Mitsch et al., 2018; Flores-Tinoco et al., 2020). Therefore, the root nodule is the point of convergence of two very important reactions namely, biological nitrogen fixation through nitrogenase machinery (Lindström and Mousavi, 2019; Signorelli et al., 2020), and carbon fixation in the photosynthetic machinery (Pinnola and Bassi, 2018; Vanlerberghe et al., 2020) (Figure 1). The union of *Rhizobium* and leguminous plants through nitrogen-fixing root nodules is an efficient nutrient cycling component in biogeochemical cycles of various ecosystems.

The rhizobium-legume symbiosis supports aboveground and belowground networks of consumers at various trophic levels (Grunseich et al., 2020; Karoney et al., 2020). By fixing nitrogen, *Rhizobium* redefines autotrophy in leguminous plants and their interactions with various consumers. This is because root infection by rhizobia triggers variations in chemical composition of host plants that may be expressed in gaseous forms such as volatile organic compounds (VOCs) (Ballhorn et al., 2013), aqueous form including cellular fluids and root exudates (Karoney et al., 2020; Tian et al., 2020), as well as solid forms like cell walls (Fournier et al., 2015; Gigli-Bisceglia et al., 2020). Such chemical changes are linked with suitability of legumes as host plants in terms of nutrient content that promotes tolerance (Karoney et al., 2020), or expression of defensive compounds in terms of antibiosis and antixenosis (Cai et al., 2017). *Rhizobium*-legume symbiosis therefore determines biodiversity and the function of various ecosystems including drylands, wetlands, savannahs, tropical rain forests, and the human-managed agroecosystems.

Currently, the world is struggling to meet food demand for a fast-growing human population projected to reach 9.7 billion by the year 2050 (UN DESA, 2017). Global demand for protein-rich food sources including legumes for human food and animal feed will continue rising (Foyer et al., 2016). Intensification of food production to meet the rising food demand is associated with depletion of soil fertility (Kopittke et al., 2019), which results in the misuse of fertilizers and pesticides (Warra and Prasad, 2020), that serves the unsustainable goal of eliminating competition from pests and pathogens (Karoney et al., 2020), while trying to attain dominance in food markets (Gonzalez, 1999; Chalam et al., 2020). The result of such unsustainable practices is increased

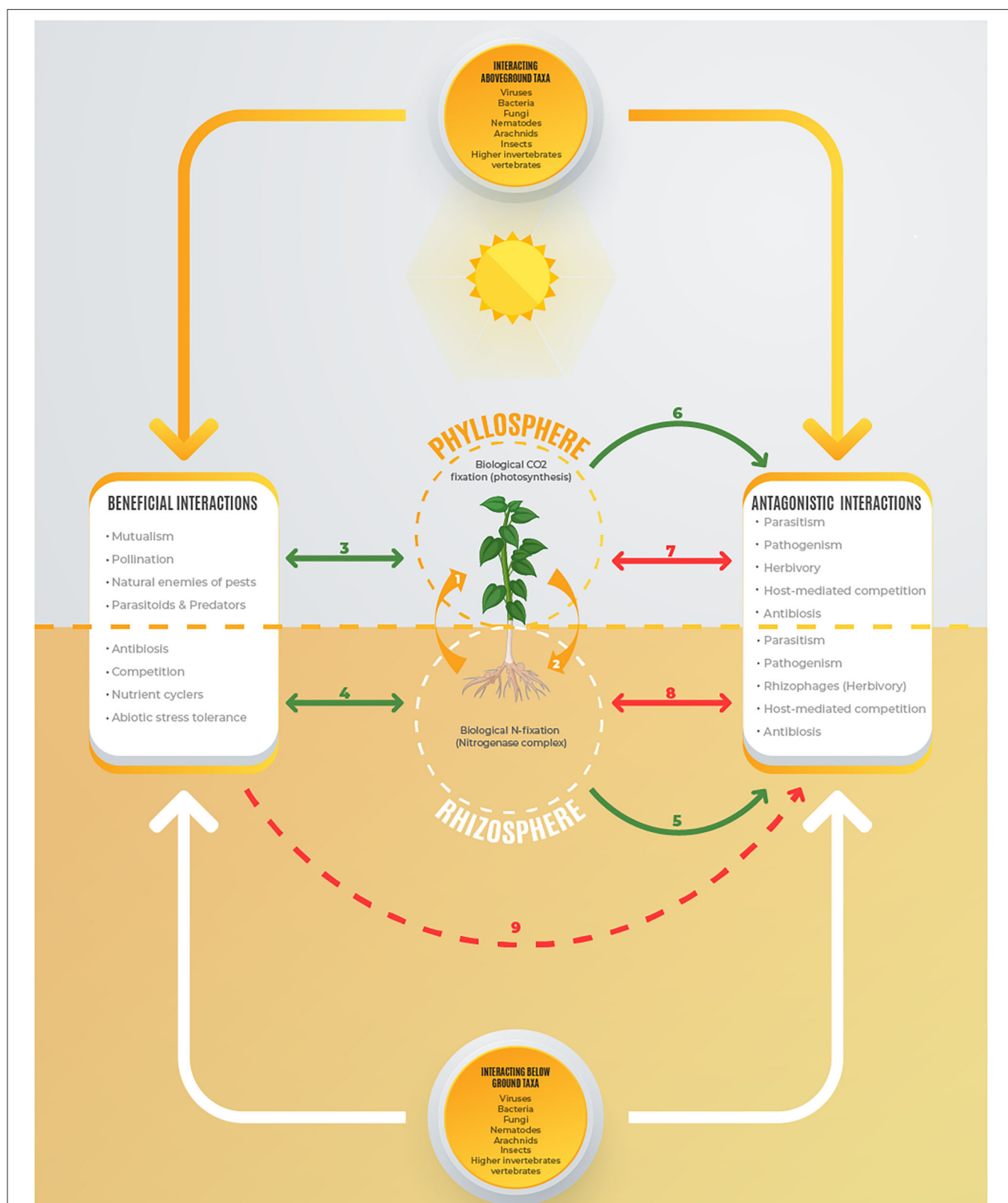
costs of production (Adolph et al., 2020), amidst a resource depleted and polluted agroecosystem that is toxic to human health and biodiversity (Mahmud et al., 2020; Warra and Prasad, 2020). These are among the reasons behind the constitution of the United Nations Sustainable Development Goals (SDGs), and in particular, to end hunger, achieve food security and improved nutrition, and promote sustainable agriculture (SDG2) (ICSU, 2017).

Rhizobia and their leguminous host plants are crucial components in attaining the SDGs through nitrogen and carbon dioxide fixation in agroecosystems (Saha and Baudh, 2020). In-depth understanding of rhizobium-legume symbioses, especially the phytochemical mechanisms and changes associated with the nitrogenase-photosynthesis reactions, lays better ground for addressing some important issues regarding Sustainable Food Systems in agroecosystems. The present article focuses on symbiotic relationships between root nodulating *Rhizobium* species and leguminous plants in biological nitrogen fixation. Phytochemical changes associated with root infection by rhizobia, and how such changes affect various microbial and invertebrate consumers have been addressed in multitrophic contexts. Such interactions have been put into the perspective of Sustainable Food Systems in agroecosystems.

## CARBON AND NITROGEN ACQUISITION BY PLANTS

### Photosynthetic Machinery and Carbon Fixation in Host Plants

Plants are autotrophs equipped with two photosynthetic machineries in the thylakoid membrane inside the chloroplasts (Nelson and Junge, 2015), with the light reaction occurring in the grana of chloroplasts producing energy molecules (Mullineaux, 2005), and the dark reaction located in the stroma of chloroplasts (Poolman et al., 2003). The light reaction in the grana has two systems known as Photosystem II (PSII) (water-plastoquinone oxidoreductase) (Wydrzynski and Satoh, 2005; Freeman, 2006) and Photosystem I (PSI) (plastocyanin-ferredoxin oxidoreductase) (Golbeck, 2006). Photosystem I is located on the outer surface of the thylakoid membrane with a reaction center called P700 that absorbs light at 700 nm to reduce  $NADP^+$  to NADPH (Webber and Lubitz, 2001). Photosystem II (PSII) is located on the inner surface of the thylakoid membrane with a reaction center called P680 that absorbs light energy at 680 nm to provide energy for photo-splitting of water molecules into protons ( $H^+$ ) and oxygen gas ( $O_2$ ) (Renger and Renger, 2008; Herbstová et al., 2012). Electrons produced in PSII are used to replace those donated by PSI to reduce  $NADP^+$  into NADPH (Haldrup et al., 2001; Roach and Krieger-Liszka, 2014). During photosynthetic reactions, ATP is synthesized from ADP and inorganic P through the process of phosphorylation (Jagendorf, 2002), which can either be cyclic phosphorylation involving only PSI (Allen, 2003; Johnson, 2011), or non-cyclic photophosphorylation involving both PSI and PSII that reduces  $NADP^+$  and  $O^{2-}$  to NADPH and  $O_2$ , respectively (Shimazaki and Zeiger, 1985; Allen, 2003).



**FIGURE 1 |** Leguminous plant with *Rhizobium*-nodulated roots. (1) The aboveground foliar system obtains gaseous CO<sub>2</sub> from the phyllosphere and fixes it into organic compounds through photosynthetic machinery and translocate them to the heterotrophic rhizobia in the root nodules; (2) Rhizobium obtains nitrogen from the  
(Continued)

**FIGURE 1 |** rhizosphere and fixes it into amino acids that are transmitted to the leguminous leaves for protein and chlorophyll synthesis; (3, 4) Beneficial mutualistic interactions between rhizobial plants with aboveground and belowground taxa with the autotroph providing organic carbon derived from photosynthesis and nitrogen fixation. (5, 6) The rhizobia plants providing food to antagonistic organisms under a situation of warfare (7, 8) and (9) beneficial biota help the rhizobial plant to suppress the antagonistic organisms.

The ATP and NADPH produced in the light reaction provide energy for the dark (carbon) reaction in the stroma that involves biofixation of carbon dioxide (Hopkins and Hüner, 2009; Buchanan, 2016). Carbon dioxide fixation can either be via the Calvin cycle in  $C_3$  plants (Raines, 2011), which includes leguminous hosts of *Rhizobium* species (Archimède et al., 2011), or the Hatch-Slack pathway for carbon dioxide fixation found in  $C_4$  plants (Osborne and Beerling, 2006). Crassulacean acid metabolism (CAM) functions in CAM plants (Males and Griffiths, 2017).

In the carbon reaction of  $C_3$  plants (Calvin cycle) that includes leguminous hosts of *Rhizobium*,  $CO_2$  that gets into plants cells via stomata is fixed into ribulose-1,5-diphosphate (RuBP) ( $C_5H_{12}O_{11}P_2$ ) under the catalyzing effect of ribulose biphosphate carboxylase (rubisco) to form two molecules of glyceric acid-3-phosphate (Wang and Lan, 2010). Glyceric acid-3-phosphate reacts with ATP to form two molecules of glyceraldehyde-3-phosphate while releasing ADP (Raines, 2003; Wang and Lan, 2010). Glyceraldehyde-3-phosphate can be synthesized into ribulose-1,5-biphosphate (RuBP) for continuation of the  $CO_2$  biofixation in the Calvin cycle (Raines, 2003; Wang and Lan, 2010), or converted into fructose-1,6-bisphosphate as a precursor for biosynthesis of glucose, sucrose, starch, or other energy-rich carbohydrates (Strand et al., 2000; Lv et al., 2017). The carbohydrates are used for generation of energy through the respiratory TCA cycle in the mitochondria (Raghavendra et al., 1994; Plaxton and Podestá, 2006). Besides foliar acquisition of carbon in the form of  $CO_2$  for photosynthesis, plants also do acquire carbon in the forms like  $CO_2$ , carbonate and organic compounds through their roots (Raven et al., 1988; Farrar and Jones, 2000).

## Nitrogen Acquisition in Host Plants

Plants require nitrogen to synthesize proteins and other complex compounds that are very important for their growth and reproduction. Nitrogen is key for the synthesis of chlorophyll and the function of photosystem I and II (Lu et al., 2001; Bassi et al., 2018). For instance, glutamate ( $C_5H_9NO_4$ ) is a nitrogenous compound that is a precursor for chlorophyll synthesis in developing leaves (Forde and Lea, 2007). Glutamate is the key compound involved in the acquisition of nitrogen by plants (Temple et al., 1998). This compound is primarily biosynthesized from pyruvate generated from the glycolytic pathway (Chesworth et al., 1998), through the breakdown of photosynthates arising from the Calvin cycle (Michelet et al., 2013). Pyruvate ( $C_3H_4O_3$ ) is converted to 2-oxoglutarate ( $\alpha$ -ketoglutarate) through the action of glutamate dehydrogenase (GDH) in the tricarboxylic acid (TCA) cycle (Qiu et al., 2019). Glutamate is also synthesized through the proline (Pro)/pyrroline 5-carboxylate (P5C) cycle

in the plant cytoplasm (Miller et al., 2009; Qiu et al., 2019). Glutamate is then used in nitrogen acquisition systems.

Plants have two nitrogen acquisition systems, the root low-affinity transport system that functions when soil N is adequate, and a high-affinity transport system that functions when N is low (Kraiser et al., 2011; Kiba and Krapp, 2016). Nitrogen is mainly acquired in the form of ammonium ( $NH_4^+$ ) in plants growing under low pH conditions and as nitrate ( $NO_3^-$ ) adapted to high pH conditions (Masclaux-Daubresse et al., 2010). Whereas ammonium is assimilated directly into amino acids, nitrate has to first be reduced to ammonium in a reaction catalyzed by nitrate reductase and nitrite reductase (Chamizo-Ampudia et al., 2017). Nitrogen in the form of ammonium ( $NH_4^+$ ) is assimilated via the glutamate synthase (GS)/glutamine oxoglutarate aminotransferase (GOGAT) synthetase pathway (Masclaux-Daubresse et al., 2006; Zhang Z. et al., 2017). In this pathway, ammonium from soil reacts with glutamate to form glutamine ( $C_5H_{10}N_2O_3$ ) (Forde and Lea, 2007). Glutamate dehydrogenase (GDH) is another enzyme that catalyzes the incorporation of ammonium into glutamate just like glutamate synthase (Grabowska et al., 2012). However, glutamate dehydrogenase has lower affinity for ammonium than glutamate synthase (Zhang Z. et al., 2017).

When soil nitrogen supply is limited, the biosynthesis and function of PS I and PS II compounds is constrained (Nunes et al., 1993; Bassi et al., 2018), and hence the provision of energy for  $CO_2$  fixation into glutamate and other products (Bascuñán-Godoy et al., 2018). Such conditions require alternative ways of acquiring nitrogen. This is whereby diazotrophic *Rhizobium* species become of significance to the plants by symbiotic nitrogen fixation through nitrogenase-catalyzed reactions.

## ACQUISITION OF CARBON AND NITROGEN BY RHIZOBIUM

Being heterotrophs, free-living rhizobia saprophytically acquire organic carbon and nitrogen in the rhizosphere (Poole et al., 2018). When soil fertility is low and the supply of organic carbon and nitrogen are limited, chances of survival of rhizobia diminish amidst intensifying competition and predation in the rhizosphere (Gabasawa, 2020) (Figure 1). Saprophytic processes that require more investment in breaking down organic compounds are weakened amidst scarcity of substrates. Scarcity of organic carbon and nitrogen in the rhizosphere favor the establishment of endophytic populations of symbiotic rhizobia in root nodules of host plants (Coba de la Peña et al., 2018). Besides providing shelter for rhizobia, root nodules also supply the symbiotic bacteria with organic carbon and other nutrients (Brewin, 2010). *Rhizobium* in the root nodules fix atmospheric  $N_2$  with the help of nitrogenase enzyme. Root nodules are facilitated with

leghemoglobin and mitochondria that scavenge for oxygen to provide microaerobic conditions that protect nitrogenase from inhibition by O<sub>2</sub> (Bergersen, 1997).

## Carbon Acquisition by Endophytic Rhizobium Species

Rhizobia require organic carbon to generate energy in the form of ATP within the bacteroid Tricarboxylic acid cycle (TCA cycle) (Lodwig and Poole, 2003). The TCA cycle of the host plant is the source of organic carbon to rhizobia in root nodules (Andersen, 2020). Endophytic rhizobia acquire carbon from host plants in the form of C<sub>4</sub>-dicarboxylates (fumarate, malate, and succinate), which can easily penetrate peribacteroid membranes of root nodules (Mitsch et al., 2018). Specifically, L-malate (C<sub>4</sub>H<sub>6</sub>O<sub>5</sub>) is the key C<sub>4</sub>-dicarboxylate that supplies carbon to symbiotic rhizobia in root nodules (Haaker et al., 1996; Poole and Allaway, 2000; Mitsch et al., 2018). Rhizobial TCA cycle functions aerobically in free-living cells (Maier, 2004), and microaerobically involving anaplerotic enzymatic pathways in endophytic bacteroids (Dunn, 1998). The ATP produced is utilized as the energy molecule for nitrogen fixation by rhizobia (Duval et al., 2013). However, there is growing information on the existence of some photosynthetic rhizobia that colonize nitrogen fixing-stem nodules (Fleischman et al., 1995; Zhang et al., 2019).

## Nitrogen Acquisition by Endophytic Rhizobium Species

Endophytic rhizobia acquire dinitrogen molecules (N<sub>2</sub>) through the gas diffusion pathway in the intercellular air spaces of root nodule cortical cells (Zeng et al., 1989; Hunt and Layzell, 1993). Nitrogen fixation reaction is catalyzed by a group of enzymes known as “nitrogenase” (Hoffman et al., 2009, 2014), which comprises of three main genetically distinct types namely Nif, Vnf, and Anf that either have molybdenum (Mo), vanadium (V), or iron (Fe) as their respective active-site central metals (Zhao et al., 2006; McGlynn et al., 2013). Mo-nitrogenase is found in all N<sub>2</sub> fixing bacteria besides being the most widely studied (Newton, 1997; Garcia et al., 2020). Nitrogen is fixed via two distinct biochemical pathways arising from Janus reactions (Harris et al., 2018), with the “distal” (D) pathway being associated with the Chatt-Schrock cycle (Husch and Reiher, 2017), and the “alternating” (A) pathway (Hoffman et al., 2014). In the diazotrophic reaction, nitrogen gas (N<sub>2</sub>) is reduced to ammonium (NH<sub>3</sub>) with the supply of energy from ATP and catalyzed by nitrogenase ( $\text{N}_2 + 8\text{e}^- + 16\text{ATP} + 8\text{H}^+ \rightarrow 2\text{NH}_3 + \text{H}_2 + 16\text{ADP} + 16\text{P}_i$ ) (Hoffman et al., 2014; Ghebreamlak and Mansoorabadi, 2020). The resulting ammonium (NH<sub>4</sub><sup>+</sup>) diffuses into root cells (Patriarca et al., 2002), and assimilated through the glutamate synthetase (GS)/glutamine oxoglutarate aminotransferase (GOGAT) pathway (Zhang Z. et al., 2017; Lea and Mifflin, 2018). Other rhizobia infect non-leguminous plants to provide plant growth promoting services through processes that do not rely on nitrogenase-catalyzed reactions (Mehboob et al., 2009).

## PHYTOCHEMICALS IN RHIZOBIUM-LEGUME SYMBIOSIS AND THEIR INFLUENCE ON CONSUMERS

The photosynthesis-nitrogen fixation machinery of leguminous plants supports complex rhizosphere (belowground) and phyllosphere (aboveground) food webs comprising of organisms from various taxonomic groups (Kempel et al., 2009; Katayama et al., 2011a,b; Zhao et al., 2014; Wu et al., 2017; Karoney et al., 2020) (**Figure 1**). Such trophic interactions primarily relate to the demand for nutrients, shelter, and reproductive space. Underlying such relationships is energy flow from the sun through complex biochemical reactions that have far-reaching effects, including the formation of subsequent generations of the organisms. Phytochemical composition of leguminous host plants exhibits variations, right from the process of rhizobial infection, multiplication into bacteroids, nitrogen fixation to senescence (Irmer et al., 2015). Such chemical compounds include chlorophylls, enzymes, photosynthates and their nutritional derivatives, plant secondary metabolites, hormones, and other signaling molecules as well as inorganic compounds (Wink, 2013; Sánchez-Chino et al., 2015; Šibul et al., 2016; Karoney et al., 2020). Various published works have linked rhizobium infection to nutritional and phytochemical changes that affect consumers in multitrophic systems (**Tables 1, 2**). Nutritional suitability of host plants is therefore influenced by rhizobium infection (Naluyange et al., 2014, 2016; Karoney et al., 2020), while prevention of overexploitation by the consumers depends on the expression of such compounds in terms of host plant resistance and tolerance (Enneking and Wink, 2000; Joosten and van Veen, 2011; Goyal et al., 2012; Goyal, 2013; Karoney et al., 2020).

Leguminous root nodules are microecosystems that host unique microbiomes consisting of consumers that benefit from resources provided by the photosynthesis-nitrogen fixation machinery. Success of root nodule symbiosis depends on how abiotic and biotic factors that determine compatibility between host plants and rhizobia take prominence over those related to antagonism. Leguminous host plants provide conducive environment that is characterized by production of resistance factors that are not harmful to mutualistic rhizobia. The leguminous root nodule also accommodates other microbes that benefit the host plant and cooperate with rhizobia. Together, the host plant, rhizobia, and other beneficial microbes within the root nodule microbiome promote their fitness over other endophytic and exophytic organisms that interact negatively through processes such as parasitism, pathogenesis, predation, and competition. Organic carbon and nitrogen produced by the photosynthesis-nitrogen fixation machinery of legumes and rhizobia supports consumers at various trophic levels. Consumers are affected by compatible relationships between host plants and *Rhizobium* species (Dean et al., 2014; Naluyange et al., 2014, 2016; Pulido et al., 2019; Karoney et al., 2020; Xu et al., 2020). Consumers are also affected by antagonisms arising from incompatible relationship between host plants and rhizobium species (Gourion et al., 2015; Clúa et al., 2018;



**TABLE 1 |** Phytochemical variations associated with *Rhizobium* species and their effects on host plants and herbivorous consumers.

Rhizobium species	Phytochemical variations	Ecological effects		References
		Effects on host plants	Effects on consumers	
<i>Rhizobium leguminosarum</i> bv. <i>viceae</i>	Increased lipids (Jasmonic acid) and flavonoids (quercetin, kaempferol) concentration	Enhances <i>Pisum sativum</i> yield through increased seed fresh and dry weights based on better seed filling	Reduced seed infection level by <i>Didymella pinodes</i>	Ranjbar Sistani et al., 2017
<i>Bradyrhizobium japonica</i>	Increased rhizobiotoxine production in cowpea ( <i>Vigna unguiculata</i> )	Increased cowpea growth	Reduced seed infection level by <i>Macrophomina phaseolina</i>	Deshwal et al., 2003b
<i>Rhizobium leguminosarum</i>	Induces pterocarpan production in pea plant ( <i>Pisum sativum</i> )	Enhances <i>Pisum sativum</i> growth	Increased resistance to <i>Fusarium solani</i>	Patel et al., 1988
<i>Rhizobium tropici</i>	Reduced production of C-based compounds (Flavonoids, phenols, and Tannins); increased production of N-based compounds (Peroxidase, ascorbate peroxidase, and lipid peroxidases in <i>Phaseolus vulgaris</i> )	Improved <i>Phaseolus vulgaris</i> tolerance to <i>Colletotrichum lindemuthianum</i>	Increased <i>Colletotrichum</i> infection in <i>Phaseolus vulgaris</i>	Karoney et al., 2020
<i>Rhizobium leguminosarum</i>	Increased nitrogen	Increased growth of <i>Phaseolus vulgaris</i>	Increased <i>Colletotrichum</i> infection and aphid attack on <i>Phaseolus vulgaris</i>	Naluyange et al., 2014, 2016
<i>Bradyrhizobium japonica</i>	Increased nitrogen levels in Soybean ( <i>Glycine max</i> )	Increased plant biomass (size and leaf number)	Increased susceptibility to spider mites ( <i>Tetranychus urticae</i> ) and other arthropods	Katayama et al., 2010, 2011a
<i>Rhizobium ensifer medicae</i>	Enhances saponins production in <i>Medicago truncatula</i>	Improves plant resistance against Pythopathogens	Deterrence of <i>Spodoptera exigua</i>	Cai et al., 2017
<i>Bradyrhizobium arachis</i>	Increased IAA and rhibotoxines production in <i>Pisum sativum</i>	Increased plant growth and biomass	Reduce infection by <i>Macrophomina phaseolina</i> on <i>Pisum sativum</i>	Deshwal et al., 2003b
<i>Bradyrhizobium</i> spp.	Enhanced Pyrrolizidine alkaloid production in <i>Crotalaria spectabilis</i>	Improves plant resistance against herbivores	Enhances plant defensive mechanisms against herbivores e.g., grazing livestock	Irmer et al., 2015
<i>Mesorhizobium</i> spp.	Increased Indolizidine alkaloids (swainsonine) production in <i>Astragalus</i> spp and <i>Oxytropis sericea</i>	Boost plant resistance against insect herbivores	Increases swainsonine production, serve as deterrent factor to <i>Frankliniella occidentalis</i> and <i>Heliothis virescens</i>	Laguerre et al., 1997; Liu et al., 2017
<i>Bradyrhizobium liaoningense</i>	Enhances production of imidaoles, pyrimidines, indoles and trigonellines in soybean,	Increased nodulation, nitrogenase activity and plant growth	Improved plant defense against fungal pathogens e.g., <i>Fusarium spp</i>	Gao et al., 2015; Shen et al., 2018
<i>Rhizobium</i> sp. (Cicer)	Increased total protein in chickpea ( <i>Cicer arietinum</i> )	Increased seed weight, yield, and total protein content	Improves plant resistance against <i>F. oxysporumf. sp. ciceris</i> (Foc)	Volpiano et al., 2019
<i>Rhizobium tropici</i> CIAT899	Enhanced production of peroxidase, catalase, and ascorbate peroxidase in <i>Phaseolus vulgaris</i>	Increase nutritive suitability to <i>Colletotrichum lindemuthianum</i>	Improve plant tolerance to <i>Colletotrichum lindemuthianum</i>	Karoney et al., 2020
<i>Rhizobium leguminosarum</i> bv. <i>viceae</i>	Increased production of jasmonic acid, kaempferol, and Quercetin in <i>Pisum sativum</i>	Improves plant resistance to pathogens	Inhibits the growth and development of <i>Didymella pinodes</i>	Ranjbar Sistani et al., 2017
<i>Rhizobium tropici</i> CIAT899	Reduced production of flavonoids, phenol, and tannins in common bean <i>Phaseolus vulgaris</i>	Increased plant susceptibility to <i>Colletotrichum lindemuthianum</i>	Increased <i>Colletotrichum lindemuthianum</i> infection on <i>Phaseolus vulgaris</i>	Karoney et al., 2020
<i>Rhizobium leguminosarum</i> bv. <i>viceae</i> (Rlv)	Triggers flavanone and chalcones production in <i>Vicia sativa</i>	Enhances the expression of nod genes increasing nodulation	Reduces the incidences of damping off caused by <i>Pythium</i> spp.	Huang and Erickson, 2007
<i>Rhizobium ensifer medicae</i>	Enhances saponins production in <i>Medicago truncatula</i>	Improved plant resistance to herbivores	Increased saponin production resulting into deterrence of <i>Spodoptera exigua</i> herbivory	Cai et al., 2017

(Continued)

TABLE 1 | Continued

Rhizobium species	Phytochemical variations	Ecological effects		References
		Effects on host plants	Effects on consumers	
<i>Rhizobium leguminosarum</i>	Increased daidzein and coumestrol production in legumes	Induced transcription of <i>nod C</i> and <i>lac z</i> genes responsible for nodulation	Protects the plant against arthropod herbivores	Khanha et al., 1999; Karowe and Radi, 2011
<i>Bradyrhizobium japonica</i>	Increased rhizobiotoxine, citrate, and catechol production	Increased iron chelation leading to increased nodulation and improved soybean resistance against pathogen	Inhibits plant infection by <i>Macrophomina phaseolina</i>	Modi et al., 1985; Guerinot et al., 1990; Deshwal et al., 2003a
<i>Rhizobium</i> strains 116,133	Increased auxins, nitrogen, and chlorophyll content in <i>Phaseolus vulgaris</i> .	Enhanced vegetative growth and plant biomass	Increased plant protection by influencing cellulase, protease, lipase and $\beta$ -1,3 glucanase productions and enhance plant defense to pytopathogens e.g., <i>Macrophomina phaseolina</i> , <i>Rhizoctonia solani</i> and <i>Fusarium solani</i>	Sara et al., 2013; Gopalakrishnan et al., 2015
<i>Rhizobium leguminosarum</i>	Increased athranilate, vibibactin and phytoalexins production in <i>Phaseolus vulgaris</i>	Enhances iron chelation, nodulation, and nitrogen fixation.	Induced plant resistance to soil pathogens e.g., <i>Fusarium</i> spp.	Rioux et al., 1986; Dilworth et al., 1998; Deshwal et al., 2003a

Benezech et al., 2020a). The function of both compatible and non-compatible relationships between host plants and rhizobia may still be affected by other beneficial or antagonistic biota and abiotic factors (Pugashetti et al., 1982; Parker, 2001; Mehboob et al., 2013; Haldar and Sengupta, 2015; Han et al., 2020). The photosynthesis-nitrogen fixation linkage of leguminous host plants and rhizobium, shapes the chemical profile and hence the community structures of both endophytic and exophytic organisms in the rhizosphere and phyllosphere, respectively. Therefore, a functional leguminous root nodule could be equated to stable and self-sufficient institution that provisions for its diverse citizens and protects its interests.

COMPATIBILITY OF RHIZOBIUM WITH HOST PLANTS

The term “symbiosis” was coined in the year 1879 by the Father of Plant Pathology, the German botanist and mycologist Professor Heinrich Anton de Bary (1831-1888), to imply “the living together of unlike organisms” (Oulhen et al., 2016). Relationships between rhizobia and leguminous plants are among the most widely known examples of symbiosis. Compatibility of rhizobium with host plants is genetically determined and highly specific (Mergaert et al., 1997; Clúa et al., 2018; Sachs et al., 2018). The key determinants of specificity in rhizobium-legume relationships are lipo-chitooligosaccharide compounds known as Nod factors secreted by rhizobium strains (Mergaert et al., 1997; Geurts and Bisseling, 2002; Soto et al., 2006), in response to flavonoid signaling compounds in root exudates (Clúa et al., 2018). Flavonoid compounds such as the flavonoid aglycones (apigenin, daidzein, kaempferol, luteolin, myricetin, and quercetin) and the flavonoid glycosides (daidzin, genistin, hesperidin, hyperoside, kaempferol-7-O-glucoside, naringin, and rutin) have been detected in root exudates of the leguminous plants *Melilotus indicus*, *Trifolium alexandrinum*, and *T. resupinatum* (Gomaa et al., 2015). Compatible interactions based on plant flavonoids and rhizobial Nod factors trigger a series of events that lead to successful infection and development of nodules that can fix nitrogen (Wang et al., 2018). Such nodules can be determinate (nodules with determinate meristematic activity) or indeterminate (nodules with indeterminate meristematic activity) (Prell and Poole, 2006; Terpolilli et al., 2012; Mao et al., 2013).

Incompatible interactions between plants and *Rhizobium* may either result in non-formation of nodules (Wu et al., 2011; Gano-Cohen et al., 2016), or if formed, the nodules cannot fix nitrogen (Oono et al., 2009; Wang et al., 2018). Such groups of “freeloaders who do not pay rent” cannot be considered to be mutualistic, but they are parasitic *Rhizobium* species (Lewin, 1982; Taha, 1993; Denison and Kiers, 2004; Oono et al., 2009; Ballhorn et al., 2016). The main factors underlying incompatible interactions between plants and *Rhizobium* are genetically expressed in the form of non-complementary Nod factors (Mergaert et al., 1997; Geurts and Bisseling, 2002; Wang et al., 2018). *Rhizobium* species having genetic symbiotic defects are incompatible with host plants (Long, 1989; Nadler et al., 1990). Besides this, the legume

**TABLE 2 |** Leguminous root exudates and their role in rhizosphere interactions with *Rhizobium* and other organisms.

Root exudates	Function in the rhizosphere	References
Sugars (monosaccharides, disaccharides, polysaccharides)	Act as chemo-attractants of rhizobia in the rhizosphere to colonize the leguminous plant roots	Igiehon and Babalola, 2018
<b>Amino acids</b>		
Arginine	Act as chemo-attractants of rhizobia in the rhizosphere to colonize the leguminous plant roots	Rasmann and Turlings, 2016
Benzoic acids	Act as chemo-attractants of rhizobia in the rhizosphere to colonize the leguminous plant roots	Rasmann and Turlings, 2016
Proline, Aspartic acids and Valine	Produced by Sesame ( <i>Sesamum orientale</i> ) inhibit egg hatching of plant parasitic nematodes thus controlling of <i>Meloidogyne</i> spp.	Bajaj et al., 1989; Ansari et al., 2019
Phenylalanine ammonia-lyase	Leads to high production of phytoalexins and glyceollins which induce <i>Glycine max</i> resistance) to <i>Pytophthora sojae</i>	Zhang C. et al., 2017
<b>High molecular weight compounds</b>		
Tannins	Chemo-attractants of rhizobia in the soil and enhance microbial interaction especially for <i>Rhizobacteria</i> and Arbuscular Mycorrhizal Fungi	Igiehon and Babalola, 2018
Luteolin	Act as chemo-attractants and increase <i>Pseudomonas putida</i> and <i>Rhizobium meliloti</i> growth rates	Maj et al., 2010; Spini et al., 2016
Quercetin	Stimulate <i>Rhizobium meliloti</i> growth and interaction with vesicular-arbuscular mycorrhizal symbioses	Spini et al., 2016
Flavonoids	Act as chemo-attractants of rhizobia stimulating their growth and nodulation in <i>Medicago trunculata</i>	Mathesius, 2019
Phytoalexins	High phytoalexins production inhibits the growth of <i>Phytophthora megasperma</i> in soybean	Ebel and Grisebach, 1988
Genistein	Signaling molecule of <i>Bradyrhizobium japonicum</i> in soy bean triggering expression of Nod genes (Nodulin) which stimulate nodulation. Increases competitiveness and symbiotic activity of <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> in red clover	Kosslak et al., 1987; Bolaños-Vásquez and Werner, 1997; Sugiyama et al., 2008
Daidzein	Signaling molecule of <i>Bradyrhizobium japonicum</i> in soy bean and stimulate the release of nod gene for nodulation process. Increases competitiveness and symbiotic activity of <i>Rhizobium leguminosarum</i> bv. <i>trifolii</i> in red clover.	Kosslak et al., 1987; Bolaños-Vásquez and Werner, 1997; Sugiyama et al., 2008
Apigenin	Signaling molecule of <i>Bradyrhizobium japonicum</i> in soy bean triggering the release of nod genes which stimulate nodulation.	Kosslak et al., 1987; Sugiyama et al., 2008
Afromosin	Signaling molecule of <i>Bradyrhizobium japonicum</i> in soy bean which stimulate nodulation.	Kosslak et al., 1987; Sugiyama et al., 2008
Coumestrol	Signaling molecule of <i>Bradyrhizobium japonicum</i> in soy bean to release the nod genes and stimulate nodulation. Increases competitiveness and symbiotic activity of <i>R. leguminosarum</i> bv. <i>trifolin</i> in red clover.	Kosslak et al., 1987; Bolaños-Vásquez and Werner, 1997; Sugiyama et al., 2008
Liquiritigenin, Naringenin and Hesperitin	Signaling molecules released by <i>Vicia sativa</i> and red clover to attract <i>Rhizobium leguminosarum</i> and trigger nod genes production boosting their competitiveness and symbiotic activities	Maj et al., 2010

host plant may produce defensive compounds including reactive oxygen species (ROS) that may inhibit *Rhizobium* nodulation (Gourion et al., 2015; Tóth and Stacey, 2015), or even the plant may be genetically non-nodulating (Matthews and Davis, 1990; Ali et al., 2014).

## ANTAGONISTIC AND BENEFICIAL RHIZOSPHERE BIOTA TO RHIZOBIUM-LEGUME SYMBIOSIS

### Antagonistic Rhizosphere Biota to Rhizobium-Legume Symbiosis

The leguminous rhizosphere is characterized by root exudation of organic compounds, enzymes, and ion exchange (Kidd et al., 2018; Preece and Peñuelas, 2020), supporting complex food webs of heterotrophic biota from various taxa (Bonkowski et al., 2009; De-la-Peña and Loyola-Vargas, 2014; Taylor et al., 2020)

(Table 2). *Rhizobium* has to survive both in the rhizosphere and host tissues where they still encounter diverse endophytic biota inside the root nodule microbiome (Taha, 1993; Omar and Abd-Alla, 2000; Sharaf et al., 2019; Taylor et al., 2020). Such rhizosphere and endophytic biota have different influences on *Rhizobium*-legume symbiosis (Checcucci et al., 2017).

### Competitors of Rhizobium Symbiosis

Competition is the contentious interaction for limited resources between organisms that is characterized by efficient resource consumption (exploitation) or harmful effects to the adversary (interference) (Ghoul and Mitri, 2016). Competition for nutrients and space is a key form of antagonistic interaction that affects rhizobia and host plants (Postma et al., 1990; Poole et al., 2018), in both independent and symbiotic states of the two organisms (Mendoza et al., 2016; Hortal et al., 2017; Lardi et al., 2017). Non-nodulating strains of *Rhizobium leguminosarum* compete with the nodulating types for infection sites, causing

delay in development and function of root nodules (Lie et al., 1988), which reduces the performance of leguminous host plants (Gano-Cohen et al., 2016). In the root nodule microbiome of *Medicago sativa*, endophytic non-rhizobium bacteria engage in competitive interactions, with *Brevibacillus brevis* to diminish the benefits of cooperation between *Sinorhizobium meliloti* with the non-rhizobial *Pseudomonas* sp. and *Paenibacillus* sp. (Hansen et al., 2020). Apart from competing for infection sites on leguminous roots (Spaink, 1995; Mendoza-Suárez et al., 2020), free-living rhizobia, and other rhizosphere microbes compete for organic carbon in root exudates (Olanrewaju et al., 2019). Sources of organic carbon in root exudates include simple and complex sugars (e.g., fructose, mannose, glucose, maltose, arabinose, and oligosaccharides), amino acids (e.g., aspartate, asparagine, glutamine, arginine, and cysteine), organic acids (e.g., ascorbic, acetic, benzoic, ferulic, and malic acids), phenolic compounds, flavonoids, enzymes, fatty acids, auxins, gibberellins, nucleotides, tannins, steroids, terpenoids, alkaloids, polyacetylenes, and vitamins (Gunina and Kuzyakov, 2015; Hayat et al., 2017; Olanrewaju et al., 2019) (Table 2). Exudation of carbon-rich compounds by leguminous plants becomes more intense under conditions of phosphorus and nitrogen deficiency in the soil (Yoneyama et al., 2012; Tawaraya et al., 2014). Such nutrient-deficient conditions instigate intensive competition for N and P between leguminous roots and rhizosphere biota (Kuzyakov and Xu, 2013). Besides helping plants fix limited nitrogen (Liu et al., 2018), rhizobia are phosphate solubilizing microbes capable of enhancing phosphorus acquisition by leguminous host plants in P-deficient rhizospheres (Qin et al., 2011; Verma et al., 2020). As endophytes, the multiplying *Rhizobium* bacteroids require organic compounds in forms such as fumarate (C<sub>4</sub>H<sub>4</sub>O<sub>4</sub>), malate (C<sub>4</sub>H<sub>6</sub>O<sub>5</sub>), and succinate (C<sub>4</sub>H<sub>6</sub>O<sub>4</sub>) (Mitsch et al., 2018), as well as various mineral elements especially molybdenum (Mo), vanadium (V), iron (Fe), and phosphorus (P) that are required for nitrogenase synthesis within the O<sub>2</sub> limited endonodular space (Rüttimann-Johnson et al., 1999; O'Hara, 2001; Rubio and Ludden, 2008; Brear et al., 2013; Hu and Ribbe, 2016). Whether intraspecific or interspecific, *in planta* competition for such nutrients is expected to occur under nutrient-deficient conditions (Oono et al., 2009).

### Pathogenic and Non-pathogenic Parasites of Rhizobium Symbiosis

Pathogenic interactions are those that involve parasitic microbes that infect and reduce the performance of their hosts (Ochieno, 2020). *Rhizobium* symbioses exist within a situation of exploitation by various forms of parasites that can either be pathogenic or non-pathogenic (Brader et al., 2017). The main pathogens that directly affect *Rhizobium* cells are bacteriophage viruses (Werquin et al., 1988; Santamaría et al., 2014; Cubo et al., 2020). Phytopathogenic viruses infect leguminous shoots and root nodules resulting in reduced nodule biomass, premature nodule decay, low leghemoglobin content, and hence impaired nitrogen fixation (Orellana and Fan, 1978; Taha, 1993; Ismail and Atef, 1998; Huang, 2001; Mangeni et al., 2020). Other phytopathogens that infect root nodules include diverse bacterial species such as *Ralstonia solanacearum*

in *Medicago truncatula* and *Mimosa pudica* (Benezech et al., 2020b; Moura et al., 2020). Fungal species such as *Cladosporium cladosporioides*, *Fusarium moniliforme*, *Fusarium oxysporum*, *Fusarium solani*, *Macrophomina phaseolina*, and *Rhizoctonia solani* are known plant pathogens (Walker et al., 2016; Batnini et al., 2020; Chen et al., 2020; Lakhra and Ahir, 2020; Poveda et al., 2020). However, non-pathogenic strains of the previously mentioned fungi with plant growth promoting effects have been isolated from leguminous root nodules of faba bean (*Vicia faba*) (Omar and Abd-Alla, 2000). While currently there is scarcity of published works on pathogenic endophytic fungi infection of leguminous root nodules, establishment of host range, and non-pathogenicity of some endophytic fungi remains a contentious subject (Ochieno, 2010, 2020; Avedi et al., 2014; Zarafi et al., 2015; Oula et al., 2020).

Root endoparasitic nematodes are pathogenic microinvertebrates that exploit resources from rhizobium-legume symbioses (Taha, 1993; Weerasinghe et al., 2005). Root knot nematodes of *Meloidogyne* spp. are examples of plant parasitic nematodes that cause galls on leguminous roots that affect rhizobial nodulation (El-Bahrawy and Salem, 1989; Wood et al., 2018; Yergaliyev et al., 2020). *Meloidogyne incognita* produces chemical factors that elicit signal transduction events that result in root knots, in a similar manner that roots nodules are formed through *Rhizobium* Nod factors (Weerasinghe et al., 2005).

Non-pathogenic parasites of *Rhizobium*-legume symbioses comprise a wide range of microbes that draw resources from symbiotic systems without causing disease to rhizobia or the host plant. These include non-pathogenic non-symbiotic bacteria and fungi that live endophytically in root nodules and other plant tissues without causing disease (Omar and Abd-Alla, 2000; Mrabet et al., 2006; Selvakumar et al., 2013; De Meyer et al., 2015; Martínez-Hidalgo and Hirsch, 2017; Hassen et al., 2018; Muresu et al., 2019). Besides the non-pathogenic bacteria, root nodules of leguminous plants such as *Hedysarum* species and *Vicia faba* host human pathogens such as *Enterobacter cloacae*, *Enterobacter kobei*, *Escherichia vulneris*, *Pantoea agglomerans*, and *Leclercia adecarboxylata* (Muresu et al., 2010; Saïdi et al., 2013; Selvakumar et al., 2013). These are non-pathogenic parasites to rhizobial host plants.

### Predation of Rhizobium-Legume Symbiosis

Predation is a form of biointeraction in which one organism (predator) eats all or part of another organisms (prey). Herbivory is a form of predation in which the prey is a plant such as legumes, while microbivory is where the prey is a microbe like *Rhizobium*. Predation of the *Rhizobium*-plant symbiosis comes both in the form of microbivory and herbivory (Ramirez and Alexander, 1980). Direct predation on *Rhizobium* cells occurs in the rhizosphere by microbivore protozoans and nematodes (Ramirez and Alexander, 1980; Postma et al., 1990; Verhagen et al., 1993; Trap et al., 2016; Jiang et al., 2017). When ingested, *Rhizobium* induces DNA damage in the intestinal cells of the bacterivorous nematode *Caenorhabditis elegans* (Kniazeva and Ruvkun, 2019). Herbivory by rhizophages belonging to various taxa that feed on leguminous roots determines the



establishment and function of *Rhizobium*-legume symbiosis within the root nodule microbiome. These include nematodes, arthropods, molluscs, and vertebrates that feed on roots of various leguminous plants (Brooks et al., 1988; Douglas and Tooker, 2012; Pereira et al., 2018; Gilarte et al., 2020). Herbivory on *Rhizobium*-plant symbiosis involves damage to root nodules and adjacent tissues by mandibulate coleopterous insects such as larval *Cerotoma arcuata* (Chrysomelidae) and *Sitona lepidus* (Curculionidae) that feed on root nodules of leguminous crops (Teixeira et al., 1996; Johnson et al., 2005; Evenden, 2018; Pereira et al., 2018). Sap-sucking organisms such as the root parasitic nematode *Meloidogyne incognita* and bean root aphid *Smynturodes betae* (Aphididae) also fall into this category of belowground herbivores (Stevenson et al., 2007; Sikora et al., 2018). Molluscs such as slugs engage in rhizophagy through feeding by scraping of leguminous roots of *medicago sativa* among other plants (Douglas and Tooker, 2012). Rhizophagy extends to vertebrate species that interfere with leguminous roots while accessing edible belowground structures (Brooks et al., 1988).

### Allelopathy and Rhizobium-Legume Symbiosis

Allelopathy (Greek: allelon=mutual, pathos=harm) is a biointeraction concept that was conceived by Professor Hans Molisch (1856-1937) around the year 1935 (Chou, 2006). Allelopathy is a mutual relationship between organisms that involves the release of secondary metabolites known as allelochemicals (Vokou et al., 2006; Farooq et al., 2020). Allelochemicals may have stimulatory (positive allelopathy) or inhibitive (negative allelopathy) effects among plants, microbes, invertebrates, and other interacting organisms (Cheng and Cheng, 2015; Mahdhi et al., 2018; Schandry and Becker, 2020). Positive allelopathy can also be referred to as “probiosis” while negative allelopathy is termed as “antibiosis” (Yunes, 2019). Allelochemicals produced by the interacting organisms are referred to as “probiotics” and “antibiotics,” respectively (Selleck, 1972; Yunes, 2019; Schandry and Becker, 2020).

Release of allelochemicals by leguminous plants can increase their vulnerability to antagonists thereby interfering with nitrogen fixing symbiosis with *Rhizobium*. For instance, cowpea *Vigna unguiculata* and faba bean *Vicia faba* are leguminous hosts of *Rhizobium* that release strigolactone allelochemicals via the carotenoid biosynthetic pathway (Matusova et al., 2005; Miyakawa et al., 2020), which stimulate germination and infection by the parasitic weeds *Striga gesnerioides* and Broomrapes *Orobancha* spp. (Bouraoui et al., 2016; Miyakawa et al., 2020). Flavonoids and strigolactones stimulate the germination of fungal pathogen spores and attraction of parasitic nematodes increasing chances of root infection (Steinkellner et al., 2007; Chin et al., 2018). Antagonistic plants can also release allelochemicals that interfere with leguminous host plants and symbiotic *Rhizobium* species in the rhizosphere (Rice, 1992; Kluson, 1995). For instance, the weed *Sonchus oleraceus* (Asteraceae) produces allelochemicals that inhibit the production of flavonoid compounds and root nodulation in the leguminous weeds

*Melilotus indicus* and *Trifolium resupinatum* (Gomaa et al., 2015).

Leguminous host plants and rhizobia also produce allelochemicals that have detrimental effects on other plants, microbes, and herbivores through negative allelopathy (antibiosis). For instance, velvetbean (*Mucuna deeringiana*), jackbean (*Canavalia ensiformis*), jumbiebean (*Leucaena leucocephala*), and wild tamarind (*Lysiloma latisiliquum*) produce phytotoxic allelochemicals that are suppressive to weeds (Caamal-Maldonado et al., 2001). The leguminous weeds *Trigonella polycerata*, *Vicia sativa*, *Lathyrus aphaca*, and *Medicago polymorpha* produce allelochemicals that are suppressive to the growth of rice (*Oryza sativa*) (Zohaib et al., 2017). Forage legumes of the genus *Desmodium* release allelochemicals that are suppressive to the parasitic weeds *Striga* spp. (Pickett et al., 2013). Furthermore, *M. deeringiana* and *C. ensiformis* allelochemicals were found to be inhibitive to plant parasitic nematodes (Caamal-Maldonado et al., 2001). Leguminous plants also release antimicrobial allelochemicals including quercetin, luteolin and other substituted flavones that inhibit pathogens in the rhizosphere (Weston and Mathesius, 2013). Rhizobia on the other hand release allelochemicals that inhibit the activities of other organisms. Such allelochemicals include anti-rhizobial peptides produced by strains of *Rhizobium*, *Mesorhizobium*, and *Sinorhizobium* associated with their enhanced nodulation capacity and competitiveness (Triplett, 1999; Naamala et al., 2016).

Leguminous host plants and rhizobia engage in positive allelopathy (probiosis) in establishing root nodule symbiosis. Leguminous root exudates contain flavonoids that are the main allelochemical attractants for *Rhizobium* symbiosis (Hassan and Mathesius, 2012; Makoi and Ndakidemi, 2012). On the other side, *Rhizobium* secretes lipo-chitooligosaccharide compounds known as Nod factors that induce root nodulation in leguminous host plants (D'haeze and Holsters, 2002; Nandhini et al., 2018). Besides flavonoids, legumes also produce strigolactone allelochemicals that enhance *Rhizobium* activities on host roots (Peláez-Vico et al., 2016; McAdam et al., 2017), while stimulating the germination of arbuscular mycorrhizal fungi that facilitate phosphorus acquisition for improved nitrogen fixation (Püschel et al., 2017; Kafle et al., 2019). Positive allelopathy also exists in the leguminous crop *Trifolium alexandrinum*, which is induced to release high level of flavonoid compounds as a resistance response toward phytotoxic allelochemicals produced by the weed *Sonchus oleraceus* (Gomaa et al., 2015).

It is worth noting that the root words of the “allelopathy” terminology, denote harmful interactions. Therefore, “positive allelopathy” should be limited to situations whereby an organism releases allelochemicals that stimulate activities of the enemy. A good example is the stimulatory effect of plant allelochemicals to parasitic weeds and microbial pathogens. Cases whereby allelochemicals are harmful to other organisms should be considered as “negative allelopathy” or “antibiosis.” Stimulatory effects of plant metabolites to symbiotic beneficial microbes such as rhizobium and arbuscular mycorrhizal fungi should not be considered to be any form of allelopathy. These should be termed as “probiosis,” a term that would better be delinked from allelopathy.

## Beneficial Rhizosphere Biota to *Rhizobium*-Legume Symbiosis

*Rhizobium*-legume symbioses are associated with rhizosphere organisms that favor their establishment and performance. The most common is the tripartite symbiotic relationship of *Rhizobium* and legumes with endophytic arbuscular mycorrhizal fungi such as *Glomus intraradices* (Scheublin et al., 2004; Scheublin and Van Der Heijden, 2006; Kaschuk et al., 2009, 2010), which enhance the acquisition of phosphorus (Tajini et al., 2012; Meng et al., 2015), while protecting the leguminous roots from attacks by microbial pathogens and root parasitic nematodes (Harrier and Watson, 2004; Wille et al., 2019). The rhizosphere also hosts a range of plant growth promoting microbes including non-nodulating bacterial species that provide services such as nitrogen fixation, nutrient cycling, growth hormone and siderophore production, and biological control while improving soil texture and water holding capacity (Martínez-Hidalgo and Hirsch, 2017; Mishra et al., 2017; Backer et al., 2018; Naik et al., 2019). For instance, apart from biological nitrogen fixation by endophytic rhizobia, other rhizosphere microbes play the roles of ammonification, nitrification, and denitrification that are part of the nitrogen cycle (Pajares and Bohannan, 2016; Kakraliya et al., 2018). Rhizosphere microbes involved in nitrification include the ammonia-oxidizing bacteria (e.g. *Nitrosomonas*, *Micrococcus*, *Europaea*, *Nitrosococcus*, *Nitrospira*, *Briensis*, *Nitrosovibrio*, and *Nitrocystis*), nitrite-oxidizing bacteria (*Nitrobacter winogradskyi*, *Nitrosococcus mobilis*, *Nitrocystis*, *Nitrospina gracilis*), and nitrite-oxidizing fungi (e.g., *Penicillium*, *Aspergillus*) and actinomycetes (e.g., *Streptomyces*, *Nocardia*) (Paungfoo-Lonhienne et al., 2017; Kakraliya et al., 2018). Legume crops such as peanut (*Arachis hypogaea*) and soybean (*Glycine max*) increase the abundance of soil bacteria and archaea, but they suppress ammonia oxidizers dominated by archaea (Paungfoo-Lonhienne et al., 2017). This is probably due to competition for ammonium between plant roots and nitrifying bacteria (Verhagen et al., 1993). Mineral phosphate solubilizing microbes include bacteria in the genus *Bacillus* and *Pseudomonas* and the fungal genera *Aspergillus* and *Penicillium* (Khan et al., 2007). Biological control by beneficial rhizosphere microbes is offered through mechanisms such as antibiosis, competition for iron, parasitism, production of extracellular cell wall degrading enzymes, and induced resistance (Whipps, 2001). Furthermore, non-nodulating bacterial species that exist in root nodules help the nodulating rhizobia in extending their host range (Wu et al., 2011). *Rhizobium*-legume symbioses may also benefit from rhizosphere invertebrates through phoretic transfer of bacterial cells by nematodes like *Caenorhabditis elegans* that are attracted by plant volatiles to enhance root nodulation (Horiuchi et al., 2005).

## RHIZOBIUM-LEGUME SYMBIOSIS AND ABOVEGROUND INTERACTIONS

### Increase in Abundance of Aboveground Consumers

Indirect interactions exist between *Rhizobium* in root nodules and consumers in the phyllosphere through host-mediated

processes (Kempel et al., 2009). *Rhizobium* infection of leguminous roots has been linked with increased colonization of common bean leaves by the fungal pathogen *Colletotrichum lindemuthianum* (Naluyange et al., 2014, 2016; Karoney et al., 2020). Also, the presence of *Rhizobium* in leguminous roots is associated with increased abundance of arthropods such as aphids and mites (Katayama et al., 2011a,b; Dean et al., 2014; Naluyange et al., 2014, 2016). Besides this, aboveground insects such as clover root weevil (*Sitona lepidus*) prefer ovipositing at the base of *Rhizobium*-infected white clover (*Trifolium repens*) to ensure food availability and hence survival of their larvae (Johnson et al., 2006). Increase in abundance of foliar consumers is due to improved nutritive suitability of rhizobial plants through symbiotic nitrogen fixation (Karoney et al., 2020). Plants infected with root nodulating nitrogen fixing rhizobia become tolerant to herbivory (Naluyange et al., 2014; Ballhorn et al., 2017; Karoney et al., 2020).

### Decrease in Abundance of Aboveground Consumers

*Rhizobium* infection of roots has been associated with inhibition of aboveground consumers through induced resistance mechanisms. Leguminous plants may either exhibit an increase in production of toxic compounds (antibiosis) (Clement et al., 1994; Soundararajan et al., 2013), or may affect the behaviors of the consumers through less attractive volatile emissions and unpleasant tastes (antixenosis) (Clement et al., 1994; Soundararajan et al., 2013). *Rhizobium*-induced antibiosis has been reported in Lima bean (*Phaseolus lunatus*) inoculated with *Bradyrhizobium elkanii* characterized by enhanced cyanogenesis that inhibits the Mexican bean beetle *Epilachna varivestis* (Coccinellidae) (Thamer et al., 2011; Godschalx et al., 2017). Nodulation of *Crotalaria* roots by *Bradyrhizobium* induces the biosynthesis of pyrrolizidine alkaloids that are toxic to grazers (Irmer et al., 2015). *Rhizobium*-induced antixenosis has been reported in Lima bean (*P. lunatus*) inoculated with *B. elkanii* that exhibits reduction in attractiveness to *E. varivestis* (Ballhorn et al., 2013), while the extrafloral nectar is less preferred by the pavement ant *Tetramorium caespitum* (Formicidae) (Godschalx et al., 2015). Apart from resistance mechanisms, rhizobia may establish dense endophytic and rhizosphere populations that compete with host plants for nutrients, and hence interfering with food supply to aboveground consumers. Such a situation may occur in plants growing under nutrient-deficient conditions (Ochieno, 2010, 2020), or when light energy for photosynthesis is not sufficient (Ballhorn et al., 2016).

### Suppression of *Rhizobium* by Aboveground Consumers

Aboveground organisms influence symbiotic activities of *Rhizobium* in leguminous roots. Infection of common bean leaves by the fungal pathogen *Colletotrichum gloeosporioides* induces plant defense responses that inhibit root nodulation by *Rhizobium* and colonization by arbuscular mycorrhizal fungi (Ballhorn et al., 2014). In this interaction, *C. gloeosporioides* in the leaves enhances root activity of polyphenol oxidase (PPO) (Ballhorn et al., 2014), an enzyme associated with plant resistance to microbial pathogens (Constabel and Barbehenn, 2008; Taranto

et al., 2017). *Rhizobium* usually evades such plant defenses in successful infections to induce root nodulation (Tóth and Stacey, 2015; Cao et al., 2017). However, polyphenol oxidase may not be responsible for suppression of *Rhizobium* nodulation, because the enzyme improves the development, structure, and function of root nodules (Webb et al., 2014). *Rhizobium* inoculation results in high concentration of nitrogen-based compounds including polyphenol oxidase, while reducing the concentration of phenolic compounds and other carbon-based metabolites (Karoney et al., 2020). The absence of polyphenol oxidase results in the accumulation of phenolic compounds (Webb et al., 2014), which are not viable sources of organic carbon for *Rhizobium* development and function compared to the C<sub>4</sub> compounds (fumarate, malate and succinate) (Mitsch et al., 2018).

An alternative explanation is that, aboveground consumers destroy leaves and interfere with photosynthesis while consuming sugars and other nutrients that are required by *Rhizobium* leading to poor root nodulation (Katayama et al., 2014). This is similar to conditions of inadequate light for photosynthesis that constrains symbiosis transforming *Rhizobium* and other mutualists into parasites (Ballhorn et al., 2016). However, there is evidence that herbivory of red alder *Alnus rubra* (Betulaceae) seedlings induces compensatory growth with enhanced root nodulation by the nitrogen-fixing *Frankia* bacteria (Ballhorn et al., 2017). This is a form of *Rhizobium*-induced tolerance to consumers in host plants (Karoney et al., 2020).

## RHIZOBIUM IN SUSTAINABLE FOOD SYSTEMS

Sustainable food systems (SFS) are currently being emphasized globally to improve food production in terms of quality and quantity in line with Sustainable Development Goals (SDGs) (ICSU, 2017). This can be achieved through methods and processes that ensure continuous profitability (economic sustainability), having broad-based benefits for society (social sustainability), with positive or neutral impacts on the natural environment (environmental sustainability) (FAO, 2018). Nitrogen-fixing *Rhizobium* species are part of plant growth promoting microbes being developed into bio-inoculants (Giller and Ronner, 2019), as part of Sustainable Food Systems (SFS) (FAO, 2018). The application of *Rhizobium* for biological nitrogen fixation (BNF) restores the function of the nitrogen and carbon cycles (Thornton et al., 2007), which are key nutrient deficiencies in human-disturbed agroecosystems (Morrow et al., 2016; Smith et al., 2016). Rhizobia symbiotically fix approximately 40 million tons of nitrogen in agroecosystems annually (Udvardi and Poole, 2013). Bio-inoculants based on symbiotic nitrogen fixing *Rhizobium* species could help minimize the misuse of synthetic nitrogenous fertilizers (Zahran, 1999), which have been linked to phytotoxicity (Naluyange et al., 2014; Delgado et al., 2016), soil acidification (Sha et al., 2020), and eutrophication of water bodies resulting in algal blooms and water hyacinth invasiveness (Naluyange et al., 2014; Onyango et al., 2020). Besides, the misuse of synthetic

nitrogenous fertilizers is a public health concern (Wang and Lu, 2020), as the unassimilated salts in consumed plant tissues and the environment have been linked with health problems including respiratory ailments, cardiac diseases, and cancers (Townsend et al., 2003; Lu et al., 2015).

The coupling of rhizobial nitrogenase activity to leguminous photosynthesis improves carbon dioxide fixation and hence carbon sequestration (Kou-Giesbrecht and Menge, 2019). Nitrogen-fixing legume crops contribute to reduced emission of greenhouse gases associated with global warming and vulnerability to the effects of climate change (Stagnari et al., 2017). Mitigation of the negative effects of atmospheric carbon dioxide can be achieved through legume-based intercropping, agroforestry, conservation agriculture, and organic farming that integrate biological nitrogen fixation by various *Rhizobium* species (Khan et al., 2011; Stagnari et al., 2017). Furthermore, *Rhizobium* species are biological control agents in Integrated Pest Management systems (IPM) (Khan et al., 2011), which act directly on pests through processes such as antibiosis and competition (Deshwal et al., 2003b; Kawaguchi et al., 2012), and indirectly by enhancing tolerance of leguminous plants to pests (Naluyange et al., 2014; Karoney et al., 2020). Biological nitrogen fixation also improves food availability and hence species richness of beneficial organisms that provide ecosystem services such as parasitoids, predators, and pollinators (Mattson Jr, 1980; Barber and Soper Gorden, 2015; Tao et al., 2017).

## FUTURE PROSPECTS

The world human population grew past 7.7 billion in the year 2019 and is expected to rise to 9.7 billion by the year 2050 (UN DESA, 2017). Feeding such a fast-growing population requires the intensification of food production systems (Corbeels et al., 2020). The Green Revolution started in the 1960s by Professor Norman E. Borlaug (1914-2009) was meant to ensure increased food production (Borlaug, 2002; Sumberg et al., 2012). However, this Green Revolution was characterized by excessive use of agrochemicals that polluted the environment besides being a threat to food security (van Emden and Peakall, 1996; Carson, 2002; Arora et al., 2020). The development of modern biotechnology industries and their marketing strategies (McCullum et al., 2003; Paarlberg, 2009), whose emphasis is perceived to require more focus on the importance of agroecological approaches (Herren et al., 2015; D'Annolfo et al., 2017), have brought up complex dimensions on the concept of Sustainable Food Systems (Zollitsch et al., 2007; Schütte et al., 2017; Ochieno, 2020). For instance, without adequate agroecological conceptualization, it may be a contentious matter to suggest that, destruction of leguminous biodiversity through herbicide-based weed management (Norsworthy et al., 2010; Corbeels et al., 2020), destroys nitrogen fixing and carbon-fixing machinery that sustain life on earth (Khan et al., 2006; Druille et al., 2015). This is because biodiversity of leguminous plants is associated with complexes of symbiotic nitrogen fixing *Rhizobium* species (Athar and Shabbir, 2008; Marwat et al., 2009).



There is need to establish agroecological management systems that support biological nitrogen fixation as part of the nitrogen cycle, as well as photosynthesis within the carbon cycle, in order to attain sustainable food systems (Tully and Ryals, 2017; Shah et al., 2020). In this case, *Rhizobium*-legume symbioses need to be an integral part of agroecosystems management strategies in the Next Green Revolution (Conway and Barbie, 1988; Arora et al., 2020). Integrated approaches that promote inflow and sufficient utilization of energy and nutrients while limiting the loss of such resources in agroecosystems need to be encouraged. This comprises Integrated Food Energy Systems (IFES) (Sachs and Silk, 1991; Bogdanski et al., 2011), especially those that include leguminous crops (Germaine et al., 2010; Bogdanski, 2012; Orr et al., 2015). Evidence already exists in yield improvement under mixed-cropping systems that include leguminous crops (Ofori and Stern, 1987; Duchene et al., 2017). For instance, agroecological technologies such as Push-Pull that rely on leguminous crops to promote cereal production while suppressing the parasitic weed *Striga hermonthica* and insect pests like the fall armyworm *Spodoptera frugiperda* and the stemborers *Chilo partellus* and *Busseola fusca* (Hailu et al., 2018). The use of organic matter such as compost and mulch to improve soil carbon, nitrogen and other soil properties that benefit *Rhizobium*-legume symbiosis need to be encouraged (Naluyange et al., 2014). Informed and better methods for the application of synthetic fertilizers and pesticides, such as those based on precision agriculture need to be developed for the promotion of *Rhizobium* symbioses (Thilakarathna and Raizada, 2018). There is need to re-examine IPM decision models such as Economic Threshold Levels (ETLs) (Capinera, 1981; Knight and Cammell, 1994), especially in situations whereby herbivore populations are boosted by *Rhizobium*-induced improved nutritive suitability and tolerance in leguminous host plants (Dean et al., 2014; Karoney et al., 2020). This will help in preventing the misuse of pest control products. This should be extended to other beneficial rhizobacteria whose interactions with herbivores have been associated with induced susceptibility in various host plants (Katayama et al., 2010, 2011a,b; Pineda et al., 2012). Resources

need to be allocated toward research and innovation in microbial genetic resources (Sharma et al., 2018), so as to identify and develop competitive and efficient *Rhizobium* strains (Irisarri et al., 2019). This should follow guidelines that deliver endophytic plant growth promoting microbes and identifying non-beneficial ones for the bio-inoculant and biofertilizer industry (Avedi et al., 2014; Ochieno, 2020). This should be complemented with research on plant genetic resources to improve *Rhizobium*-legume symbiosis with nutrient use efficiency. Regardless of the technology used to develop such plants and microbes, their function as biotic factors in agroecosystems need to be well-integrated into Sustainable Food Systems conceptual frameworks (Hansen et al., 2017; Afzal et al., 2020). There is need to address disparities in research partnerships related to *Rhizobium*-legume symbioses and other similar plant and soil associated technologies (Giller, 2020; Minasny et al., 2020; Ochieno, 2020). Investment in research on various aspects of *Rhizobium*-legume interactions is necessary for the application of such plant growth promoting microbes for Sustainable Food Systems. Emphasis should be on the application of modern technologies in unraveling the composition and function of root nodule microbiomes in relation to rhizosphere microbiomes of various ecosystems.

## AUTHOR CONTRIBUTIONS

DO conceptualization and drafting the manuscript. EK drafting the manuscript and tables. EM, EN, and VN drafting the manuscript and imaging. DB and SS drafting the manuscript. All authors contributed to the article and approved the submitted version.

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# Phosphorus Nutrition and Growth of Cotton Plants Inoculated With Growth-Promoting Bacteria Under Low Phosphate Availability

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The low availability of phosphorus (P) in the soil drastically limits the world productivity of crops such as cotton. In order to contribute sustainably to the solution of this problem, the current study aimed to evaluate the capacity of phosphate-solubilising bacteria to improve plant growth and its relationship with physiological parameters, as well as the shoot P content in cotton plants in a soil with low P availability amended with rock phosphate. The results showed that, of the six plant growth-promoting bacteria strains evaluated under greenhouse conditions, the *Rhizobium* strain B02 significantly promoted growth, shoot P content and photosynthetic rate. This strain also improved the transpiration rate and the relative content of chlorophyll but without significant differences. Remarkably, *Rhizobium* sp. B02 had a more significant effect on plant growth compared to the P nutrition. Furthermore, the effect of its inoculation was more pronounced on the roots' growth compared to the shoot. Finally, application of *Rhizobium* strain B02 showed the capacity to optimize the use of low-solubility fertilizer as the rock phosphate. These findings could be associated with the metabolic activities of plant growth promotion exhibited by phosphate-solubilising strains, such as phosphate solubilisation, production of indole compounds and siderophores synthesis. In conclusion, this research provides evidence of the biotechnological potential of the *Rhizobium* genus as phosphate-solubilising bacteria with multiple plant growth-promoting activities capable of improving the plant growth and phosphate nutrition of non-leguminous crops such as cotton in soil with low P availability amended with rock phosphate.

**Keywords:** phosphate-solubilising bacteria, PGPB, rock phosphate, indole compounds, siderophores

## INTRODUCTION

Phosphorus (P) is an essential element for various plant metabolic processes and contributes to the formation of cellular biomolecules such as adenosine triphosphate (ATP), nucleic acids and proteins (Heuer et al., 2017). The biological role of this macronutrient is significant, but its low availability (~0.1%) in the soil, estimated in 5.7 billion hectares of land worldwide, severely limits the yield of crops such as cotton, corn, rice, soybeans and wheat in tropical soils (Granada et al., 2018). In addition to this, P is derived from finite resources such as P-rich rock in the form of

phosphate, which has been 83% exploited in its world reserves (Magallon-Servín et al., 2019). Despite this problem, regular applications of phosphate fertilizers are required to maintain crop yields; nevertheless, its application is increasing rapidly—around 2.5% per year under intensified agriculture due to the growing demand for food (Bashan et al., 2013).

Unlike many countries with agricultural potential, Colombia has not shown substantial progress in the management of nutrients like P from the soil to the plant nor in optimizing the efficiency of the use of fertilizers (Dhir, 2017). For example, cotton (*Gossypium hirsutum* L.) is a widely planted crop (~10,000 ha) in Colombia, mainly under soils of the order Vertisol and Inceptisol, but its production is being affected by the high costs of the excessive use of phosphate fertilizers (Martínez-Reina and Hernández, 2015). One of the causes associated with this problem is that soils where cotton has been planted in the last 20 years present excessive accumulation of P fixed to soil minerals (Vargas et al., 2019). Cotton is one of the most critical and sensitive fiber-producing crops to P, as this macronutrient plays an indispensable role during the early developmental phase, production of biomass and final yield of the cotton crop (Amin et al., 2017). Specifically, P deficiency in cotton plants causes slow shoot development and flower buds, dark green leaves, flower bud necrosis, and yellowing of older leaves (Li et al., 2020). Therefore, new strategies to improve the efficiency of the use of phosphate fertilizers and reduce their application, while maintaining high cotton crop yields, have become one of the greatest challenges in Colombia.

As the efficiency of the use of P is composed of two major components, P uptake and utilization efficiency, some researchers have already reported that an effective strategy is the integration of the increase of the availability of P in the soil through microbial action and the improvement of genotypes with high-absorption efficiency of P (Mai et al., 2018; Iqbal et al., 2019). Currently, studies in plant growth-promoting bacteria (PGPB) have become a focus of soil science research for plant nutrition and agriculture (Rai et al., 2020). Within the PGPB, there is a bacterial group with the ability to affect the dynamics of phosphorus in the soil and increase its availability that is denominated as phosphate-solubilising bacteria (PSB), and the genera *Pseudomonas*, *Bacillus*, *Azotobacter*, and *Rhizobium* are the most reported with this metabolic capacity (Alori et al., 2017). Among those bacterial genera, the role of *Rhizobium* strains on P cycling in plant nutrition is the least explored. To provide P to plants, PSB alter the sorption balance of P in the soil and solubilises and mineralises P in unavailable inorganic and organic forms (Richardson and Simpson, 2011). In some studies, PSB have shown biofertilising potential, allowing the reduction of 33–75% of the dose of soluble phosphate fertilizers in different crops (Sahandi et al., 2019; Rosa et al., 2020). Likewise, PSB have also shown the ability to optimize the efficiency of low P solubility fertilizers such as rock phosphate and compost (Estrada-Bonilla et al., 2021). However, there are limited reports on the potential of PSB in cotton cultivation, since the major focus of development has been on nitrogen nutrition and phytostimulation (Pereg and McMillan, 2015; Diaz et al., 2019).

Curiously, several results of studies on PSB have shown discrepancies between the methodologies used and the results obtained *in vitro* and *in vivo* assays (Kishore et al., 2015; Pii et al., 2015; Granada et al., 2018). The main inconsistencies that have been found are focused on effective selection of PSB and the appropriate environmental conditions to evaluate if PSB contribute to plant nutrition and plant growth from the laboratory to the field. Two of the most common criteria proposed by various researchers (Collavino et al., 2010; Bashan et al., 2013) to evaluate and show the impact of PSB are as follows: (i) a high capacity to increase soluble P from sources with a very low degree of solubility, e.g., rock phosphate, hydroxyapatite, metal-phosphorous compounds and organic phosphate esters, associated with physicochemical parameters (organic matter and pH, among others) of the soil, and (ii) a plant growth-promoting effect by metabolic activities in soils deficient in available P. Based on these two criteria, as well as the P fertilization challenge in cotton crops and the scarce information about PSB inoculation in the same context, the objective of this research was to study how PSB inoculation influences plant growth, P nutrition and physiological parameters of cotton plants on soil with limited P available and amended with an insoluble source like rock phosphate.

## MATERIALS AND METHODS

### Bacterial Strains and Preparation of Inoculants

In this study, we used the PGPB strains SP20, N8, N9, G56, G58, and B02, which were provided by the microorganism collection of Colombian Corporation for Agricultural Research (AGROSAVIA), Colombia. Strains G56, G58, and B02 were isolated from nodules of *Vigna unguiculata* (Mendoza and Bonilla, 2014), while strains N8 and N9 were isolated from the rhizosphere of silvopastoral systems composed of *Pennisetum clandestinum*, *Plantago major*, and *Lolium perenne* (unpublished data). Regarding SP20 strain, it has been reported to have various phenotypes required for colonization of plant root surface and endurance in the rhizosphere and was isolated from the cotton rhizosphere (Amaya-Gómez et al., 2020). These strains were selected because in previous experiments they proved capable of promoting the growth of cotton seedlings under *in vitro* conditions (Supplementary Figure 1). For the preparation of the inocula, the strains were grown on yeast-mannitol agar plates (Vincent, 1970) under standard conditions, i.e., 30°C for 24 h of incubation. Each bacterial inoculum ( $\sim 10^9$  CFU mL<sup>-1</sup>) was produced aerobically under standard conditions on a rotary shaker (150 rpm) in yeast-mannitol broth.

### *In vivo* Screening of Plant Growth Promotion With Phosphorus-Deficient Soil

Greenhouse experiments were carried out at the research center Nataima of AGROSAVIA in Espinal, Tolima, Colombia (4°11'28.39" N latitude and 74°57'38.69" W longitude). The soil type used in the study is sieved airdried Inceptisol soil (Soil Science Division Staff, 2017) typical of the region where the



cotton crop is cultivated. In pots, 1 kg of unsterile soil with low P content was placed (pH: 6.12, organic matter: 1.69 mg kg<sup>-1</sup>, P: 12.28 mg kg<sup>-1</sup>, effective cation exchange coefficient: 6.90 cmol kg<sup>-1</sup>, Ca: 4.79 cmol kg<sup>-1</sup>, Mg: 1.74 cmol kg<sup>-1</sup>, K: 0.23 cmol kg<sup>-1</sup>, Na: < 0.14 cmol kg<sup>-1</sup>). Four cotton seeds (*Gossypium hirsutum* L.) of the M-123 variety were sown per pot. A completely randomized experimental design was carried out with eight treatments, three replicates and two independent experiments.

The treatments were as follows: (i) complete fertilization without bacterial inoculation [urea, diammonium phosphate (DAP) and potassium chloride (KCl)], (ii) control treatment with fertilization using urea and KCl replacing DAP with rock phosphate (RP) (9 mg kg<sup>-1</sup> P<sub>2</sub>O<sub>5</sub> total) without bacterial inoculation, and (iii–viii) fertilization with urea, RP and KCl, and inoculation with each of the PGPB strains evaluated. Based on soil analysis and conventional fertilization rates recommended in the cotton crop [urea (75 kg ha<sup>-1</sup>), DAP (50 kg ha<sup>-1</sup>) and KCl (50 kg ha<sup>-1</sup>)], 5 mL pot<sup>-1</sup> of the following concentrations were applied: 6.3 g L<sup>-1</sup> of urea, 16 g L<sup>-1</sup> of DAP, and 5 g L<sup>-1</sup> of KCl. Regarding RP, 0.152 g pot<sup>-1</sup> was applied, which is equivalent to the DAP doses. Inoculation and fertilization, according to the treatments, were carried out 8 and 13 days after sowing, respectively.

Inoculation was carried out by applying 5 mL of each inoculum or sterile YM broth (uninoculated treatment) to the rhizosphere. After 30 days at 18–38°C and ~55% humidity, the relative chlorophyll content measurement was carried out employing a SPAD-502 chlorophyll meter (Konica Minolta, Tokyo, Japan) when the plants had reached the main growth stage (the fifth leaf unfolded) (Munger et al., 1998). Likewise, gas exchange measurements were carried out as follows: net photosynthesis (P<sub>n</sub>) and transpiration rate (E), between 09:00 and 11:00 h with the LI-6400 XT infrared gas analyser (Lincoln, NE, USA). The evaluation parameters were established at 400 μmol s<sup>-1</sup> for the flow rate, with a concentration of 400 μmol of CO<sub>2</sub> and a photonic flux density of 1,200 μmol of light photons m<sup>-2</sup> s<sup>-1</sup> (Chastain et al., 2016). Then, the length of the root and the shoot were measured. Plant tissues were oven-dried separately at 60°C for 48 h to measure their dry weights. Shoot tissues were ground to powder and wet oxidized in a solution of nitric acid and 30% hydrogen peroxide (4:2, v-v). Digests were analyzed for P concentration by the molybdenum blue method (Murphy and Riley, 1962).

## Genome Sequencing

The total genomic DNA of the selected PGPB with the highest plant growth-promoting effect was extracted using the QIAamp DNA Mini Kit (Qiagen, Canada). The concentration of the DNA was measured using a Qubit Fluorometer (ThermoFisher Scientific, United States) through the Quant-iT dsDNA HS Assay Kit following the manufacturer's instructions. Genome library preparation was done with the Nextera XT (Illumina) and the sequencing was conducted on a MiSeq (Illumina) with 250 bp pair end reads at the Microbial Genomics Laboratory of the Molecular Genetics and Antimicrobial Resistance Unit at Universidad El Bosque, Bogotá, Colombia. Read quality was evaluated using FastQC; low quality reads were removed

using Trimmomatic v0.32. High quality reads were assembled into contigs using IDBA-UD. Taxonomic mapping was then performed using MyTaxa. From these results, evolutionary associations with nearby complete genomes stored in NCBI were inferred by calculating the Average Nucleotide Identity (ANI) value using FastANI (Santos-Torres et al., 2021).

## Screening of Plant Growth-Promoting Activities

The *in vitro* characterization consisted of biochemical tests to determine if the selected strain exhibited plant growth-promoting traits related to the improvement of P nutrition. For the following assays, a bacterial suspension (~10<sup>9</sup> CFU mL<sup>-1</sup>) in sterile saline solution (0.89% w v<sup>-1</sup>), washed twice and centrifuged (5,220 xg for 10 min) from an inoculum produced under the standard conditions mentioned above, was used. First, the ability of the strains to increase the availability of P was evaluated in both P solubilisation and mineralisation. In solubilisation, two types of insoluble sources of P at 5 g L<sup>-1</sup> were used: tricalcium phosphate and RP. For this, experiments were performed in 250 mL Erlenmeyer flasks filled with 75 mL of NBRIP medium supplemented with each P source (Nautiyal, 1999). Bacteria were inoculated by adding 7.5 mL aliquots of the bacterial suspension. The medium inoculated with sterile water was used as a control treatment. After the culture was incubated for 5 days for tricalcium phosphate and 12 days for RP at 30 ± 1°C at 200 rpm on a rotary shaker, the liquid medium was centrifuged at 16,000 xg for 10 min. The culture supernatant was then used to evaluate the P released into the solution using the molybdate blue colorimetric method (Fiske and Subbarow, 1925). The P quantities were calculated from a standard curve of soluble phosphate (KH<sub>2</sub>PO<sub>4</sub>). For mineralisation, qualitative detection of phytase production was carried out by growing the cultures (50 μL) from bacterial suspension in a solid NBRIP medium supplemented with 1% (w v<sup>-1</sup>) sodium phytic acid (C<sub>6</sub>H<sub>18</sub>O<sub>24</sub> × NaH<sub>2</sub>O, Sigma-Aldrich, Germany) for 15 days, as described by Kerovuo et al. (1998).

As a second feature, the synthesis of indole compounds with and without the addition of tryptophan was estimated using the colorimetric assay based on the modified Salkowski reagent (Glickmann and Dessaux, 1995). Bacteria were inoculated by adding 7.5 mL aliquots of the bacterial suspension in 250 mL Erlenmeyer flasks filled with 75 mL of tryptic soy broth (TSB) supplemented with 100 mM of tryptophan. After the culture was incubated for 3 days at 200 rpm, the liquid medium was centrifuged at 16,000 xg for 10 min, and supernatants obtained were mixed with the Salkowski reagent in a 4:1 ratio for 20 min under dark conditions. Indole compounds were spectrophotometrically determined at 535 nm. Finally as third trait, the production of siderophores was qualitatively detected by the method of Schwyn and Neilands (1987). For this, 50 μL bacterial suspension was inoculated in plates containing agar Chrome Azurol S (CAS). These plates were incubated at 30°C for 5 days and observed for orange color formation around each colony. Each test was performed in a completely randomized design, with three replicates and two independent experiments.

## Data Analysis

The data were subjected to one-way ANOVA and multivariate analysis of variance (MANOVA) to examine the significance of differences and variability at the 95% confidence level ( $p < 0.05$ ), respectively, using the SPSS 22.0 programme (SPSS Inc., Chicago, IL, USA). Moreover, treatment means were compared by applying the Duncan's test. The correlation between the variables evaluated in *in vivo* screening was conducted through a principal component analysis (PCA) with a biplot using the software PAST 3.03 (Hammer et al., 2001). The graphics were performed using the GraphPad Prism 8 software (Graphpad, San Diego, CA).

## RESULTS

### Greenhouse Study

As expected, the application of the DAP fertilizer had a more positive impact on all the parameters measured in cotton plants than RP in the absence of PGPB inoculation. In contrast, when analyzing all treatments, we observed significant increases of 36 and 21% in shoot dry weight with DAP and B02+RP compared to the treatment RP uninoculated (**Figure 1A**). In the shoot length, improvements of 13 and 7% were found when DAP and B02+RP were applied compared to the control (**Figure 1B**). In root development, uninoculated treatment plants with RP exhibited a biomass of  $1.86 \text{ g plant}^{-1}$ . In contrast, DAP and B02+RP treatments produced higher biomass with values of 3.36 and  $3.25 \text{ g plant}^{-1}$ , representing significant increases of 81 and 75%, respectively (**Figure 1C**). The application of B02+RP significantly promoted the root length by 34% with a value of  $24.73 \text{ cm plant}^{-1}$  (**Figure 1D**). Meanwhile, DAP promoted length by 24% ( $23.02 \text{ cm plant}^{-1}$ ) compared to the control ( $18.44 \text{ cm plant}^{-1}$ ). The other biological treatments did not differ significantly from the control. In P nutrition, a beneficial effect was observed when B02, SP20, and N8 were inoculated. For example, the shoot P content in control treatment increased by 12 and 9% with DAP and B02+RP (**Figure 1E**). Regarding the physiological parameters, the photosynthetic rate increased by 15 and 14% in the SP20+RP and DAP treatments (**Figure 2A**). For the chlorophyll measurement, a 9% increase was observed with B02+RP and DAP (**Figure 2B**). In addition, increases of 12, 8, and 6% were evidenced with N8+RP, DAP, and SP20+RP in transpiration rate (**Figure 2C**).

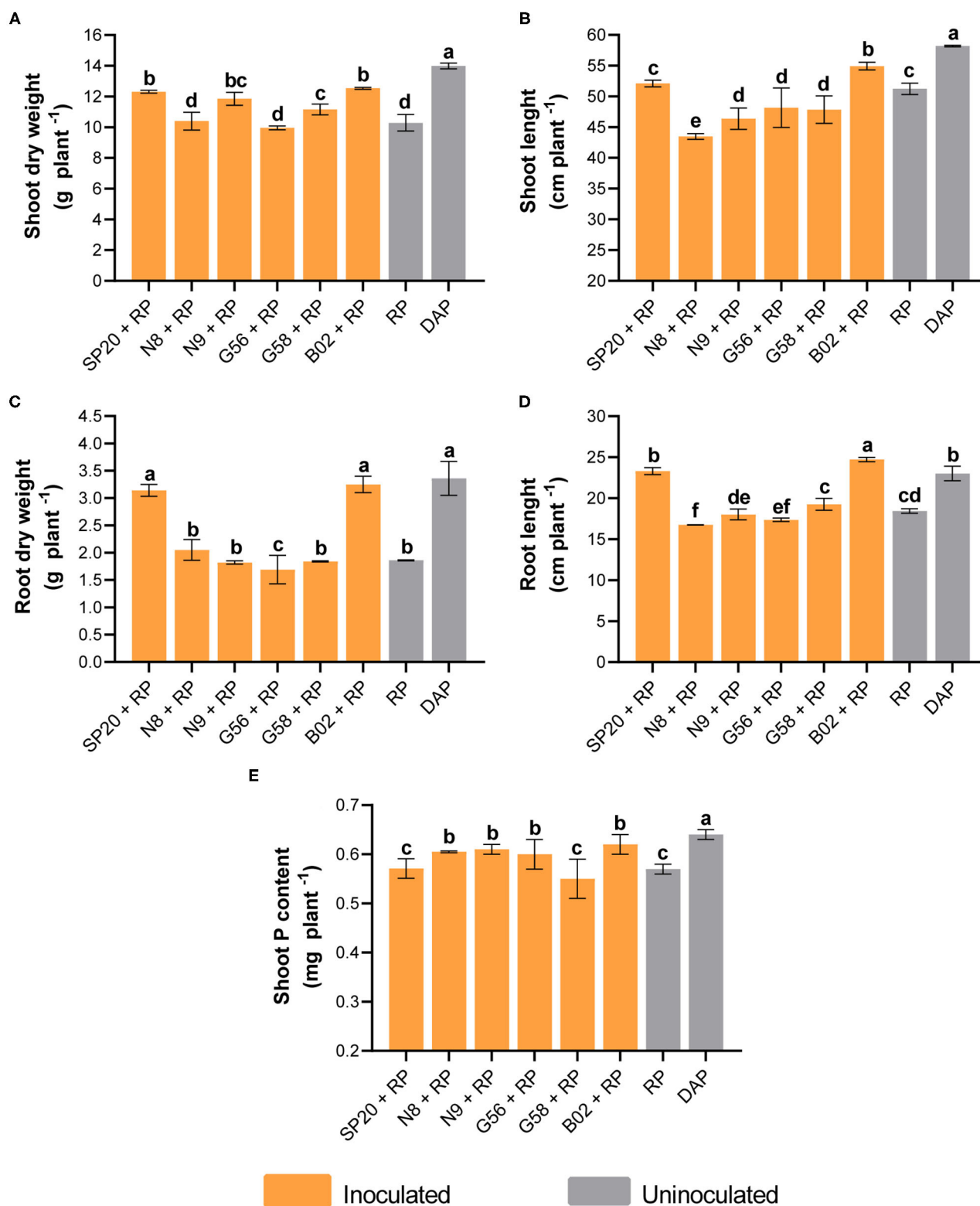
PCA analysis (**Figure 3**) allowed us to evidence the effect of PGPB application in a more integrated way between the eight response variables initially raised. The first two components of PCA, i.e., PC1 and PC2, explained ~64% of the experiment variation. PC1 accounted for 48.55% and was associated positively with all parameters evaluated. PC2 accounted for 15.34% and showed a positive correlation with photosynthetic rate, P content and transpiration rate and negatively with the other parameters. The angle included between the arrows pointing at two variables determined the correlation between the parameters as follows: sharp angles defined positive correlations, squared angles defined a null correlation, and obtuse angles defined negative correlations

(de Almeida Carvalho-Estrada et al., 2020). Based on this, we observed a positive relationship between the three growth parameters (shoot dry weight, root length, and shoot length) and between the photosynthetic rate and P content. In addition, the position over the two dimensions on the graph indicates how the variables clustered and revealed that all treatments were grouped into two major clusters. Cluster 1 was composed of uninoculated treatment amended with DAP, B02+RP, and SP20+RP, while Cluster 2 was composed of the other strains with RP and the uninoculated treatment amended with RP. The relative position of clusters with respect to the arrows for each parameter suggests that the treatments in Cluster 1 exerted the greatest beneficial influence on plant growth, which corroborates what was evidenced when analyzing each parameter in the one-way ANOVA. Remarkably, the MANOVA analysis (**Table 1**) confirmed that there were significant differences between the treatments of inoculation with B02 and SP20 compared to the uninoculated treatment with RP, but that there were no significant differences with the treatment with DAP application. Among the strains B02 and SP20, it was observed that B02 caused the greatest plant growth-promoting effect in five parameters of the eight evaluated. Hence, our data indicate that B02 was the strain with the highest increase of soil P lability and was selected for the following trials. Based on the P-limiting conditions of the experiment and the improvement in the physiological parameters and P content, this strain is considered PSB.

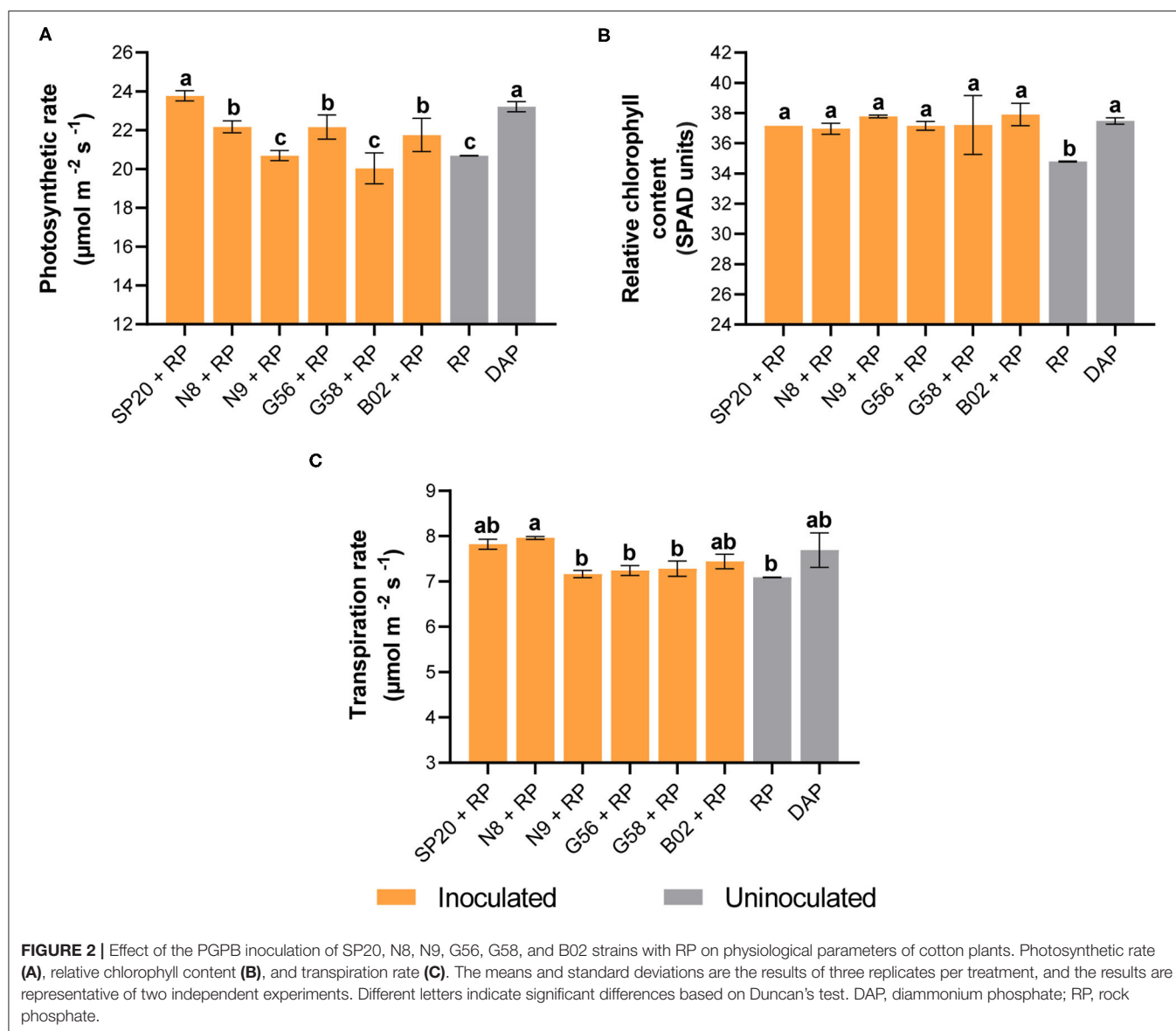
### Phylogeny and Characterization of Plant Growth-Promoting Features of Selected PSB

Strain B02 has a genome size of 4.99 Mb and belongs to the *Rhizobium* genus ( $p$ -value: 0.024). Nevertheless, it probably does not belong to any species stored in the NCBI, since the closest species were *Agrobacterium pusense* GCF013285525 (formerly *Rhizobium pusense*) and *Rhizobium oryzihabitans* GCF010669145 with an ANI values of 87 and 85%. The genome is available at the NCBI genome database under the de accession number SAMN16969919 in the bioproject number PRJNA627728.

We observed that the B02 strain showed several plant growth-promoting activities (**Table 2**). This strain was active in P solubilisation using tricalcium phosphate and RP as P sources. We found approximately 10 times more P soluble in tricalcium phosphate than RP. The observed values were  $0.41 \text{ mg PO}_4^{-3} \text{ mL}^{-1}$  for tricalcium phosphate and  $0.5 \text{ mg PO}_4^{-3} \text{ mL}^{-1}$  for RP. Regarding organic P mineralisation, we did not observe halos around the colonies as a positive indicator of sodium phytate hydrolysis; therefore, B02 strain showed that they are not capable of producing phytases. In the production of indole compounds, the strain exhibited this activity. Interestingly, we observed the B02 strain also produce indole compounds without tryptophan addition, though the highest amount (more than double) was measured with tryptophan addition. Finally, the strain showed the capacity for producing siderophores through orange color formation around each colony.



**FIGURE 1 |** Influence of the PGPB inoculation of SP20, N8, N9, G56, G58, and B02 strains with RP on plant growth and shoot P content of cotton plants. Shoot dry weight (A), shoot length (B), root dry weight (C), root length (D), and shoot P content (E). The means and standard deviations are the results of three replicates per treatment, and the results are representative of two independent experiments. Different letters indicate significant differences based on Duncan's test. DAP, diammonium phosphate; RP, rock phosphate.



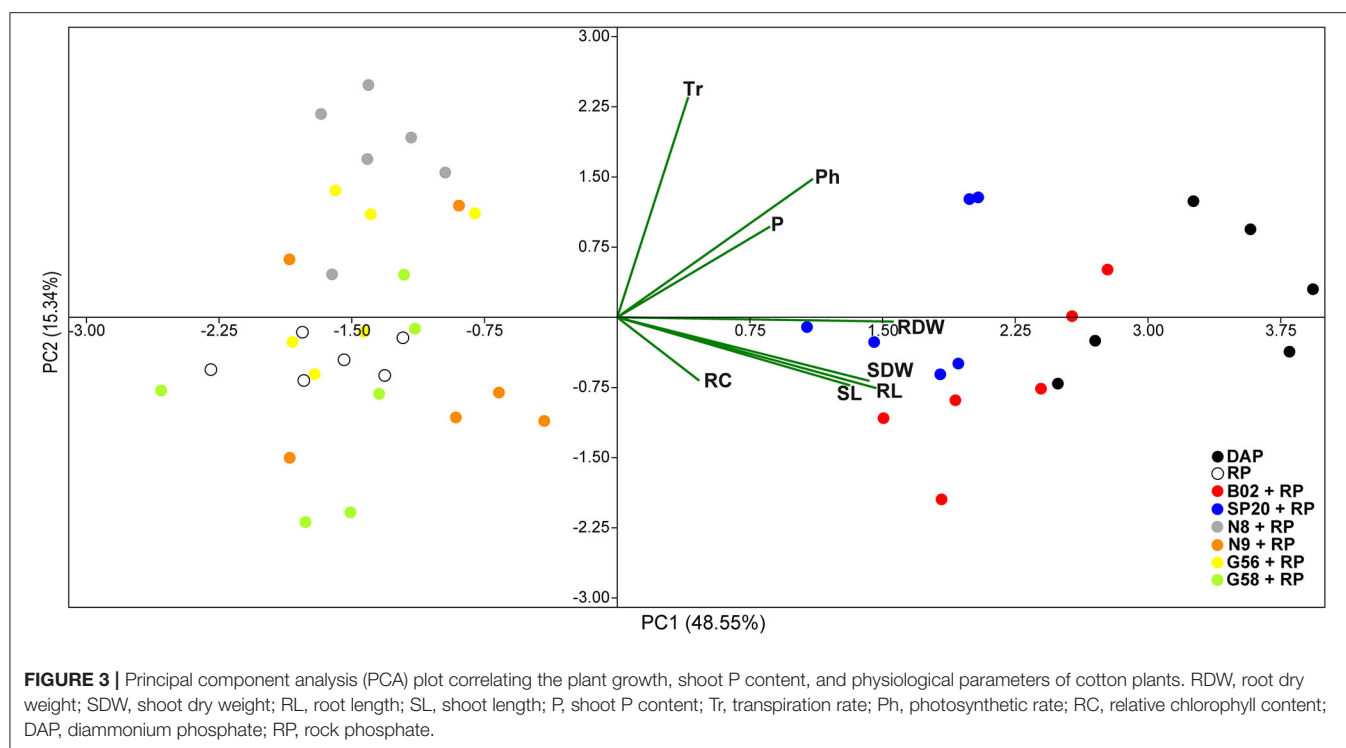
## DISCUSSION

There is a global demand for developing sustainable crop production, where adequate use of phosphate represents a challenge in the agricultural sector worldwide (Bindraban et al., 2020). Due to the environmental concerns associated with improving the efficient use of phosphate sources such as DAP fertilizer with high solubility and RP with low solubility, the study and appropriate selection of PGPB with biofertiliser potential acquire more and more relevance (Wan et al., 2020). In this research, evidence of the plant growth-promoting effects of PSB inoculation using RP as P source on cotton was shown in P-deficient soils.

Previously, the plant growth-promoting capacity of our strains was evaluated under controlled *in vitro* conditions on cotton plants (Supplementary Figure 1). The six strains

showed the ability to significantly ( $p < 0.05$ ) promote cotton seedling length between 10 and 71%. The above showed the positive interaction between plant and microorganism. Then, the greenhouse experiment was carried out in a nutritionally deficient soil with the application of an insoluble source like RP. The results showed that bacterial inoculation caused significantly different responses in the plants. Of the six strains evaluated under greenhouse conditions, inoculation of *Rhizobium* sp. B02 exerted a significant influence ( $p < 0.05$ ) on plant morphometric parameters (biomass and length), shoot P content and photosynthetic rate of cotton. In the other parameters, i.e., transpiration rate and relative chlorophyll content, increases were observed but without significant differences. As the sampling time is a determining condition to assertively select bacterial strains and is one of the most criticized aspects (Bashan et al., 2020), we measured these findings in the growth stage of





three true leaves (30 days), during which cotton plants require more nutrients, especially P, and respond positively to their availability (Grant et al., 2001; Iqbal et al., 2020).

Interestingly, when comparing the results of both biomass and length obtained in the treatments with bacterial inoculation plus RP with respect to the control (RP uninoculated), we inferred that the positive influence was more pronounced on the development of the root compared to shoot. Furthermore, based on the PCA obtained between plant parameters, we deduced that the inoculation of B02 and SP20 had a higher effect on growth parameters in contrast to parameters related to P nutrition and plant physiology. Likewise, the inoculation of *Rhizobium* sp. B02 with RP application generated a similar response without significant differences to the application of DAP fertilizer uninoculated in cotton plants, which allows us to infer that the individual inoculation of this strain could partially replace the DAP application.

Several studies have reported on the use of *Rhizobium* sp. as a successful strategy to improve the growth of legumes, such as bean, lentil, pea, chickpea and soybean (Wang et al., 2019). Interestingly, over the last few years, many studies have reported the presence of *Rhizobium* in the rhizosphere, endosphere and phyllosphere of non-leguminous crops (Díez-Méndez and Menéndez, 2021). However, the number of publications on the inoculation of *Rhizobium* sp. on cotton is very scarce. For example, Hafeez et al. (2004) showed that the inoculation of rhizobia improves seedling emergence, nutrient absorption ( $K^+$  and  $Ca^{+2}$ ) and cotton growth. To our knowledge, no reports demonstrate whether the single use

of *Rhizobium* sp. beneficially influences cotton development in phosphorus-deficient soils.

A determining condition in this study was the soil characteristics. The soil used had low available P ( $12.28 \text{ mg kg}^{-1}$ ). According to Ramírez and Kloepper (2010), this condition is necessary to observe plants respond to an increase in soluble P generated by microbial action. Another parameter of the soil, which is often forgotten in PSB studies, is the P sorption index, which represents the retention capacity of P in soil. Previous findings have shown that the effectiveness of PSB is controlled by the sorption capacity of P in the soil, demonstrating an inverse relationship (Osorio and Habte, 2015). In our study, the soil used belongs to the order of the inceptisols, which are characterized by having an average P sorption capacity (andisols > inceptisols > entisols) (Brenner et al., 2019). This condition allows the released P ions to be moderately absorbed by the soil components, reducing their availability to the plant, which does not show the full effectiveness of the biological activity of the strains. However, under this average level of P sorption, a beneficial effect of *Rhizobium* strain B02 on cotton plants growth was observed. Additionally, the soil was not sterilized and was obtained from fields where cotton is regularly planted. The objective of using these conditions was to demonstrate the effect of plant growth promotion of the PSB by simulating real commercial crop conditions (Romero-Perdomo et al., 2017).

In the biochemical tests carried out for the PSB selected, we show that *Rhizobium* sp. B02 have multiple plant growth-promoting features. Our results suggest that B02 strain is capable of solubilising tricalcium phosphate and RP. Tricalcium phosphate was used because calcium is immobilized in soils with

**TABLE 1** | *P*-values of the MANOVA analysis at  $\alpha = 0.05$  between treatments evaluated under greenhouse conditions.

	DAP	RP	SP20+RP	N8+RP	N9+RP	G56+RP	G58+RP	B02+RP
<b>DAP</b>		0.02	0.13	0.01	0.01	0.01	0.02	0.16
<b>RP</b>	0.02		0.04	0.15	0.27	0.30	0.46	0.02
<b>SP20+RP</b>	0.13	0.04		0.02	0.03	0.02	0.04	0.14
<b>N8+RP</b>	0.01	0.15	0.02		0.29	0.37	0.16	0.01
<b>N9+RP</b>	0.01	0.27	0.03	0.29		0.25	0.56	0.02
<b>G56+RP</b>	0.01	0.30	0.02	0.37	0.25		0.18	0.01
<b>G58+RP</b>	0.02	0.46	0.04	0.16	0.56	0.18		0.03
<b>B02+RP</b>	0.16	0.02	0.14	0.01	0.02	0.01	0.03	

DAP, diammonium phosphate; RP, rock phosphate.

**TABLE 2** | Plant growth-promotion mechanisms exhibited by *Rhizobium* sp. B02.

Strain	PO <sub>4</sub> <sup>3-</sup> (mg mL <sup>-1</sup> )		Indole compounds (μg mL <sup>-1</sup> per OD <sub>630</sub> unit)			Sider. (+/-)
	TCP	RP	Phytases (+/-)	Without Trp.	With Trp.	
B02	0.41 ± 0.02	0.05 ± 0.010	-	37.51 ± 4.16	136.36 ± 10.47	+

Data are presented as mean ± standard deviations. Means and standard deviations are the results of three replicates per biochemical analysis, and the results are representative of two independent experiments. (+) indicates the presence of the activity and (-) indicates the absence of the activity. Trp: tryptophan; TCP: tricalcium phosphate; RP: rock phosphate; Sider: siderophores.

a pH between 6 and 8 (Estrada et al., 2013), like the soil used in this study. In addition, we added RP, an insoluble source of P, to activate and evaluate the metabolic features of the strain in relation to P. These results suggest that the strain solubilised the insoluble P associated with both soil and insoluble fertilizer (RP), which improves cotton plant P absorption. Therefore, it is possible to suggest that *Rhizobium* sp. B02 increased the efficiency of the RP fertilizer. Regarding the synthesis of siderophores, B02 showed positive results for their production. Siderophores are mainly associated with the sequestration of iron in the soil, which may increase P availability (Pii et al., 2015). Furthermore, we evaluated the synthesis of indole compounds, an activity that stimulates various processes at the root level of the plant (Moreno-Galván et al., 2020). We observed that the strain exhibits this activity, and that tryptophan improves their production. Interestingly, B02 produce indole compounds in the absence of tryptophan, revealing different biosynthetic pathways for their production (Patten and Glick, 1996). Moreover, according to Amaya-Gómez et al. (2020), *Rhizobium* strain B02 is able to move, survive various concentrations of H<sub>2</sub>O<sub>2</sub>, form biofilms and metabolize different carbon substrates as phenotypes required for colonization of plant root surfaces and endurance in the rhizosphere. In that study, the strain B02 was identified by partial 16S rRNA gene sequencing.

A possible explanation for these findings could be based on a theoretical relationship between the *in vitro* and *in vivo* results. Based on Ramírez and Kloepper (2010) and Romero-Perdomo et al. (2017), the indole compounds produced by the strains stimulate the length of the cotton root, allowing greater exploration of the soil and higher absorption of nutrients. As P is limited, the phosphate solubilisation and the siderophores production of the strains increase its availability. Additionally, the enhancement of the transpiration rate could also have

contributed to the improvement of the shoot P content in the inoculated plants, providing additional influences on the movement of P toward the roots. This process is mediated by the P function at the aquaporin level, since the phosphorylation process regulates the activity and the number of aquaporins that are directly related to the hydraulic conductivity and water absorption in roots (Zhou et al., 2013). The synergy of these plant growth-promoting activities could also have a beneficial influence on the chlorophyll content and photosynthetic capacity of cotton plants. Hence, plants could absorb more light energy to drive photosynthesis, which is related to the significant increase and the positive correlation evidenced in this parameter. This is consistent with previous reports in other plants (Sahandi et al., 2019; Wu et al., 2019). Furthermore, several findings have shown that the P supply generated by the increase in the photosynthetic rate directly influences the accumulation of dry matter, increasing its dry weight (Liu et al., 2020). To prove the biological contribution of these metabolic activities, research with mutant strains will be required (Rilling et al., 2019). The present description of the plant growth-promoting abilities of *Rhizobium* strain B02 corroborates that *Rhizobium* sp. is well-known for being a P solubiliser, a synthesis of indole compounds and a producer of siderophores (García-Fraile et al., 2012; Estrada-Bonilla et al., 2021).

Additionally, we did not perform a statistical correlation between *in vitro* results and *in vivo* results in the plant. Previous reports have shown that the number of growth-promoting features is not directly proportional to the effect on the plant and that the measurement of the capacity of each mechanism, such as P solubilising and mineralising under *in vitro* conditions, does not occur similarly in soil (Raymond et al., 2020). According to Patel et al. (2010), the soil provides very different conditions than a solid or liquid medium under controlled conditions.

Moreover, the soil has different physical properties (adsorption, absorption, etc.) that can affect the behavior of microorganisms. A finding that corroborates this suggestion was found by Collavino et al. (2010). They found low statistical correlations between solubilisation halos and soluble P in both solid and liquid NBRIP medium with the P content in plant tissue.

Based on these findings, we show that PSB inoculation improves the growth, the P content of plant tissue and the physiological parameters in plants. For this, selection criteria such as strains that release soluble P from sources with a very low degree of solubility associated with environment conditions and strains that promote plant growth in soils deficient in available P are suggested. Although over the years, the taxonomy of the genus *Agrobacterium* and *Rhizobium* has undergone various revisions due to the transfer of species, the taxonomic analysis obtained with the sequencing of the genome of the B02 strain indicates a possible new species of the genus *Rhizobium* (Mousavi et al., 2015; Delamuta et al., 2020). Therefore, polyphasic taxonomic studies are necessary to carry out, such as housekeeping genes *recA* and *atpD*, to confirm the position of the B02 strain as distinct from the recognized *Rhizobium* species (Flores-Félix et al., 2020). *Rhizobium*, among many other PGPB, are the most studied microorganisms—mainly for their ability to form an effective symbiosis with leguminous crops in order to transform atmospheric nitrogen into assimilable nitrogen—the presented results show the biotechnological potential of this genus as a PSB in cotton plants. Likewise, this work provides evidence on the potential of PSB inoculation to increase the efficient nutritional use of P for developing more sustainable cotton production.

## CONCLUSION

In this research, the inoculation of *Rhizobium* sp. B02 together with the application of RP in cotton plants, improves growth, shoot P content, photosynthetic rate, transpiration rate and relative chlorophyll content in soil with low P availability. Furthermore, this strain showed potential as phosphate-solubilising bacteria to increase the efficient use of RP. These findings might be associated with the ability of the strains to solubilise both tricalcium phosphate and rock phosphate, to synthesize indole compounds, and to produce siderophores. This work is a first approximation to optimize the efficient use of P

in cotton cultivation through the application of PSB and low-solubility sources such as RP.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found here: <https://www.ncbi.nlm.nih.gov/genbank/>, SAMN16969919.

## AUTHOR CONTRIBUTIONS

FR-P and RB: conceptualization. GE-B and RB: funding acquisition and supervision. FR-P, IB, and JM-L: methodology. FR-P and GE-B: analysis and interpretation of the data. FR-P, IB, JM-L, GE-B, and RB: writing—original draft. FR-P, GE-B, and RB: writing—review & editing. All authors have made a direct and intellectual contribution to the manuscript.

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# The Current and Future Role of Microbial Culture Collections in Food Security Worldwide

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Food security is the pillar of nutritional wellbeing for food availability, and is necessary to satisfy all physiological needs to thus maintain the general wellbeing of populations. However, global agricultural deficiencies occur due to rapid population growth, causing an increase in competition for resources; such as water, land, and energy, leading to the overexploitation of agro-ecosystems, and the inability to produce a suitable quantity of efficient food. Therefore, the development of sustainable agro-biotechnologies is vital to increase crop yield and quality, reducing the negative impacts caused by intensive non-sustainable agricultural practices. In this way, the genetic and metabolic diversity of soil and plant microbiota in agro-ecosystems are a current and promising alternative to ensure global food security. Microbial communities play an important role in the improvement of soil fertility and plant development by enhancing plant growth and health through several direct and/or indirect mechanisms. Thus, the bio-augmentation of beneficial microbes into agro-ecosystems not only generates an increase in food production but also mitigates the economic, social, and environmental issues of intensive non-sustainable agriculture. In this way, the isolation, characterization, and exploitation of preserved beneficial microbes in microbial culture collections (MCC) is crucial for the *ex situ* maintenance of native soil microbial ecology focused on driving sustainable food production. This review aims to provide a critical analysis of the current and future role of global MCC on sustainable food security, as providers of a large number of beneficial microbial strains with multiple metabolic and genetic traits.

**Keywords:** agriculture, biological control agents (BCAs), microbial inoculants, sustainability, climate change, plant growth-promoting microorganisms (PGPM)

## INTRODUCTION

The rapid growth of the human population will increase the current food demand over the coming years, but intensive non-sustainable agricultural practices place food security, the economy, and the environment at a risk worldwide. Thus, in recent years, global society has faced new challenges related to the development of ecological, efficient, and sustainable alternatives to satisfy increasing

food demand (Martínez-Castillo, 2016). For example, the abrupt changes in climatic conditions at a global scale have had immense negative impacts on agricultural production. The Intergovernmental Panel on Climate Change (IPCC) predicts a global average temperature increase of 1.4 to 5.8°C during the current century (2001–2100), decreasing the production of major cereal crops such as maize (20–45%), rice (20–30%), and wheat (5–50%) (Arora, 2019). In addition, climate change will modify the lifecycle stages and the development rates of phytopathogens and pests (Gupta et al., 2018), which will reduce annual agricultural production by up to 30% (Sharip et al., 2012).

On the other hand, soil degradation is the most significant environmental problem for food production, causing poverty and hunger in developing countries (Pereira et al., 2017). Approximately 52% of the land used for agriculture worldwide is moderately or severely affected by soil degradation (ELD, 2015), due to erosion, salinization, acidification, contamination, or compaction (Kopittke et al., 2019). Soil erosion is the largest contributor to land degradation worldwide, leading to the loss of 75 billion tones of fertile soil per year with an annual economic cost of about USD 400 billion (ELD, 2015; International Atomic Energy Agency (IAEA), 2015). Thus, farmers have relied on the use of high amounts of synthetic agro-inputs to fertilize crops and maintain phytosanitary control, which negatively impact human and environmental health (Villarreal-Delgado et al., 2018). In this sense, in the past 40 years, the use of these agro-inputs in agriculture has increased drastically, i.e., nitrogen (N) has increased by 7.4 times but the yield has only increased by 2.4 times in the same period, indicating that crops have reduced their ability to use N efficiently (Hirel et al., 2011), which increases the economic and environmental cost of food production (Sharip et al., 2012). Therefore, the development of innovative and sustainable agro-biotechnologies focused on solving environmental, economic, and social issues associated with current intensive non-sustainable agricultural practices is needed to contribute to food security (Cano et al., 2017).

Through time, microorganisms have been studied due to their fundamental importance in the maintenance of multiple functions and ecosystem services in the biosphere (Delgado-Baquerizo et al., 2016). Microorganisms are an essential part of human wellbeing, participating in medicine, agriculture, aquaculture, food industry, and biotechnology, among others (Weng et al., 2005; Díaz-Rodríguez et al., 2017). Since microbiota is found in every place on the planet, it plays vital roles in ecosystems, such as; (i) social and ecological sustainability, (ii) adaptation and mitigation of climate change, (iii) as a biotechnological resource for humanity, (iv) water cycling and nutrients, and (v) the increase of food production (Kalia and Gupta, 2005; Pajares et al., 2016).

In agro-ecosystems, microbial communities interact with crops through direct or indirect action mechanisms, regulating their growth and productivity by increasing tolerance to abiotic and biotic stress, plant nutrition, and antagonism against phytopathogenic agents (Santoyo et al., 2019). This particular group of microorganisms are commonly named plant growth-promoting microorganisms (PGPM). PGPM inhabit soil and plants, colonizing roots in  $10^5$ – $10^7$  colony forming units (CFU) per gram of fresh root. These interactions have beneficial impacts

on health and plant growth (Mishra et al., 2017; Valenzuela-Aragon et al., 2018).

Currently, some members of several microbial genera have been studied for their ability to produce phytohormones, and to solubilize phosphates, such as *Micrococcus*, *Pseudomonas*, *Ralstonia*, *Enterobacter*, *Pantoea*, *Acinetobacter*, *Bacillus*, *Aeromonas*, *Burkholderia*, and *Microbacterium* (Lara and Negrete, 2015; Ali et al., 2017). Additionally, others are related to the tolerance of different types of biotic and abiotic stress, such as *Pseudomonas*, *Bacillus*, and *Azospirillum* for salinity stress; *Burkholderia* and *Rhizobium* for water stress; and *Azotobacter* and *Bacillus* for nutrient uptake efficiency (Rokhzadi and Toashih, 2011; Choudhary et al., 2016). Moreover, a group of microorganisms known as biological control agents (BCA) have shown the ability to inhibit the growth of phytopathogens and mitigate the incidence of plant diseases, i.e., *Bacillus amyloliquefaciens* and *Microbacterium oleovorans* have demonstrated the ability to control *Fusarium verticillioides* (Etcheverry et al., 2009); *Trichoderma asperellum* controls *Fusarium* wilt; *Metschnikowia pulcherrima* inhibits the development of *Botrytis cinerea*, *Alternaria alternata*, and *Penicillium expansum* (Köhl et al., 2019); bacteriophages control *Erwinia amylovora* and *Ralstonia solanacearum*; and agriphage controls *Pseudomonas syringae* and *Xanthomonas campestris* (Olanrewaju et al., 2017).

However, the agro-ecosystems are undergoing accelerated deterioration worldwide due to erosion, loss of organic carbon, nutrient depletion, soil sealing, climate change, and other threats, generating a loss of those promising PGPM genera (FAO, 2015a). Therefore, the conservation of this biological diversity is essential for its re-incorporation into agro-ecosystems. Thus, the role of microbial culture collections (MCC) is crucial in achieving this goal (Valenzuela-Ruiz et al., 2018). In this way, the emergence of biological concerns, world food insecurity, and the continuous discovery of new microbial species or subspecies (de los Santos Villalobos et al., 2019) creates the need not only to preserve these microorganisms but also to study them for the development of new agro-biotechnologies (de los Santos-Villalobos et al., 2018). Unfortunately, at present, almost all promising microorganisms are not fully exploited and correctly preserved, which impedes achieving a positive impact on the food security of the world in a sustainable way.

This review aims to highlight the importance of soil microbial resources for the development of sustainable agricultural practices, as well as to highlight the role of MCC as reservoirs and providers of PGPM. Finally, we critically analyze the current status and perspectives to increase the use, exchange, and exploitation of promising microbial strains preserved in these culture collections for sustainably contributing to global food security.

## GLOBAL AGRICULTURAL ISSUES FOR FOOD SECURITY

It is estimated that 38.5% of the surface of the planet is dedicated to agriculture, which has increased 1% per year, while food production has increased between 2 and 4% per year (FAO,

2017c). In addition, around 800 million people in the world, or 1 in every 9 humans, suffer from hunger; also, more than two million people suffer from nutrient deficiency, which is known as “hidden hunger” (FAO, 2017a). This is strong evidence of the global food security crisis, which is understood as the failure of nutritional wellbeing through the availability of food necessary to satisfy all physiological needs and maintain the health of the population (Wheeler and Von Braun, 2013). On the other hand, it is estimated that the global population will increase up to 10 billion people by 2050; therefore, agricultural production will have to increase between 50 and 100% to meet global food demands (FAO, 2017c).

The constant increase in the global human population has caused an increment in competition for resources such as water, land, and energy, leading to the overexploitation of agro-ecosystems. This scenario results in the inability to produce enough nutritious and equitable food, awakening the need to develop sustainable alternatives (Godfray et al., 2010; FAO, 2017b). For example, during the green revolution (1960–1980), crop production patterns changed considerably to meet the food demand of the growing world population between 1950 and 1998 (from 2.6 to 5.9 billion people, respectively) (Sunding and Zilberman, 2001). Thus, intensive non-sustainable agricultural practices such as the increased use of synthetic fertilizers and pesticides, large-scale irrigation, and new varieties of high-yield crops were implemented (Gliessman, 2002; Matson, 2012). However, food demand is still on the rise, and intensive non-sustainable agricultural practices have failed to continuously enhance crop yields and quality, generating serious *in* and *ex situ* environmental, economic, and social consequences (Matson, 2012; FAO, 2019).

Intensive non-sustainable agriculture exerts significant internal and external effects. There are two types of external effects: (1) outside the agro-ecosystem, where there is a depletion of groundwater and environmental contamination by the usage of synthetic agro-inputs; and (2) global effects, which result in greenhouse gas emissions, and animal, plant, and human diseases. In addition, as an internal effect (in the agro-ecosystem) soil degradation causes salinization, the reduction of organic matter content, and lowers plant nutrient use efficiency (Lal, 2015; Struik and Kuyper, 2017). For example, <40% of applied nitrogen fertilizers are used efficiently by plants, where the remaining 60% results in volatilization, accumulation in soils, leaching in rivers, lakes, and streams, among others (FAO, 2017d; Chandini et al., 2019). Moreover, according to Tsiafouli et al. (2015), intensive non-sustainable agriculture reduces the microbial diversity and population, which leads to processes of biological and chemical degradation in the soil. At present, soil degradation is increasing at a rate of 5–7 million hectares per year worldwide (International Atomic Energy Agency (IAEA), 2014), and is largely due to its exploitation to satisfy the rising food demand (Maura and Febles, 2018). This reduces the global food productivity by 12%, increasing food prices by 30% (ELD, 2015).

On the other hand, climate change presents a huge challenge for agriculture. It is estimated that by 2050 the temperature of the planet will increase by an average of 3.5–4.7°C, causing

warm winters and summers in many countries. This will have negative consequences for water supply, cooling, and food production (Leahy, 2019). However, agriculture also has a part in the contribution to global climate change through the production of synthetic agro-inputs, an activity that uses a lot of energy by burning fossil fuels and emitting CO<sub>2</sub> into the atmosphere. Agriculture contributes 20% of the CO<sub>2</sub>eq emissions globally, while at the regional level, Africa and Latin America contributed the largest amount to the total CO<sub>2</sub>eq emissions due to agriculture, with a share of ~60% (FAO, 2020). In this way, climate change has different negative effects on ecological and physiological events in crops, such as changes in the soil microbial ecology, plant-microbiome interactions, plant growth rates, alterations in the distribution regimes, and proliferation of new phytopathogens, pests, and weeds (Mall et al., 2017). These problems will lead to increased crop production costs with lower yields (Nelson et al., 2009; Thomson et al., 2010). Thus, one of the main challenges for agriculture is the adaptation to anthropogenic and natural changes to increase crop yields by sustainable agro-biotechnologies (Foley, 2011), where one of the most promising alternatives is the use of the genetic and metabolic diversity of native microbiota in agro-ecosystems.

## MICROBIAL DIVERSITY IN AGRO-ECOSYSTEMS

Soil is the thin layer that covers the Earth, made up of organic substances, living organisms, air, water, and mineral particles. Soil is a vital natural resource from which most of the global food is produced (Hartemink, 2016). Food production requires essential nutrients, metabolites, and water, among others, that are provided by soil to plants. Thus, soil fertility is directly related to the quantity and quality of produced food (FAO, 2015b). In addition, this matrix provides ecosystem services, such as water purification, degradation of pollutants, flood and climate regulation, food, fiber, and fuel supply, carbon retention, nutrient cycling, a source of pharmaceuticals, and genetic resources (Adhikari and Hartemink, 2016). Soil contains a large reservoir of microorganisms ( $1 \times 10^9$  microbial cells g<sup>-1</sup> dry soil) and microbial diversity ( $1 \times 10^5$  microbial species g<sup>-1</sup> dry soil) (Bodelier, 2011; Bhattarai et al., 2015). These microbial communities are responsible for carrying out between 80 and 90% of its biological processes, including biogeochemical cycles (indispensable for maintaining the equilibrium of agro-ecosystems), organic matter decomposition, soil formation, primary production, climate regulation, and disease control, among others (Nannipieri et al., 2017; Saccá et al., 2017).

Besides, microbial communities play an important role in improving soil fertility, where microbial genera such as *Azotobacter*, *Azospirillum*, *Helio bacterium*, *Bradyrhizobium*, *Bacillus*, *Gluconacetobacter*, *Methylobacterium*, *Nitrosomonas*, *Nitrobacter*, *Klebsiella*, and *Pseudomonas* are involved, for example, in the N cycle including N<sub>2</sub> fixation, nitrification, denitrification, and ammonification (Pajares and Bohannan, 2016). On the other hand, microorganisms are involved in the land-atmosphere carbon (C) exchange, through the balance



between respiration and photosynthesis by carbon fixers, such as autotrophic (chemoautotrophic and photoautotrophic) microbes (Gougoulas et al., 2014). These communities are also important in the phosphorus (P) cycle, due to their ability to solubilize phosphates, i.e., fungal (some species of *Aspergillus* and *Penicillium*) and bacterial (some species of *Bacillus*, *Micrococcus*, and *Pseudomonas*) genera are involved in phosphate solubilization by the production of organic acids and the excretion of protons during  $\text{NH}_4^+$  assimilation (Mullen, 2019). Thus, in recent years, more attention has been paid to the function of microorganisms in agro-ecosystems, since they play a fundamental role in plant health and food production, as well as the improvement of soil fertility (Johansson et al., 2004).

## THE ROLE OF PLANT GROWTH-PROMOTING MICROORGANISMS (PGPM) IN FOOD PRODUCTION

The use of beneficial microbes, named plant growth-promoting microorganisms (PGPM), as microbial inoculants (biofertilizers) is a sustainable alternative to improve crop yields. PGPM (rhizobacteria, soil or endophytic bacteria, endo- or ectomycorrhizal fungi, cyanobacteria, and many others) can colonize soil and plants in significant quantities ( $10^5$ - $10^7$  CFU per gram of fresh root) and exert beneficial effects on plants through several mechanisms (Mishra et al., 2017). They can improve nutrient uptake, plant growth, and plant tolerance to abiotic and biotic stress, as well as biocontrol agents against plant pathogens and pests (Gangwar et al., 2017; Valenzuela-Ruiz et al., 2018).

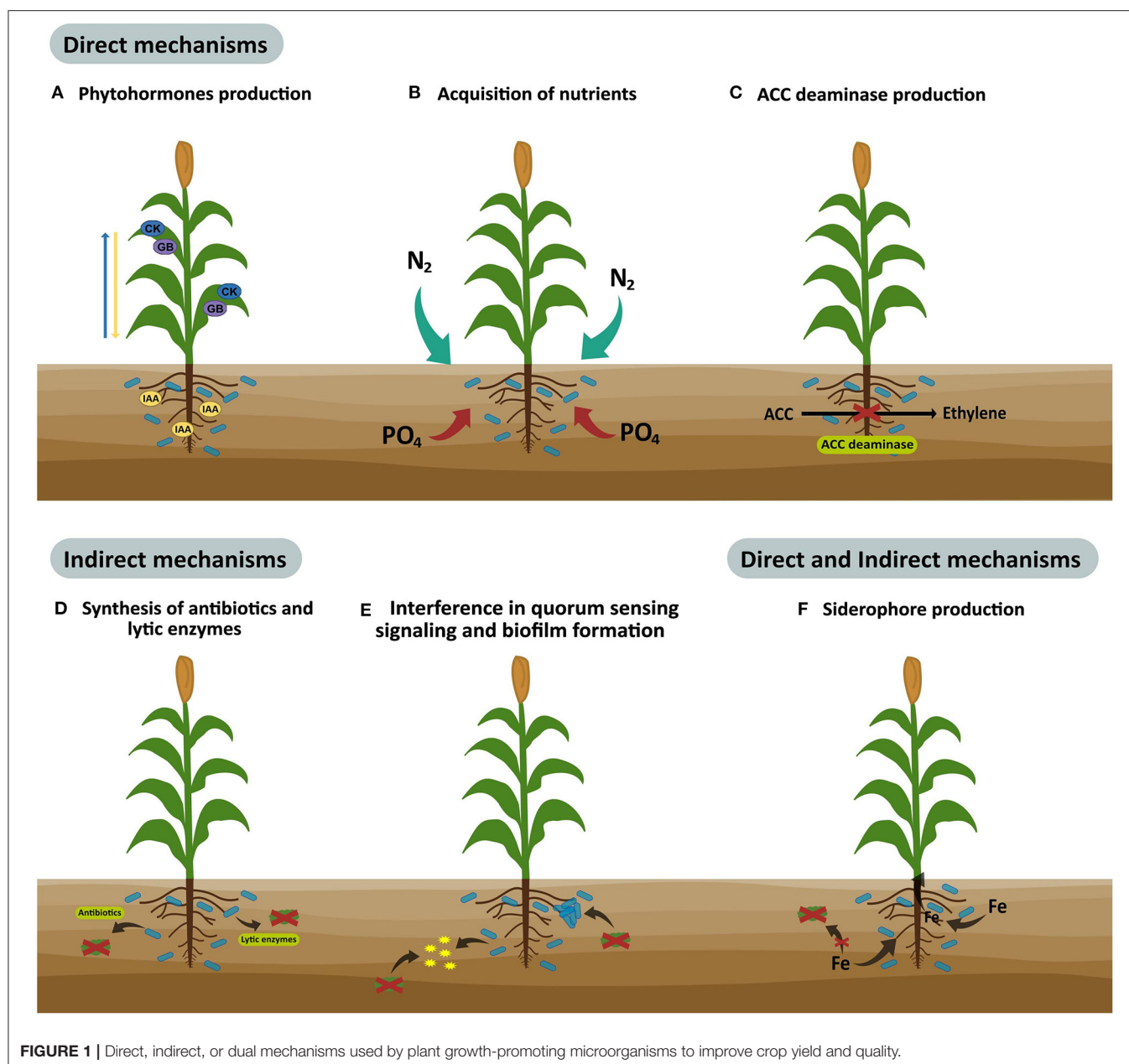
Microbial inoculants are biodegradable bio-products that contain living or inactive cells of PGPM with the ability to colonize the rhizosphere or inner part of plants, and perform growth-promoting effects on plants (Umesha et al., 2018; Singh et al., 2019). These bio-products are an eco-friendly way to improve the growth of plants by reducing the damage caused by phytopathogens or pests and improving their resistance to abiotic stress (Chávez-Díaz et al., 2020). The use of PGPM in agriculture has gained increasing interest and is currently a sustainable alternative for global food production due to their positive impacts on plant growth (Parewa et al., 2018), mitigation of the pollution generated by agrochemicals, and the reduction of soil degradation (Dubey et al., 2017; Mishra et al., 2017).

In this sense, the earliest microorganisms used as inoculants were the “rhizobia,” diazotrophic bacteria able to colonize the rhizosphere and establish nodules in the roots of host plants (Ciancio et al., 2016; Berg et al., 2017). Currently, several microbial genera are used in the formulation of microbial inoculants due to their metabolic diversity, i.e., many *Bacillus* species induce growth promotion in plants, control phytopathogens, and are spore-forming bacteria-resistant to stressful conditions (Villarreal-Delgado et al., 2018; Ibarra-Villarreal et al., 2021). Some members of the genus *Klebsiella* and *Rhizobium* can fix nitrogen, solubilize organic and inorganic phosphates, produce 1-aminocyclopropane-1-carboxylate (ACC)

deaminase, and produce phytohormones (Suliasih and Widawati, 2019). A large number of bacteria of the genus *Azospirillum* can produce phytohormones, polyamines, and fix atmospheric nitrogen (Cassán et al., 2014; Puente et al., 2018). Some strains of *Enterobacter* are biocontrol agents against phytopathogens, while some *Arthrobacter* strains can degrade a wide variety of compounds, including aromatic molecules, pesticides, and organochloride (Fernández-González et al., 2017). Besides, it has been reported that some *Burkholderia* species can bioremediate pollutants and are applied to biocontrol phytopathogens, can fix atmospheric nitrogen, and promote plant growth (Bolívar-Anillo et al., 2016). Some strains of *Serratia* induce plant growth by stimulating phytohormone production and phosphate solubilization, as well as inducing systemic resistance, enhancing salinity tolerance, and fixing atmospheric nitrogen (Devi et al., 2016; Singh and Jha, 2016; Moreno et al., 2018). However, these positive effects on crops caused by PGPM depend on the soil and climate conditions, crop genotype, plant phenological stage, root exudates, agricultural practices, the ability of PGPM to colonize the soil and plant tissues, and the application modes of beneficial strains, among others (Valenzuela-Aragon et al., 2018).

PGPM improve plant growth and health through direct, indirect, or dual mechanisms (Figure 1). They are considered to be direct mechanisms when bacteria begins to synthesize metabolites or when they increase the availability of nutrients to plants, the most common mechanisms being the production of phytohormones and ACC deaminase, nitrite production, sulfide oxidation, organic phosphate mineralization, and inorganic phosphorus solubilization. On the other hand, indirect mechanisms are related to the inhibition and elimination of phytopathogens through the competition for space and nutrients, the production of antibiotics, antimicrobial substances or lytic enzymes, interference in quorum sensing signaling, induced systemic resistance, and biofilm formation. Furthermore, there are mechanisms with dual activity (direct and indirect), where the most common is the production of siderophores, molecules that sequester iron making it available for the plants but less available to phytopathogens (Moreno et al., 2018; Parewa et al., 2018; Santoyo et al., 2019).

PGPM can exert more than one growth promotion mechanism and may be used in different stages of the plant growth cycle (Gupta et al., 2015; Parewa et al., 2018). For example, strains of *Pseudomonas fluorescens* are the most used bacteria for preventing plant diseases through antibiotics production, which also stimulate plant growth by amino acids, specific growth promoters, and the synthesis of hormones. Additionally, strains of *Bacillus subtilis* promote plant growth by phytohormones and cause resistance against *Fusarium oxysporum* in tomatoes, and *Rhizoctonia solani* and *Phytophthora nicotianae* in tobacco plants (Villarreal-Delgado et al., 2018). Also, *Trichoderma* species produce phytohormones and solubilize phosphates, generating growth-promoting effects, and the control of pathogenic fungi in soil (Peláez-Álvarez et al., 2016; Leal-Almanza et al., 2018). Arbuscular mycorrhizal fungi (AMF) are the most prevalent PGPM found in agricultural soils (Nadeem et al., 2014; Nath and Meena, 2018). These are root obligate symbiotrophs that provide essential nutrients, pathogen protection, and



**FIGURE 1** | Direct, indirect, or dual mechanisms used by plant growth-promoting microorganisms to improve crop yield and quality.

stress tolerance to the host plant (Begum et al., 2019). AMF, symbiotic, and free-living PGPM have beneficial effects that increase plant health, growth, and yields, under optimal or stressful conditions (Nadeem et al., 2014). Thus, synergistic interactions between AMF and other PGPM can enhance the nutrient uptake and growth-promoting effects, in some cases to a greater extent compared to individual microbial strains. For example, these microbial interactions improve the phosphorus bioavailability and absorption by plants (Nanjundappa et al., 2019), a mechanism used by these beneficial microbial consortia for solubilizing phosphates is organic acid production (acetic, lactic, gluconic, formic, and oxalic acids, etc.), which solubilize

phosphate rocks by acidification processes. Moreover, co-inoculated PGPM can produce chelating substances effective for calcium, aluminum, and iron causing the liberation of inorganic phosphate (Beltrán, 2014; Prabhu et al., 2019). On the other hand, the production of inorganic acids (sulfuric acid, nitric acid, and carbonic acid) by beneficial microbial consortia is involved in phosphate solubilization, but in the absence of acid production, the phosphate solubilization occurs by proton extrusion with nitrogen. The organic phosphate-solubilization by the co-inoculation of beneficial microbes is carried out by enzyme activity, once phosphatase and C-P lyase hydrolyze ester bonds of phosphonates (Prabhu et al., 2019).

Several different studies have suggested that microbial inoculants based on compatible microbial consortia may confer an advantage over formulations based on single strains (Egamberdieva et al., 2013). This is due to the ability for combining different functional metabolites and capabilities to improve soil quality and crop yield (Thilagar et al., 2016). For example, the co-inoculation in black bean (*Phaseolus vulgaris*) with *Trichoderma atroviride* and *Bacillus subtilis* showed a significant growth promotion in plant-dry biomass (43% over the control) compared to un-inoculated plants; while the individual inoculation of *T. atroviride* or *B. subtilis* increased only 2% or 34% in this trait, respectively (Yobo et al., 2011). Similarly, the co-inoculation of *Trichoderma asperellum* and arbuscular mycorrhizal fungi (*Gigaspora margarita* and *Acaulospora tuberculata*) showed a significant increase in plant height (>20 cm), as well as root and shoot fresh weights (>4.7 and >6.9 g/plant, respectively) compared to the un-inoculated treatment (Tchameni et al., 2011). The co-inoculation of *rhizobia*-*Azospirillum* in legumes showed a greater production of flavonoids in plants, which triggered the expression of the *Rhizobium* nod gene (Marks et al., 2013; Puente et al., 2018). Puente et al. (2019) confirmed that soybean seeds inoculated (at sowing) with *Bradyrhizobium japonicum* E109 and later foliar-sprayed with *A. brasilense* Az39 showed higher fresh and dry shoot biomass, more nodules, higher leghemoglobin levels, and increased nitrogen and protein content in grains, in comparison with the *B. japonicum* E109-alone treated seeds.

At present, several successful applications of microbial inoculants have been reported, with an increase of up to 10–30% in crop yields, and reductions of the amount of applied chemical fertilizers by up to 50% (Alori and Babalola, 2018; Parewa et al., 2018). In this way, the use of microbial inoculants is growing. The global market for biofertilizers is projected to reach 2.6 billion USD at a CAGR of 14.42% by the end of the year 2023 (Market Research, 2018). Thus, the extensive use of microbial inoculants is an alternative to improve soil fertility, crop quality, and yields, and contribute to mitigating the effects of climate change, as well as to increase the development of regional economies by the establishment of sustainable agribusiness (de los Santos-Villalobos et al., 2018). In this way, the preservation of isolated PGPM and their re-incorporation into agro-ecosystems is vital to maintain their ecological functions (Wood et al., 2015), for which their safeguarding in certified MCC makes them available for bioprospecting (de los Santos-Villalobos et al., 2018; Toader et al., 2019). Therefore, these MCC should focus on the study of metabolic, molecular, and functional traits of promising PGPM for developing sustainable agro-biotechnological alternatives (Smith, 2003; de los Santos-Villalobos et al., 2018).

## CONSIDERATIONS AND CHALLENGES IN THE USE OF MICROBIAL INOCULANTS

PGPM can be used as biofertilizers and/or biopesticides, depending on their genetic and metabolic diversity. However, several microbial strains can exhibit different results in the same plant species and vice versa. In this way, there are several

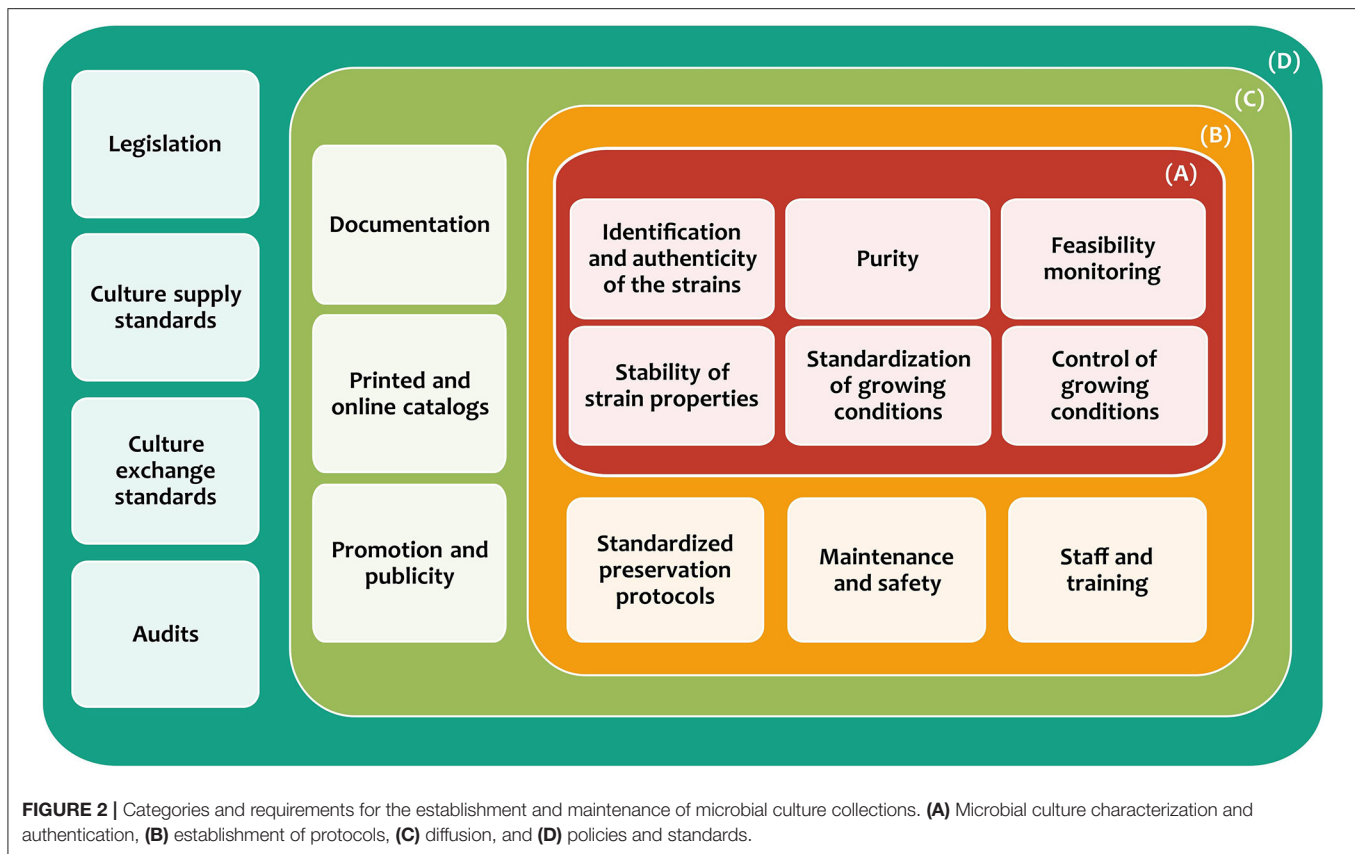
successful experiments at the laboratory and/or greenhouse level; however, those promising results sometimes are highly variable in the field, due to the complexity of the agro-ecosystem (Timmusk et al., 2017). Thus, it is important to select a specific microbial inoculant according to agroecosystem conditions, crop genotype, agricultural practices, climatic conditions, and expected benefits (Martínez-Viveros et al., 2010). The microbial inoculant efficiency depends on the establishment of its PGPM in the host plant and the rhizosphere, which is based on the inoculated PGPM-plant-microbiota competition mediated through the plant exudates (Jin et al., 2019). Besides, the climatic condition is another crucial factor involved in microbial inoculant effectiveness in the field. According to global estimates, abiotic factors lead to crop yield losses of 50%, temperature variations are the main contributor (27%); however, salinity (10%), drought (9%), and other forms of stress (4%) also affect crop yields and the success of microbial inoculants (de los Santos-Villalobos et al., 2018; Kaur et al., 2018; Ibarra-Villarreal et al., 2021). Still, little is known about the factors that control the competition of microbial species in the field; therefore, it is critical to have a higher number of cells to compete with native microorganisms and produce an increase in productivity (Compant et al., 2019). The estimated PGPM concentration required to cause positive effects in plants cannot be established as a general standard because it varies from one species to another; however, some microbial inoculants contain from  $10^7$  to  $10^9$  CFU/g of bioproduct, another influential factor is the lack of common international standards (Zayed, 2016).

On the other hand, the success of microbial inoculants depends on their application modes (plant tissues, rhizosphere, seeds, and/or bulk soil). These modes should be defined according to the type of crop, soil properties, climatic conditions, and agricultural practices in the field (Patil and Solanki, 2016; Kaushik and Djiwanti, 2019). Thus, it is important to highlight that the use of microbial inoculants in the field under different biotic and abiotic factors is a promising agro-biotechnological alternative to contribute to food safety sustainably.

## ROLE OF MCC IN THE PRESERVATION OF THE BENEFICIAL MICROBIAL DIVERSITY

The progress of microbiology brought the need to establish collections of microbial cultures to study and *ex situ* preserve the biodiversity in ecosystems and the distribution of promising microbial strains to produce goods and services. Subsequently, advances in molecular biology stimulated the development of intensive programs for bioprospecting all these microorganisms (Altier, 2013).

MCC, also known as biological resource centers (BRC), are primary suppliers of culturable microorganisms, replicable parts of these (i.e., DNA, genomes, and plasmids), and viable but not yet culturable microorganisms in biological or environmental matrices (Kurtzman and Labeda, 2009; Smith et al., 2014). According to Smith et al. (2014), the general concept of an MCC or BRC includes “the provision of services and repositories of the living cells, genomes of organisms, and information



relating to heredity and the functions of biological systems.” The importance of MCC has been recognized by the Organization for Economic Cooperation and Development (OECD), which is a promoter of policies to improve economic wellbeing and social welfare at an international level (Smith et al., 2014). Therefore, these MCC have two main functions: (i) the conservation of agro-ecosystems through the isolation and preservation of the microbial diversity, providing a biological safeguard service; and (ii) the study and easy access of microorganisms, reference strains, and microbial resources to the public to generate biotechnological strategies (Mahilum, 2009).

The first MCC that provided a public service was established in Prague in 1890 by Professor Frantisek Král. Some of the strains that were deposited for the first time in this culture collection are still available (Smith et al., 2014). Later, other culture collections were created, such as the Mycothèque de l'Université Catholique de Louvain (MUCL) established in 1894 in Belgium, the Collection of the Centraalbureau voor Schimmelcultures (CBS) in 1906 in Holland, both specialized in fungi. Then, the American Type Culture Collection (ATCC) was established in the United States in 1925, which preserves different types of microorganisms (Sharma et al., 2017). Until 1920, the main role of MCC was initially for their value in carrying out both taxonomic and epidemiological studies; now culture collections have adopted new technologies to characterize and add value to the services they offer. In addition, with the advancement of biochemical and physiological studies, the conservation of

microorganisms has been improving (Malik and Claus, 1987; Sharma and Shouche, 2014).

Thus, specific requirements have been established to maintain and ensure the quality and diffusion of all services provided by MCC (Smith, 1996; González and Jiménez, 2013), which can be divided into four categories (**Figure 2**). The first category is *microbial culture characterization and authentication* (A), which has the purpose of strain identification, as well as their preservation. This should ensure contamination-free cultures, the survival of at least 70% of cells, and stable maintenance of the preserved microorganisms (Escobar et al., 2016). The second category is the *establishment of protocols* (B), which focuses on the standardization of the protocols for the culture collections operation, from the preservation of different types of microorganisms to the maintenance of specialized equipment. Besides, it is necessary to have trained staff in cutting-edge technologies for the preservation, growth, and identification of microbes. The third category is *diffusion* (C), which is focused on all aspects related to achieving a greater scope and accessibility of the generated information (documentation and catalogs) to the general public. The last category is *policies and standards* (D), which seeks compliance with national and international laws, regulations, and policies about the safety, shipping, exchange of microorganisms, and other matters (González and Jiménez, 2013; Sharma and Shouche, 2014).

At present, the World Federation for Culture Collections (WFCC) is the main organization that coordinates the activities



**TABLE 1** | Examples of microbial culture collections around the world.

Culture collection name	Acronym	Country	Website
American Type Culture Collection	ATCC	United States of America	<a href="https://www.atcc.org">https://www.atcc.org</a>
German Collection of Microorganisms and Cell Cultures GmbH	DSMZ	Germany	<a href="https://www.dsmz.de">https://www.dsmz.de</a>
The National Collection of Type Cultures (NCTC) for bacteria	NCTC	United Kingdom	<a href="https://www.phe-culturecollections.org.uk/collections/nctc.aspx">https://www.phe-culturecollections.org.uk/collections/nctc.aspx</a>
Coordinated Collections of Microorganisms/LMG Bacteria Collection	LMG	Belgium	<a href="https://bccm.belspo.be/catalogues/lmg-catalogue-search">https://bccm.belspo.be/catalogues/lmg-catalogue-search</a>
Refik Saydam National Type Culture Collection	RSKK	Turkey	<a href="http://www.hsk.gov.tr">http://www.hsk.gov.tr</a>
NITE Biological Resource Center	NBRC	Japan	<a href="https://www.nite.go.jp/en/nbrc/index.html">https://www.nite.go.jp/en/nbrc/index.html</a>
All-Russian Collection of Microorganism	VKM	Russia	<a href="http://www.vkm.ru/Collections.htm">http://www.vkm.ru/Collections.htm</a>
Centraalbureau voor Schimmelcultures, Fungal and Yeast Collection	CBS	The Netherlands	<a href="https://wi.knaw.nl">https://wi.knaw.nl</a>
The Canadian Center for the Culture of Microorganisms	CCCM	Canada	<a href="http://cccm.botany.ubc.ca/">http://cccm.botany.ubc.ca/</a>
The Mexican Culture Collection of Microorganisms recognized as a biological resource center (BRC) by CONABIO	CDBB-500	México	<a href="http://cdbb.cinvestav.mx/cdbb/index.html">http://cdbb.cinvestav.mx/cdbb/index.html</a>
Colección de Microorganismos Edáficos y Endófitos Nativos	COLMENA	México	<a href="http://www.itson.mx/colmena">http://www.itson.mx/colmena</a>
The Brazilian Collection of Environmental and Industrial Microorganisms	CBMAI	Brazil	<a href="https://cbmai.cpqba.unicamp.br">https://cbmai.cpqba.unicamp.br</a>
Bioresource Collection and Research Center	BCRC	Taiwan	<a href="http://www.bcrc.firdi.org.tw/">http://www.bcrc.firdi.org.tw/</a>
Collection Nationale de Cultures de Microorganismes	CNCM	France	<a href="https://research.pasteur.fr/en/team/national-collection-of-cultures-of-microorganisms/">https://research.pasteur.fr/en/team/national-collection-of-cultures-of-microorganisms/</a>
Agricultural Research Service Culture Collection	NRRL	United States of America	<a href="http://nrri.ncaur.usda.gov/">http://nrri.ncaur.usda.gov/</a>

of MCC worldwide (Sharma et al., 2017). It aims to promote and support the establishment and monitoring of MCC, by providing an information network between affiliated collections and users (Federación mundial de Colecciones de Cultivo, 2010). The WFCC supports the World Data Center of Microorganisms (WDCM) for the compilation of culture collection data, management, services provided, and most recent research with an online international database (Sharma et al., 2017). Currently, the WDCM lists 802 collections in 78 regions, of which 295 are located in Asia, 257 in Europe, 202 in America, 42 in Oceania, and 18 in Africa. The country with the largest number of MCC is Brazil (86), followed by Thailand (66) and China (48) (WDCM, 2020a). These MCC provide different services, such as: consulting, strain distribution, strain identification, patent deposits, storage service, and training (Sharma and Shouche, 2014; WDCM, 2020a). In this way, around the world, culture collections play a fundamental role in the preservation of microbial diversity, and the accessibility of axenic and stable promising strains for agriculture, genetics, industrial and medical microbiology, marine biology, and food safety, among others (Table 1) (Sharma et al., 2017).

The conservation of biodiversity and genetic resources provide essential support for emerging eco-efficient biotechnologies, in both the developed and developing world (OECD, 2009). The most economically powerful countries with the greatest scientific-technological development have created highly-specialized institutions to harbor large culture collections (approximately 98% of known microorganisms are preserved) such as the United States of America, China, Belgium, Japan, and India (WDCM, 2020a). Besides, many countries or individual institutions obtain official support for these culture collections; however, the establishment of MCC requires a constant source of funding that not all of them can obtain. Also, the records that include MCC must be digitized for use in international schemes (CGREA, 2007). For example, in the region of Latin America and the Caribbean, there are valuable specialized MCC created and maintained by universities and research institutes; however, there is not a systematized information source about their progress, limitations, efforts, and initiatives.

Globally, several MCC are recognized for their role in the preservation of microorganisms with international or regional importance, which has a high impact on biotechnology at all

levels, including food security (Table 1). For example, WDCM (at an international level) promotes the development of culture collections of microorganisms supporting the existing ones by providing advice and assistance (González and Jiménez, 2013). Similarly, ATCC safeguards and distributes standard reference microorganisms since they have a wide variety of high-quality biological materials (microorganisms, cell lines, and bioproducts) intended for scientific research (ATCC, 2020). One more example is the Colección de Microorganismos Edáficos y Endófitos Nativos (COLMENA), which is focused on the preservation, characterization, classification, and re-incorporation of native microorganisms to agro-ecosystems in Mexico, to reduce the loss of microbial diversity associated with food production (de los Santos-Villalobos et al., 2018). At present, a total of 3,272,734 microorganisms have been registered in the WDCM, of which 1,429,816 are bacteria (43.7%); 872,094 are fungi (26.6%); 39,491 are viruses (1.2%); and 33,020 are cell lines (1.0%) (WDCM, 2020a). Also, the WDCM has elaborated a catalog of reference strains for easy access, which are recommended for their use in quality control, regulated by different ISO standards. For this, the WDCM assigns a unique identification number to every reference strain, while the culture collection acronyms followed by a number are used as an identifier (at the strain level) for its global identification and tracking (Wu et al., 2016). This catalog contains a total of 196 reference strains of 131 species of bacteria (Table 2), fungi, and yeast (Table 3) (WDCM, 2019). Currently, according to scientific literature, many microbial species preserved in the WDCM could be grouped as PGPM, due to their large diversity of metabolic capabilities with wide agro-biotechnological applications. However, the number of AMF preserved in MCC is lower; probably due to limitations in the specific conservation methodologies since these microorganisms depend on their host plant to complete their life cycle, which makes it difficult to grow in pure synthetic media (Lalaymia et al., 2013). Thus, only two MCC (registered in WFCC) are specialized in these types of microorganisms holding 1,302 AMF strains in total (WDCM, 2020b). Therefore, it is necessary to improve short and long-term preservation methodologies for these microorganisms, which (like others PGPM) play a fundamental role in the growth regulation and nutrients acquisition by plants in ecosystems (Lalaymia et al., 2013; Begum et al., 2019).

On the other hand, the offered services by most of the MCC around the world are limited to the preservation, deposit, and transfer of microorganisms, and do not thoroughly study the strain capabilities; usually, this is left only to interested researchers or professionals. For this reason, more MCC focused, not only on microbial preservation, but also dedicated to the research, teaching, and characterization of promising strains for biotechnological applications are needed (de los Santos-Villalobos et al., 2018). Furthermore, only a few culture collections have a public catalog containing information about preserved microorganisms grouped by function, biosafety, taxonomy, and biotechnological applications, among others. This is a strong limitation for the extensive use of PGPM as microbial inoculants since it complicates the selection of promising strains. For this reason, MCC must also make the biological data of

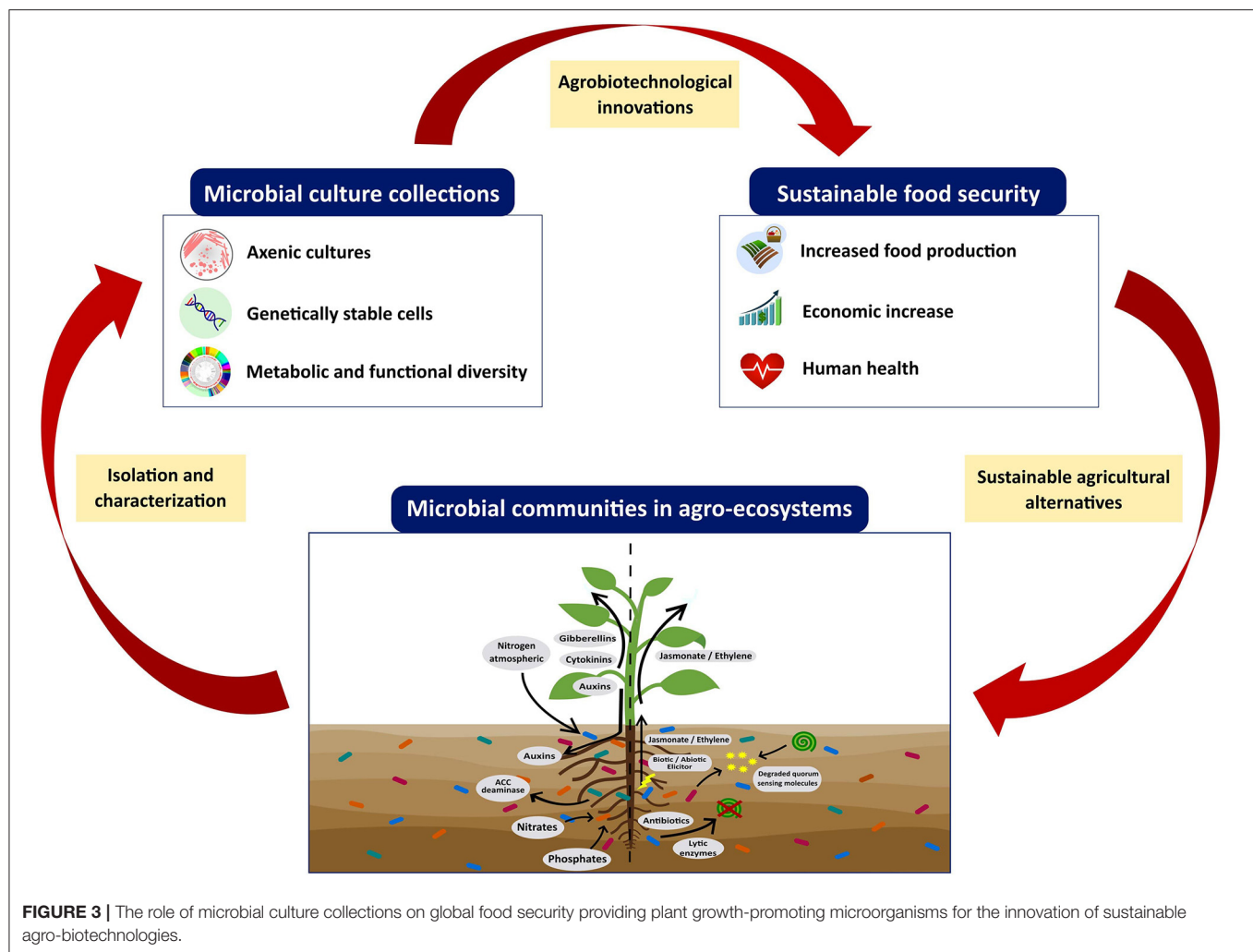
**TABLE 2 |** The number of bacterial species in the WDCM reference strain catalog that have identifier numbers for the reference strains (WDCM, 2019).

Genus	Species Identifiers		Genus	Species Identifiers	
Bacteria					
Acinetobacter	1	3	Lactococcus	1	18
Aerococcus	1	13	Legionella	3	25
Aeromonas	3	29	Leuconostoc	1	10
Arcobacter	3	22	Listeria	6	54
Bacillus	8	92	Micrococcus	1	16
Bifidobacterium	3	13	Morganella	1	11
Brochothrix	1	11	Pantoea	1	9
Campylobacter	4	45	Pediococcus	2	20
Camobacterium	2	18	Photobacterium	1	9
Citrobacter	1	18	Proteus	1	9
Clostridium	5	41	Pseudomonas	4	120
Cronobacter	2	7	Rhodococcus	1	17
Desulfotomaculum	1	6	Salmonella	6	60
Enterobacter	1	20	Shigella	2	20
Enterococcus	3	109	Staphylococcus	4	146
Escherichia	2	77	Streptococcus	2	18
Geobacillus	1	10	Thermoanaerobacterium	1	4
Hafnia	1	11	Vibrio	6	57
Klebsiella	4	34	Weissella	1	11
Lactobacillus	8	102	Yersinia	4	32

**TABLE 3 |** The number of filamentous fungi and yeasts species in the WDCM reference strain catalog that have identifier numbers for the reference strains (WDCM, 2019).

Genus	Species Identifiers		Genus	Species Identifiers	
Filamentous fungi and yeasts					
<i>Aspergillus</i>	5	37	<i>Penicillium</i>	5	18
<i>Candida</i>	1	30	<i>Pleurotus</i>	1	4
<i>Cladosporium</i>	1	4	<i>Rhizopus</i>	1	3
<i>Coniophora</i>	1	7	<i>Saccharomyces</i>	1	26
<i>Eurotium</i>	2	5	<i>Serpula</i>	1	2
<i>Gloeophyllum</i>	1	5	<i>Trametes</i>	1	11
<i>Lentinus</i>	1	2	<i>Trichophyton</i>	1	3
<i>Mucor</i>	1	2	<i>Wallemia</i>	1	2
<i>Oligoporus</i>	1	6	<i>Zygosaccharomyces</i>	1	3

microorganisms more accessible to the community, including (i) taxonomy, (ii) source of isolation, including the biological and environmental context in which strains were found, (iii) evolution, (iv) metabolic-functional traits, and (v) ecological relationship. All these data should be accessible to the general public through an online catalog (databases) with open access to make it easier to transfer microbial strains (Caktu and Turkoglu, 2011; Cruz-Leyva et al., 2015). However, at present, only 53% of MCC have a digital catalog of preserved microorganisms (WDCM, 2020a), the digitization and update of information for each preserved microorganism is one of the major limitations of culture collections. Thus, the expected positive impacts of preserved PGPM in MCC to global food security will be reached when, at least, three stages are linked: (i) the isolation of



microbial strains in agro-ecosystems, (ii) the preservation of these strains in an MCC, and (iii) bioprospecting these promising strains to transfer and develop sustainable agro-biotechnological alternatives (Figure 3). Finally, rules and procedures have been established for the transfer and exploitation of genetic resources preserved in MCC, such as (i) the Budapest Treaty that mentions the International Recognition of the Deposit of Microorganisms for the Patent Procedure, established in 1980 (Organización Mundial de la Propiedad Intelectual (OMPI), 2017); and (ii) the Nagoya Protocol (implemented in October 2014), which aims for the fair and equitable sharing of the benefits derived from the use of genetic resources (Sharma et al., 2017). The Budapest Treaty regulates the process of patent deposits in an international framework; thus, a patent deposit made with one International Depositary Authority (IDA) is sufficient and recognized by all member states of Budapest. Nonmember countries may also accept deposits according to the Budapest Treaty as per the norms of the country (Bussas et al., 2017). On the other hand, the Nagoya Protocol is an agreement that complements the Convention on Biological Diversity. It provides a transparent and legal framework to effectively implement the beneficial

sharing between providers and users of genetic resources, by contributing to the establishment of more predictable conditions to access the genetic resources, conservation, and sustainable uses of biodiversity (Convention on Biological Diversity, 2015).

## CONCLUSIONS

The concern for sustainable food production that meets the needs of the global human population has become a crucial issue in the agricultural sector. Thus, the development of new sustainable agro-biotechnological alternatives to increase crop yields and quality, and restore soil fertility, is vital to satisfy the current and future food demand worldwide. In this way, several PGPM represent a feasible and eco-friendly strategy to combat challenges of food security and climate change, due to their metabolic and genetic diversity. Regarding the agro-biotechnological potentials of PGPM, microbial communities in agro-ecosystems have become more relevant in recent years for the development of new microbial inoculants. These bioproducts can increase nutrition and stress tolerance in

crops. Thus, the use of microbial inoculants can contribute to reducing the economic, environmental, and health costs of food production, but there are many considerations and challenges to address in order for them to be effective in the field. Besides, since there is no single universal solution for all these issues, the integration of microbial inoculants with other technologies (sustainable soil management, new genetic varieties of crops, and efficient use of agro-inputs, among others) are necessary to enhance the expected positive effects.

In this sense, the success of the extensive use of microbial inoculants depend on the isolation, identification, and characterization of promising PGPM, which should be preserved in MCC for (i) the *ex situ* conservation of these biological components of agro-ecosystems, (ii) the study of these microbial resources, and (iii) the transfer of these biological cultures to develop new agro-biotechnologies. Thus, it is essential to solving bottlenecks in the functioning of MCC, mainly the digitization, easy access, and dissemination of biological information for each preserved microbial strain (including metabolic and genetic diversity). This will lead to the extensive use of PGPM for the development of new strategies to increase sustainable food production. Thus, the role of global MCC is vital to provide biological resources that directly support soil health, sustainable food generation, and the wellbeing of the human population.

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## AUTHOR CONTRIBUTIONS

Conceptualization, supervision, and validation were developed by SS-V. All authors contributed to manuscript revision, and have read and approved the submitted version. All authors wrote, visualized, and edited the manuscript.

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# Contrasting Expression of Rhizobial Phytase in Nodules of Two Soybean Cultivars Grown Under Low Phosphorus Availability

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Phosphorus deficiency can be a major limitation to legume growth when plant nitrogen nutrition depends on symbiotic nitrogen fixation. One possible approach to overcome this constraint is the selection of plant and rhizobial genotypes capable of metabolizing complex forms of phosphorus in the nodules. The aim of this research was to study the rhizobial phytase transcript abundance in nodules of two soybean cultivars (*Glycine max* (L.) Merr.) grown under two different phosphorus conditions in hydroaerobic conditions. An *in situ* RT-PCR of a rhizobial phytase was performed in microtome sections of soybean nodules of two cultivars growing under phosphorus sufficiency vs. phosphorus deficiency. The results showed that the plant cultivar may influence the level of transcript abundance of the bacterial phytase and in consequence affect the phosphorus use efficiency of nitrogen-dependent *Bradyrhizobium* spp.-soybean symbioses. Thus, the selection of a good combination of plant and rhizobial genotypes should be a priority when breeding for phosphorus deficiency is performed.

**Keywords:** nodule RT-PCR *in situ*, phytase, phytate mineralization, rhizobia, soybean, symbiotic nitrogen fixation, sustainability

## INTRODUCTION

Phosphorus (P) is the second limiting element for plant growth after nitrogen (N), thus being an essential nutrient for agricultural production. Total soil P, in both inorganic and organic forms, is abundant in most soils (Richardson, 2001). However, only 1–5% of the total P is dissolved in the soil solution as orthophosphate ions,  $\text{HPO}_4^{2-}$  or  $\text{H}_2\text{PO}_4^-$ , and absorbed by plants (Richardson and Simpson, 2011; Khan et al., 2013). Despite being often overlooked, soil organic P (Po) comprises 30–50% of soil total P (Hinsinger et al., 2015), whether as part of the biomass or associated to the soil organic matter (Maougal et al., 2014a). Phytates, in particular, may represent up to 80% of the total Po (Quiquampoix and Mousain, 2005). However, organic forms of P are unavailable to plants unless mineralization takes place (Lazali et al., 2013). Phytases are enzymes responsible for the mineralization of phytates. They are classified according to their hydrolytic mechanism,



the substrate specificity, their protein structure and optimal pH: histidine acid phosphatase (HAP) (EC 3.1.3.8),  $\beta$ -propeller phytase (BPP) (EC 3.1.3.8), cystein phytase (CPhy) and purple acid phytase (PAP) (EC 3.1.3.2) (Mullaney and Ullah, 2003; Jorquera et al., 2008a). There are a lot of rhizospheric bacterial genera that can mineralize phytate such as *Enterobacter*, *Pseudomonas*, *Burkholderia*, *Bacillus* and *Klebsiella*, among others (Yoon et al., 1996; Richardson and Hadobas, 1997; Richardson, 2001; Hill et al., 2007; Jorquera et al., 2008b; Maougal et al., 2014a; Ramesh et al., 2014). Several plant species have the ability to increase the activity of phytases in the rhizosphere in response to P deficiency (Li et al., 1997).

P has a crucial role for symbiotic nitrogen fixation (SNF) and the subsequent ammonium assimilation, which take place in legume nodules; both processes are highly energy-consuming and depend on the energy status of nodules (Araújo et al., 2008; Lazali et al., 2016). P deficiency comprises a limitation for SNF in legumes (Graham et al., 2003), resulting in a decrease in SNF and, consequently, in slow plant growth (Israel, 1987). Previous reports showed that mutations on three *Sinorhizobium meliloti* P transport systems, OrfA-Pit, PstSCAB, and phoCDET, failure to obtain sufficient P for growth during the infection process, forming “empty nodules,” which contain very few infected cells, in the alfalfa (*Medicago sativa*) symbiont and fail to fix  $N_2$  (Bardin et al., 1996; Yuan et al., 2006). Although, the expression of one of these transports could be necessary and sufficient for SNF in alfalfa (Yuan et al., 2006). The content of P is greater in nodules than in other plant organs under conditions of either P sufficiency or deficiency (Tang et al., 2001; Schulze et al., 2006). Legumes allocate different type of P sources in nodules, and leaves start to deplete before nodules, when they grow under P deficiency (Suliman et al., 2010). It has been reported for different legumes, such as *M. sativa*, *Medicago truncatula*, soybean (*Glycine max*), common bean (*Phaseolus vulgaris*), pea (*Pisum sativum*), and *Sophora flavescens*, that nodules become a P sink under P deficient growing conditions (Sa and Israel, 1991; Kouas et al., 2005; Suliman et al., 2013a; Hu et al., 2018).

For the above-stated reasons, P deficiency is a major limitation to legume production in which N nutrition depends on SNF (Araújo et al., 2008). Effective approaches to P deficiency include: (1) increasing P acquisition by mechanisms such as root exudation and morphology (Gahoonia et al., 2007; Ao et al., 2010; Cheng et al., 2011); (2) inoculation with mycorrhizal fungi or with P-solubilizing and/or mineralizing bacteria or fungi (Rosas et al., 2006; Bucher, 2007; Elkoca et al., 2007; Harvey et al., 2009); (3) selecting plant genotypes with greater enzymatic activity, capable of metabolizing phosphate compounds in the nodules (Araújo et al., 2008; Lopez-Arredondo et al., 2014; Drevon et al., 2015; Castro-Guerrero et al., 2016); (4) selecting specific rhizobia with P-mineralizing abilities, capable of metabolizing complex forms of P in the nodules, such as phytate (Lazali et al., 2016).

The present study analyzes this last approach, aiming to test if one of the two strains used to inoculate Uruguayan soybean (SEMIA 587) could mineralize the phytate present in soybean nodules, increasing P content and therefore the efficiency of the SNF. In Uruguay, SNF has been upheld as a long-term public policy since the 1960's (Altier et al., 2013). As a result

of research and outreach policies, 100% of the farmers have adopted the use of inoculation technology (Lindström et al., 2010). In Uruguay, there are two officially recommended strains for soybean inoculation, *Bradyrhizobium elkanii* strain SEMIA 587 and *Bradyrhizobium elkanii* strain SEMIA 5019 (syn. 29w) (Olivera et al., 2016). Specific references of these two strains can be found in Hungria et al. (1998). First, we tested the ability of the strain SEMIA 587 to mineralize phytate *in vitro* and afterwards, we studied the rhizobial phytase transcript abundance in soybean nodules for a better understanding of the role of this enzyme in the acquisition and cycling of phytate in soybean. We also addressed whether the transcript abundance levels significantly vary between two cultivars.

## MATERIALS AND METHODS

### Growth Curves

The ability of the strain *Bradyrhizobium elkanii* SEMIA 587 to use phytate was tested at pH 6 and 7 by inoculating the following Angle medium (Angle et al., 1991) modified by Richardson and Hadobas (1997): 1 mM  $KNO_3$ , 2 mM  $MgSO_4 \cdot 7H_2O$ , 4 mM  $CaSO_4$ , 55 mM glucose, 50 mg  $L^{-1}$  thiamine-HCl, 0.5 mL  $L^{-1}$  Fe-citrate 1%, 0.2 mL  $L^{-1}$  micronutrient solution containing per liter (2.82 g  $H_3BO_3$ , 98 mg  $CuSO_4 \cdot 5H_2O$ , 3.08 g  $MnSO_4 \cdot H_2O$ , 0.29 g  $NaMoO_4 \cdot 2H_2O$ , 4.41 g  $ZnSO_4 \cdot 7H_2O$ ) and 6 mM Na-phytate (Sigma, ref P0109). The optical density at 600 nm was measured every 24 h during seven days (168 h). The above-mentioned medium supplemented with 1.5 mM  $KH_2PO_4$ , instead of Na-phytate, was used as control. Three replicates for both growing conditions were used.

### Rhizobial HAP Phytase Gene Primers Design

The design of the rhizobial HAP phytase primers was performed online at the National Center of Biotechnology Information (NCBI, <http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The highest hit by BLASTp to rhizobiaceae group of a known amino acid sequence of the *Escherichia coli* HAP phytase (GenBank accession number: AAN28334.1) was obtained with a conserved hypothetical HAP-type phytase of *Azorhizobium caulinodans* (GenBank accession number: 5692057 AZC\_4319). The gene sequence of this hypothetical HAP-type phytase was obtained from *A. caulinodans* full genome (GenBank accession number: AP009384.1). Thereafter, the following pair of primers were designed with the primer-BLAST tool from this HAP gene-sequence: forward primer (HAPfor), 5'CAGTTCACGCCAAAGATGCC3'; reverse primer (HAPrev), 5'CGCGTATGGTCCATCCTGAA3'. Reverse primer containing three consecutive mismatched bases near its 3' terminus can hybridize to the target RNA at a temperature (in this study: 42°C) which is not high enough for DNA denaturation. Furthermore, due to the lower affinity between this mismatched primer and genomic DNA (gDNA), it is a very inefficient primer for gDNA amplification at high annealing temperatures (in this study: 62°C). Consequently, the mismatched primer can be used to selectively amplify the newly synthesized complementary DNA

(cDNA), even in the presence of high quantities of gDNA (Koo and Jaykus, 2000).

### **In vitro RT-PCR of Rhizobial Phytase Transcripts**

HAP designed primer pair along with a primer pair that amplifies BBP described by Farhat et al. (2008) (BPPfor, 5'GATGCAGCTGATGATCCTGCG3'; BPPrev, 5'ATTTTCTCCGTCCTGTGCGAC3'), were used to amplify *B. elkanii* SEMIA 587 phytases expressed in presence of phytate as unique P source. *B. elkanii* SEMIA 587 RNA and gDNA were extracted with the *RNeasy Plus* and *DNeasy Plant Mini Kits* (QIAGEN), respectively. RNA was extracted from *B. elkanii* SEMIA 587 cultivated in Angle medium supplemented with Na-phytate and  $\text{KH}_2\text{PO}_4$ .

Retrotranscription reactions were carried out in 25  $\mu\text{L}$ , but first, 1  $\mu\text{g}$  RNA and 3  $\mu\text{L}$  of 10  $\mu\text{M}$  HAP or BPP reverse primers were incubated at 70°C during 5 min and at 4°C for 2 min. Then, 5 X Buffer (Promega), 10 mM of each dNTP, 0.1% bovine serum albumin (BSA), and 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase H- (Promega), were added and incubated at 42°C during 60 min.

A touchdown PCR was performed with HAP and BPP primers and with cDNA and gDNA as templates, to test if at different temperatures it was able to amplify a phytase. PCR reactions were carried out in 25  $\mu\text{L}$  volumes containing 1  $\mu\text{L}$  target, 10 X Buffer (Invitrogen), 50 mM  $\text{MgCl}_2$ , 10 mM of each dNTP, 10  $\mu\text{M}$  forward primer (HAP or BPP), 20  $\mu\text{M}$  reverse primer (HAP or BPP), 0.1% bovine serum albumin (BSA), 5 U Taq Polymerase (Invitrogen), under the following thermal conditions: 95°C for 2 min, 30 cycles of 95°C for 30 s, 70°C for 45 s, and gradually reduced 1°C till reaching 60°C, 72°C for 45 s, with a final extension at 72°C for 3 min.

When PCR products were obtained in the touchdown PCR, a gradient PCR was performed with cDNA and gDNA as templates, to obtain the optimal hybridization temperature of primers. PCR reactions were carried out in 25  $\mu\text{L}$  volumes containing 1  $\mu\text{L}$  target, 10 X Buffer (Invitrogen), 50 mM  $\text{MgCl}_2$ , 10 mM of each dNTP, 10  $\mu\text{M}$  forward primer, 20  $\mu\text{M}$  reverse primer, 0.1% BSA, 5 U Taq Polymerase (Invitrogen), under the following thermal conditions: 94°C for 3 min, 35 cycles of 94°C for 30 s, 64–59°C for 45 s, 72°C for 45 s, with a final extension at 72°C for 3 min. Consequently, the optimal hybridization temperature of primers HAP was 62°C.

All amplification products were checked by electrophoresis in 1% agarose gel.

### **Hydroaerobic Culture of Soybean**

Two commercial soybean cultivars, namely N5909 (Nidera) and SR532 (Santa Rosa), were used in this study. SR532 and N5909 have a short and long production cycle, respectively. *B. elkanii* SEMIA 587 was used to inoculate these two cultivars. Before sowing, the soybean seeds were surface sterilized, first with 95% ethanol for 3 min and afterwards, with 3% NaOCl for 3 min more. Then, seeds were rinsed five times with sterile distilled water. Later, seeds were imbibed in sterile distilled water for 4 h, and then transferred for germination on sterile 15 g  $\text{L}^{-1}$  agar-water plates (Beyhaut et al., 2006). After germination, inoculation

was performed by 30 min soaking 10-day-old seedlings in a suspension of *B. elkanii* SEMIA 587 containing  $\sim 10^8$  cfu  $\text{mL}^{-1}$ . The inoculum was prepared by growing the strain SEMIA 587 in yeast extract mannitol broth during four days at 28°C at 120 rpm.

Twenty inoculated seedlings were transferred into each container, with base dimensions 0.2 × 0.4 m and height 0.2 m, refilled weekly with 40 L of Vadez et al. (1996) nutrient solution. In order to avoid initial N deficiency and ensure optimal nodulation, urea was supplied at 1 mmol  $\text{plant}^{-1}$  during the initial 15 days of growth. Thereafter, the plants were grown in N-free solution. They were grown under both deficient and sufficient P supply in hydroaerobic conditions with a permanent flow of 400  $\text{mL min}^{-1}$  of compressed air to ensure the oxygenation of the culture solution (Hernandez and Drevon, 1991). P was supplied weekly in the form of  $\text{KH}_2\text{PO}_4$ , at 75 vs. 250  $\mu\text{mol plant}^{-1}$  as P deficiency vs. P sufficiency, respectively (Vadez et al., 1996). The pH was adjusted to 6.8 with 0.2 g  $\text{L}^{-1}$   $\text{CaCO}_3$  (Hernandez and Drevon, 1991). The experiment was carried out in a greenhouse, 28/20°C under 16/8 h day/night cycle, with an additional light supply of 400  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  and 70% relative humidity during the day.

### **Shoot, Root and Nodule Biomass**

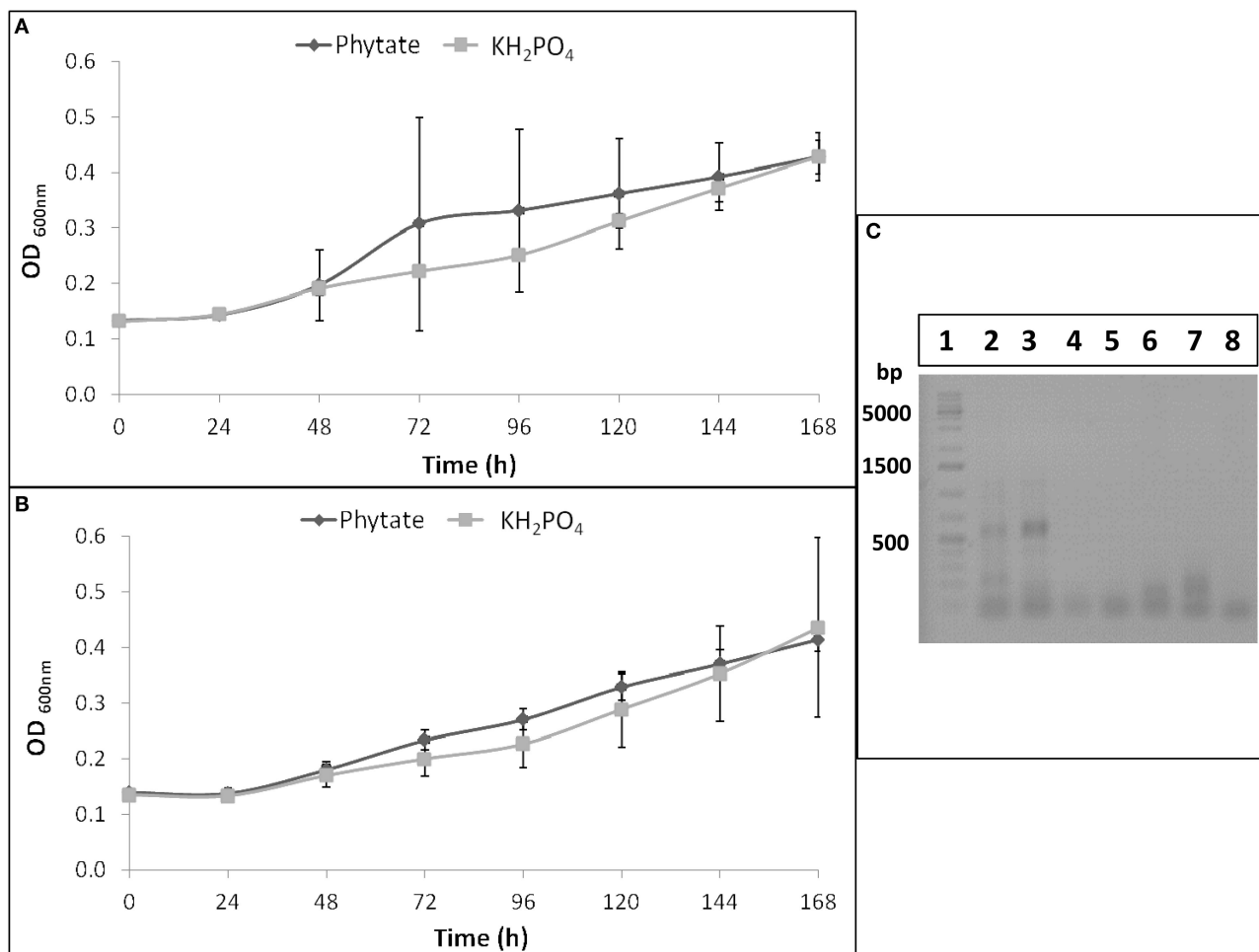
Forty two days after transfer (DAT), soybean plants were harvested and shoot, roots and nodules were separated. Dry weights (DW) were determined after drying at 70°C for three days. Shoot accumulated P was determined by the vanadomolybdate method in Laboratorio Oriental (<http://labo.com.uy/>).

The efficiency in use of the rhizobial symbiosis (EURS) was estimated by the slope of the regression model of shoot biomass as a function of nodule biomass ( $y = ax + b$ ), where  $a$  corresponds to the EURS and  $b$  corresponds to the plant biomass production without nodules (Zaman-Allah et al., 2007).

### **In situ RT-PCR of Rhizobial Phytase Transcripts**

The *in situ* RT-PCR was made according to Lazali et al. (2013) and Maougal et al. (2014b). Three nodules of 3 mm diameter of three plants of each cultivar and P treatment were carefully detached from roots at 42 DAT. They were thoroughly washed with diethyl pyrocarbonate (DEPC) treated water, then fixed in 4% paraformaldehyde, 45% ethanol and 5% acetic acid, kept for 2 h under vacuum, and stored overnight at 4°C. Fixed nodules were extensively rinsed with two baths of DEPC treated water during 5 min each, two baths of 1 X phosphate buffered saline (PBS, 5 mM  $\text{Na}_2\text{HPO}_4$ , 300 mM NaCl, pH 7.5) during 10 min each, one bath of 1 X PBS plus 0.2% glycine during 10 min and one last bath with 1 X PBS during 10 min.

Thereafter, the fixed nodules were dehydrated in baths of increasing concentrations of ethanol and butanol: one bath of 50% ethanol during 30 min; two baths of 70% ethanol during 30 min and 1 h; one bath of 70% of ethanol overnight at 4°C; two baths with 100% ethanol for 1 h; and in the end, at least, three baths with 100% butanol during one week. Then, the nodules were included in paraffin and cut into transversal 12  $\mu\text{m}$  thick sections using a microtome (Micro-cut H1200 Vibrating Microtome). The resulting sections were placed on slides treated



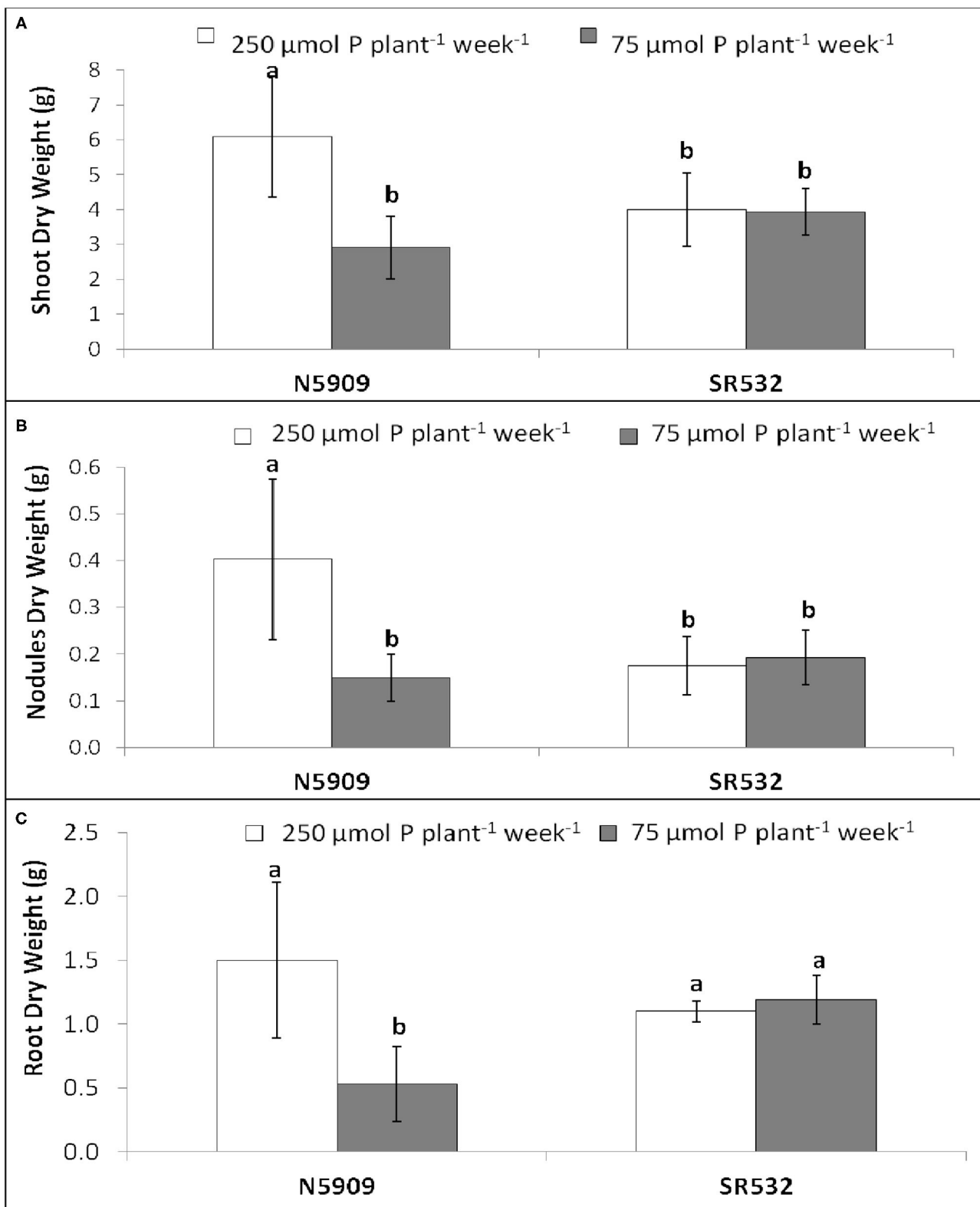
**FIGURE 1 |** *B. elkanii* SEMIA 587 growth curves in Angle medium at pH 6 (A) or at pH 7 (B) supplemented with 1.5 mM KH<sub>2</sub>PO<sub>4</sub> (light gray) or with 6 mM Na-phytate (dark gray). Data represent average and standard deviation of three replicates. (C) RT-PCR or PCR products of *B. elkanii* SEMIA 587 grown in Angle medium supplemented with Na-phytate or with KH<sub>2</sub>PO<sub>4</sub> as P source in 1% agarose gel. Lane 1, GeneRuler 1 kb Plus DNA Ladder (Thermo Scientific); Lane 2, RT-PCR performed with HAP primers and cDNA as template obtained from SEMIA 587 cultivated with phytate as P source; Lane 3, RT-PCR performed with HAP primers (and BSA) and cDNA as template obtained from SEMIA 587 cultivated with phytate as P source; Lane 4, RT-PCR performed with BPP primers and cDNA as template obtained from SEMIA 587 cultivated with phytate as P source; Lane 5, PCR performed with HAP primers (and BSA) and gDNA as template obtained from SEMIA 587 cultivated with phytate as P source; Lane 6, PCR performed with BPP primers and gDNA as template obtained from SEMIA 587 cultivated with phytate as P source; Lane 7, RT-PCR performed with BPP primers (and BSA) and cDNA as template obtained from SEMIA 587 cultivated with KH<sub>2</sub>PO<sub>4</sub> as P source; Lane 8, RT-PCR performed with BPP primers and cDNA as template obtained from SEMIA 587 cultivated with KH<sub>2</sub>PO<sub>4</sub> as P source.

with silane to increase cell adhesion. Thereafter, the sections were deparaffinated through three baths of Safesolv (Q Path®) for 10 min each, one bath of Safesolv during 5 min; three baths of 100% ethanol during 10 min each; one bath of 70% ethanol during 5 min; one bath of 50% ethanol during 5 min. This was continued by section rehydration with one bath of 1 X PBS during 10 min, one bath of 1 X PBS plus glycine 0.2% during 2 min, one bath of 1 X PBS during 10 min and one bath of 1X PBS for 5 min at 65°C.

The first cDNA strand was synthesized from total RNA of nodular sections, that were incubated at 42°C during 1 h in 100 µL reverse transcriptase mix containing 5 X Buffer (Promega), 10 mM of each dNTP, 10 µM HAPrev, 0.1% BSA, and 200 U M-MLV reverse transcriptase H- (Promega).

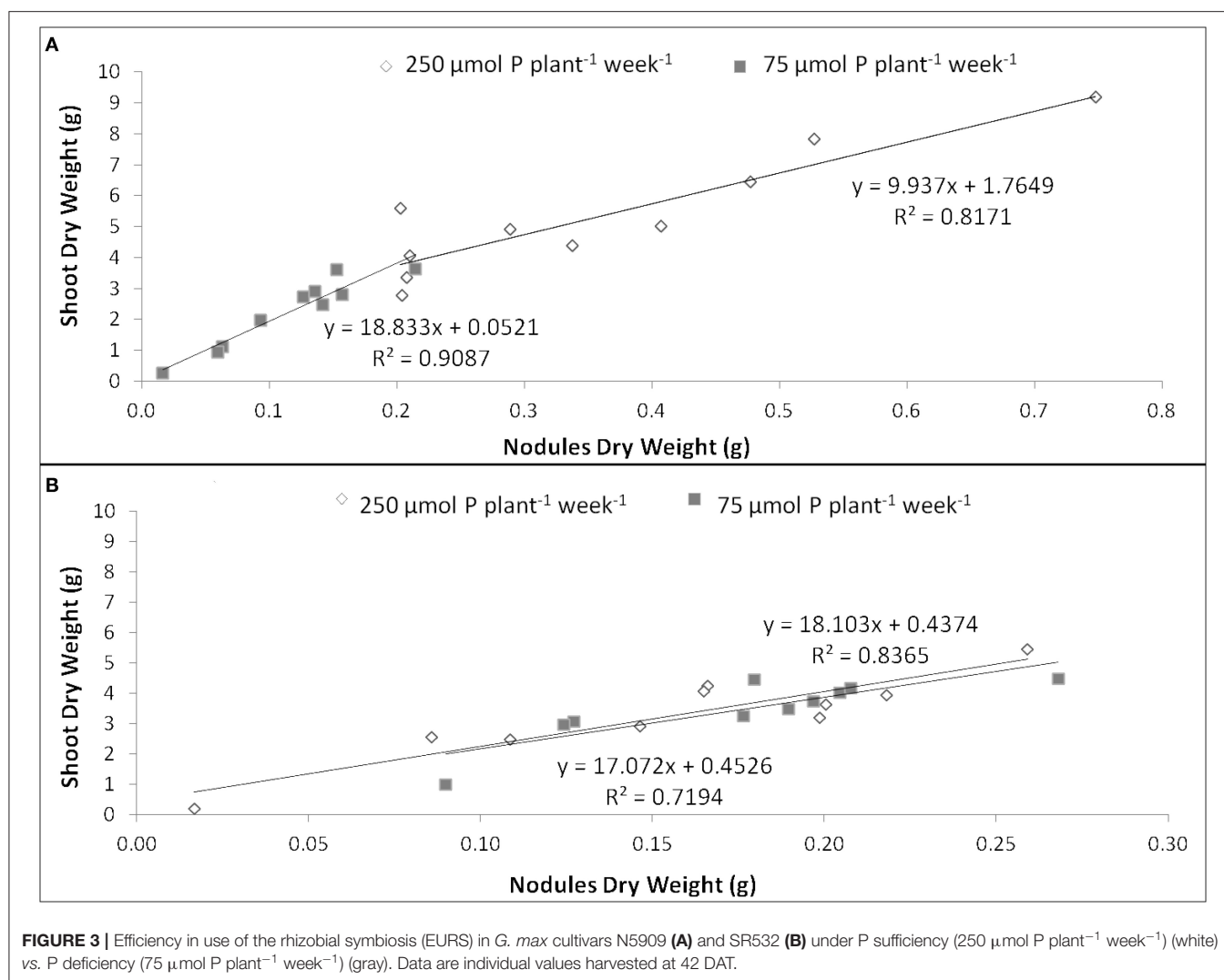
Negative controls (NRT) were prepared by omitting the reverse transcriptase. Afterwards, the reverse transcriptase mix was removed, by adding two times 1 mL of 1 X PBS and incubating the slides two times more in 50 mL of 1 X PBS during 10 min. PCR reactions were carried out in 100 µL volumes containing 10 X Buffer (Invitrogen), 50 mM MgCl<sub>2</sub>, 10 mM of each dNTP, 1 mM digoxigenin-11-uridine-triphosphate (Dig-11-dUTP), 10 µM HAPfor, 20 µM HAPrev, and 5 U Taq polymerase (Invitrogen), under the following thermal conditions: 35 cycles of 94°C for 30 s, 62°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 3 min.

For detection of the amplified cDNA in the nodules, the PCR mix was removed, and samples were washed in 100 µl of blocking solution under gentle agitation in the dark



**FIGURE 2 | (A)** Shoot dry weight, **(B)** nodules dry weight, and **(C)** root dry weight of *G. max* cultivars N5909 and SR532 inoculated with *B. elkanii* SEMIA 587 under P sufficiency (250  $\mu\text{mol P plant}^{-1} \text{ week}^{-1}$ ) (white) vs. P deficiency (75  $\mu\text{mol P plant}^{-1} \text{ week}^{-1}$ ) (gray). Data represent average and standard deviation of ten replicates harvested at 42 DAT. Treatments with a common letter are not significantly different ( $p > 0.05$ ).



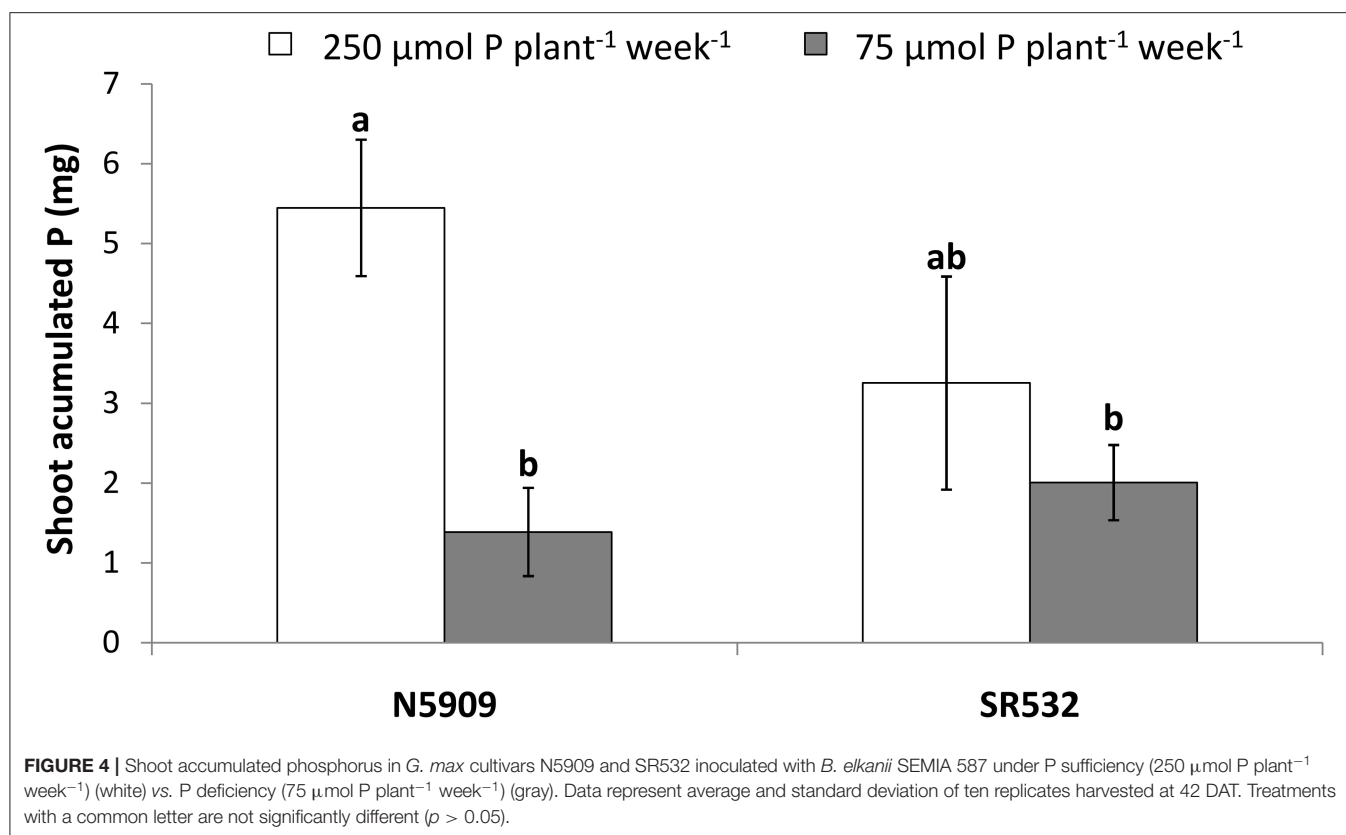


at 37°C. The blocking solution was replaced by 100 μl of alkaline phosphatase conjugated anti-dioxigenin Fab fragment (Roche Diagnostics) diluted 1:1000 in 2% BSA. The samples were incubated at room temperature for 90 min, then washed three times to remove excess antibody. Detection of alkaline phosphatase was carried out using an ELF-97<sup>®</sup> endogenous phosphatase detection kit (Molecular Probes). The ELF substrate was diluted 1:40 in the alkaline detection buffer (Molecular Probes), vigorously shaken, and then filtered through a 0.22 μm filter (Millex<sup>®</sup>-GV, Millipore) to remove any aggregates of the substrate that may have formed during storage. Samples were incubated in 20 μL ELF substrate–buffer solution for 20 min in the dark and transferred to washing buffer (1 X PBS, 25 mM EDTA, and 5 mM levamisole, pH 8.0). Three washings of 1 min were performed. Samples were mounted for observations with a BX61<sup>®</sup> microscope (Olympus) equipped with an epifluorescence condenser, a Hoechst/DAPI filter set configured with an excitation filter of 360–370 nm, a dichroic

mirror of 400 nm and an emission of 420 nm, and a gray View II<sup>®</sup> camera (ORCA AG). Image analysis was performed using ImageJ software (Schneider et al., 2012) as an image analysis program. The intensity of the fluorescent signal emitted by the rhizobial phytase transcript was measured as number of green pixels per image.

## Statistical Analysis

Shoot, nodules and root DW, shoot accumulated P and intensity of the fluorescent signal emitted by the rhizobial phytase transcript were analyzed through analysis of variance (ANOVA) and tested for differences between cultivars and P growing conditions using a *post-hoc* Tukey's HSD test ( $p < 0.05$ ) in R3.1.3 software (<https://www.r-project.org/>) using the “agricolae” package (de Mendiburu, 2020). The relationship between nodule and shoot biomass was tested by regression analysis.



## RESULTS

### Phytate Mineralization by *B. elkanii* SEMIA 587

*B. elkanii* SEMIA 587 showed similar growth curves when it was cultivated in Angle medium supplemented with Na-phytate or  $\text{KH}_2\text{PO}_4$ , as P sources, at both pH evaluated (6 and 7) (Figures 1A,B). Therefore, this strain was able to mineralize phytate and to use it as a P source.

Two primers pairs, that amplify two types of phytases, HAP and BPP, were tested through a retro-transcription and a subsequent touchdown PCR in *B. elkanii* SEMIA 587. An amplification product was obtained when SEMIA 587 was cultivated with Na-phytate as P source and when HAP primers were used, but not when BPP primers were used (Figure 1C). When SEMIA 587 was cultivated with  $\text{KH}_2\text{PO}_4$  as P source, no amplification products were obtained either when HAP or BPP primers were used (Figure 1C). Additionally, we demonstrated that HAP gene was not amplified from gDNA with these HAP primers (Figure 1C), due to the lower affinity between the mismatched primer and the gDNA at the used annealing temperature (62°C).

### Efficiency in Use of the Rhizobial Symbiosis for Plant Growth

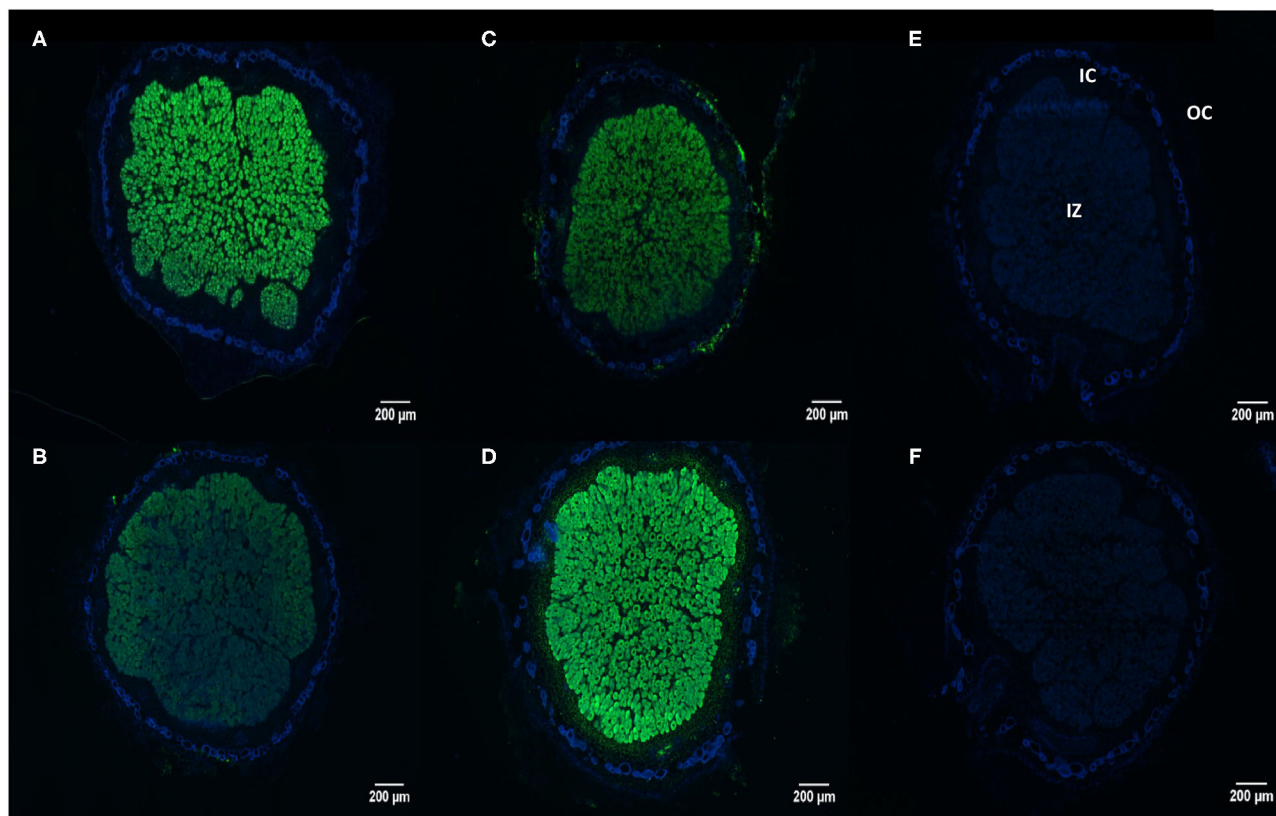
Cultivar N5909 cultivated under P sufficiency showed more shoot, nodules and root DW than under P deficiency ( $p = 0.0001$ ,  $p = 0.0007$ ,  $p = 0.0300$ , respectively), whereas no significant differences of shoot, nodules and root DW under P sufficiency and P deficiency in cultivar SR532 were found (Figure 2).

The EURS was estimated as the regression slope of shoot biomass as a function of nodule biomass, whenever the correlation between both parameters was significant (up to  $R^2 = 0.719$ ). Under P sufficiency, SR532 was the most efficient, and showed similar EURS under P deficiency, whereas N5909 increased its EURS under P deficiency (Figure 3).

Cultivar N5909 accumulated more P in its shoots under P sufficiency than under P deficiency ( $p = 0.0008$ ), whereas cultivar SR532 did not show any significant difference in shoot accumulated P between P treatments (Figure 4).

### Rhizobial Phytase Transcript Abundance in Soybean Nodules

Using the *In situ* RT-PCR technique we were able to show the transcript abundance of the rhizobial phytase in nodular sections of soybean cultivars N5909 and SR532 inoculated with *B. elkanii* SEMIA 587 cultivated under P sufficiency and P



**FIGURE 5 |** *In situ* RT-PCR localization of rhizobial phytase transcripts in nodule sections of *G. max*-*B. elkanii* SEMIA 587 symbiosis, under P sufficiency ( $250 \mu\text{mol P plant}^{-1} \text{ week}^{-1}$ ), (A) cultivar N5909; (B) cultivar SR532; and under P deficiency ( $75 \mu\text{mol P plant}^{-1} \text{ week}^{-1}$ ), (C) cultivar N5909; (D) cultivar SR532. Negative controls (without reverse transcriptase) for: (E) cultivar N5909; (F) cultivar SR532. Transcripts are colored in green and nodular tissues in blue. IZ (infected zone), IC (inner cortex), OC (outer cortex).

deficiency (Figures 5A–D). The NRT nodules not subjected to reverse transcription did not display any fluorescent signal (Figures 5E,F), confirming that fluorescence was not due to methodological artifacts. The rhizobial phytase was transcribed in all bacteroidal symbiosomes in the infected zone, with a high transcript abundance rate since the fluorescent signal, as assessment of number of transcripts, was intense for all the treatments (Figures 5A–D).

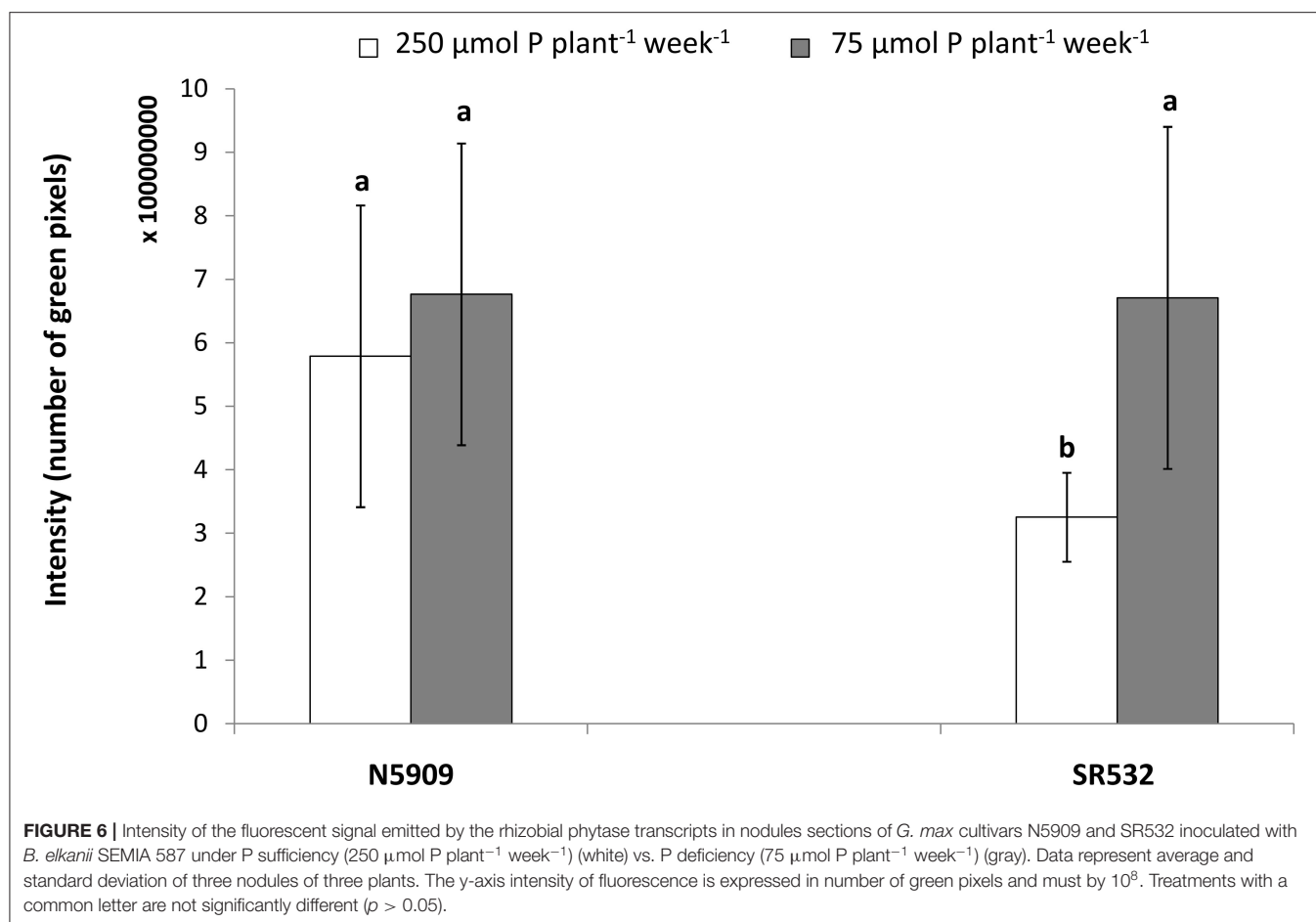
In the case of cultivar SR532, the fluorescent signal was more intense under P deficiency in comparison to P sufficiency ( $p < 0.0001$ ), whereas in the case of cultivar N5909, there were no significant differences between the intensity of the fluorescent signal under P deficiency or sufficiency. The intensity of the fluorescent signal in SR532 under P deficiency was equal to the intensity of this signal in N5909 under both P conditions (Figure 6). The ANOVA showed a significant interaction between the factors P supply and cultivar for the variable intensity of fluorescent signal ( $p = 0.0010$ ).

## DISCUSSION

The present study shows for the first time that a rhizobial strain could mineralize phytate and use it as a P source and that we

could locate and observe the distribution of the transcripts of a rhizobial phytase within nodules of  $\text{N}_2$ -fixing soybean plants, thanks to the *in situ* RT-PCR methodology carried out. This method has proven suitable to quantify the level of transcripts in prokaryote (Maougal et al., 2014b) and in eukaryotes cells (van Aarle et al., 2007; Bargaz et al., 2012; Lazali et al., 2013), and in the case of the present study, we were able to quantify the expression of rhizobial phytase transcripts with this method. The rhizobial phytase transcripts were localized only in the bacteroidal cells. It has been shown that P in *G. max* nodules was higher in the infected zone in comparison to the cortical region (revised by Hu et al., 2018). Thus, the high level of transcripts in the infection zone may respond to bacteroidal requirement for metabolism and survival, as well as bacteria multiplication (Bargaz et al., 2012; Lazali et al., 2013).

Legumes nodules are plant tissues with high concentration of P and when plants are under P-depletion, they follow a strategy to maintain SNF and viable leaf tissue as long as possible, expressing several P cycling genes in nodules (Cabeza et al., 2014). Plant phosphatase transcripts have been previously shown as affected by plant available P (Araújo et al., 2008; Bargaz et al., 2012; Lazali et al., 2013). Previously, the transcript abundance and activity of the PAP phytase of common beans was shown to increase when plants were grown under P deficiency, independently of the plant



genotype (Araújo et al., 2008; Lazali et al., 2013). In addition, Bargaz et al. (2012) demonstrated that the transcript abundance and activity of the plant phosphoenol pyruvate phosphatase (PEPase) increased under P deficiency for several common bean genotypes. Contrary, the two soybean cultivars performed contrastingly when grown under low- and high-P supply. While cultivar N5909 did not show differences in intensity of the fluorescent signal under low- and high-P, cultivar SR532 showed a high transcript abundance of the rhizobial phytase in the nodules when grown under P deficiency. Moreover, the transcript abundance of the rhizobial phytase in the nodules of SR532 under P sufficiency seemed to be repressed compared to the transcript abundance in nodules of N5909 under the same conditions. These facts suggest that the transcript abundance rate of the rhizobial phytase depends on the plant genotype and P level, although further testing of other soybean genotypes is required. Additionally, the high transcript abundance level of the rhizobial phytase in the nodules of the cultivar SR532 under P deficiency, suggests that this enzyme contributes to the adaptation of this cultivar to P deficiency. Cultivar SR532 under P deficiency shows a correlation of high bacterial phytase transcripts with high EURS, suggesting a high regulation between EURS and the P content. Overall, our results suggest that the high tolerance of

SR532 to P deficiency, as compared to N5909, is associated with better capacity to maintain SNF under low P supply based on the ability to increase the rhizobial phytase transcript abundance under such condition. Phytases might play a major role for internal plant metabolism rather than for obtaining P from the soil phytate (Tang et al., 2006). Phytate seems to act as a phosphate storage (Raboy, 2003) and rhizobial phytases can mineralize the phytate present in the soybean nodules, increasing the concentration of orthophosphates ions necessary for the SNF (Lazali et al., 2013).

Although further studies are required to identify new rhizobial strains with phytase activity and to test their interaction with various soybean genotypes, the differential transcript abundance shown in this research suggests that this phenomenon can contribute to the external and internal use efficiency of phytate for SNF. Sulieman et al. (2013b) found that different strains of *S. meliloti* showed different symbiotic efficiency with *M. truncatula* growing under different P levels. Thus, the identification and selection of an efficient combination of rhizobia and soybean genotype might play an important role in soybean breeding and production, aiming to reduce synthetic P fertilizer inputs and to improve sustainability in soils with low available P. We propose to consider the ability of a strain



to mineralize phytate at the moment of evaluate new N<sub>2</sub> fixing rhizobial strains, and to evaluate how the plant genotype influences this process, looking for combinations of rhizobia and plant that enhances P metabolism, thus SNE, under low available P soils, to select the best adapted symbiosis to P deficient soils.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

VC performed the experimental work, analyzed data, and wrote the manuscript. LA and CT coordinated the experimental work. NA, EB, and J-JD conceived, planned the study, and helped

to write the manuscript. All authors read and approved the final manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Bioinoculants as Promising Complement of Chemical Fertilizers for a More Sustainable Agricultural Practice

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Plant Growth Promoting Rhizobacteria (PGPR) represent a heterogeneous group of bacteria, which have been characterized for their ability to influence the growth and the fitness of agricultural plants. In the quest of more sustainable practices, PGPR have been suggested as a valid complement for the agronomical practices, since they can influence several biochemical and molecular mechanisms related to the mineral nutrients uptake, the plant pathogens suppression, and the phytohormones production. Within the present work, three bacterial strains, namely *Enterobacter asburiae* BFD160, *Pseudomonas koreensis* TFD26, and *Pseudomonas lini* BFS112, previously characterized on the basis of distinctive PGPR traits, were tested to evaluate: (i) their persistence in soil microcosms; (ii) their effects on seeds germination; (iii) their possible influence on biochemical and physiological parameters related to plant growth, fruit quality, and plant nutrient acquisition and allocation. To these aims, two microcosms experiments featuring different complexities, i.e., namely a growth chamber and a tunnel, were used to compare the effects of the microbial inoculum to those of chemical fertilization on *Cucumis sativus* L. plants. In the growth experiment, the *Pseudomonas* spp. induced positive effects on both growth and physiological parameters; TFD26, in particular, induced an enhanced accumulation of mineral nutrients (Fe, Ca, Mn, Ni, Zn) in plant tissues. In the tunnel experiment, only *P. koreensis* TFD26 was selected as inoculum for cucumber plants used in combination or in alternative to a chemical fertilizer. Interestingly, the inoculation with TFD26 alone or in combination with half-strength chemical fertilizer could induce similar (e.g., Ca accumulation) or enhanced (e.g., micronutrients concentration in plant tissues and fruits) effects as compared to plants treated with full-strength chemical fertilizers. Overall, the results hereby presented show that the use of PGPR can lead to comparable, and in some cases improved, effects on biochemical and physiological parameters of cucumber plants and fruits. Although these data are referred to experiments carried out in controlled condition, though different from an open filed cultivation, our observations suggest that the application of PGPR and fertilizers mixtures might help shrinking the use of chemical fertilization and potentially leading to a more sustainable agricultural practice.

**Keywords:** PGPR - plant growth-promoting rhizobacteria, *Cucumis sativus* L., fertilization practices, ionic analysis, sustainable agriculture, fruit quality

## INTRODUCTION

The excessive and continuous application of chemical fertilizers has often compromised soil chemical and biological properties causing pollution of soil ecosystems and detrimental effects on human health (Zhang et al., 2018). Quite often, agricultural soil suffers chemical and biological degradation due to nutrient and biodiversity depletion (Kopittke et al., 2019). Thus, such inappropriate soil fertility often leads to the compulsory use of pesticides and chemical fertilizers as well as to high costs of agricultural management in order to ensure an adequate crop productivity. In the presented scenario, the quest of more sustainable agricultural practices has drawn the attention to the biological potential of the interactions between crops and microorganisms inhabiting the rhizosphere (Pii et al., 2015b; Mimmo et al., 2018). It is well-known that microorganisms have a key role in many biotic mechanisms essential to preserve the biological and physiochemical balance in soil (Alegria-Terrazas et al., 2016). Thus, the investigation on the dynamics underlying the plant-microbiome interactions are crucial to achieve sustainable innovations in agriculture and has recently become a main scientific Research Topic. In particular, research efforts have been focused on that group of bacteria commonly termed Plant Growth-Promoting Rhizobacteria (PGPR) that naturally inhabits in close association with roots and benefits their host improving their growth by several mechanisms (Saharan and Nehra, 2011). The growth enhancements brought about by PGPR can be the result of either indirect actions (e.g., preventing the harmful effects of phytopathogenic organisms *via* the production of antagonistic substances or thanks to the stimulation of the plant systemic resistance), or direct mechanisms, such as the production of bioactive molecules (e.g., phytohormones) and/or enhancing the nutrient bioavailability at the rhizosphere (Crecchio et al., 2018). Several pieces of research have also highlighted that PGPR might contribute to improve plants growth by increasing their tolerance to abiotic stressors (Jetiyanon and Kloepper, 2002; Zhang et al., 2008a, 2010; Dimkpa et al., 2009; Chithrashree et al., 2011; Marastoni et al., 2019) and by modulating the physiological and biochemical activities of plants, especially those underlying the acquisition of mineral nutrient (Zhang et al., 2008b; Xie et al., 2009; Pii et al., 2015b, 2016b, 2018; Scagliola et al., 2016; Kolega et al., 2020). In this context, the employment of PGPR, alone or in combination with synthetic fertilizers, with the aim of enhancing nutrients acquisition in plants and, more in general, of increasing the use efficiency of endogenous soil sources of nutrients, might help to shrink the use of agrochemicals, as also foreseen by the *green deal* concept for an increasingly sustainable agriculture. In this sense, several studies have demonstrated that the inoculation of PGPR, in complete or partial replacement of chemical fertilizers, increases growth and yield of many commercial plants such as tomato (Hernandez and Chailloux, 2004), strawberry (Bona et al., 2015), curcuma (Kumar et al., 2016), cucumber (Saeed et al., 2015), sunflower (Arif et al., 2016), and cotton (Gomathy et al., 2008; Dhale et al., 2010; Nayalini et al., 2010).

It is also worth mentioning that PGPR inoculation in crops has been shown to impact the production of secondary

metabolites, thus affecting the overall nutraceutical value of the primary agricultural products. Indeed, several authors assessed that PGPR inoculation positively affected the quality of both fruit (e.g., mulberry, strawberry, citrus, apricot sweet cherry, and raspberry) (Eşitken et al., 2002, 2003, 2005, 2006; Orhan et al., 2006; Pii et al., 2018) and aromatic herbs (Banchio et al., 2009; Erika et al., 2010; Santoro et al., 2011; Heidari and Golpayegani, 2012; Cappellari et al., 2013, 2019a,b; Kolega et al., 2020) in terms of health-promoting compounds. However, very often the effects produced by PGPR inoculation on plant growth as well as on the qualitative and quantitative profile of secondary metabolites have been observed to be strongly dependent on the specific combination of plant species, microbial strain, and cultivation conditions. This possibly indicates the existence of specific interaction mechanisms, which can eventually lead to different outcomes in crops (Cappellari et al., 2019a).

On the basis of these premises, the aim of the present research was to assess the impact of different PGPR strains on cucumber plants grown in soil-based microcosms. In a previous work (Pii et al., 2016a; Scagliola et al., 2016), 80 bacterial colonies have been isolated from the rhizosphere of barley (*Hordeum vulgare* L.) and tomato (*Solanum lycopersicon* L.) plants grown in a calcareous soil, naturally featuring a low Fe availability. The bacterial strains were characterized for their PGPR traits, namely solubilization of phosphate, production of siderophores, and indole-3-acetic acid (Scagliola et al., 2016). Very often, it is reported in the literature that the outcomes of the microbe-plants interaction are strongly dependent on both the microbial strain and the plant species. With the aim of investigating whether the growth promotion properties of the isolated strains were restricted to barley and tomato or not, we decided to carry out further experiments on a different horticultural plant. Among the 80 bacterial colonies, 12 selected isolates, concurrently showing the best PGPR traits, were tested *in vivo* on hydroponically grown cucumber (*Cucumis sativus* L.) plants, in order to evaluate their ability to influence the biochemical and molecular mechanisms underpinning the Fe acquisition mechanisms at root level (Scagliola et al., 2016), as previously demonstrated for the model PGPR *Azospirillum brasilense* (Pii et al., 2015c, 2016b). On the base of the results presented by Scagliola et al. (2016), the three strains showing the best PGP features in the *in vivo* experiment were chosen to inoculate soil-grown cucumber plants. Therefore, the present experiments represent the natural prosecution of the research undertaken, from hydroponic growth system to soil-based systems.

## MATERIALS AND METHODS

### Plant Growth-Promoting Rhizobacteria Growth Conditions and Inoculation

The three strains used in this work, identified as described below, are BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*. They were routinely grown at 28°C in Nutrient Broth (Oxoid) for 24 h at 120 rpm. The strains, able to solubilize phosphates



and to produce siderophores and indole-3-acetic acid, had been previously isolated from the rhizosphere of barley and tomato plants, grown in a calcareous soil by the RHIZOtest system (Bravin et al., 2010), and characterized on the basis of PGP features (Scagliola et al., 2016).

## 16S rDNA Gene Sequencing

DNA extraction was performed by thermal shock, heating the PGPR colonies in 50  $\mu$ L of distilled water at 95°C for 15 min (Maniatis et al., 1982). The 16S rDNA gene PCR amplifications were carried out using two couples of universal primers E8F-E926R and E786R-E1541R (Baker et al., 2003). The PCR mix (50  $\mu$ L) contained 50 pmol of each primer, 10 nmol of each 2'-deoxynucleoside 5'-triphosphate, 3 U of Taq DNA polymerase (EuroTaq; EuroClone), 2.5 mM  $MgCl_2$ , and 25–50 ng of template DNA. All PCR amplifications were performed using a MyCycler<sup>TM</sup> thermal cycler (Bio-Rad Laboratories Inc.). Each PCR product was analyzed by electrophoresis in a 1.5% (w/v) TEAE agarose gel. Bands of interest, 918 and 755 bp long, were also excised from the gel and were purified using the Wizard<sup>®</sup> SV Gel and PCR Clean-Up System kit (Promega). The amplicons were sequenced from both ends by Eurofins Genomics (Milan, Italy). 16S rDNA sequences were aligned using the BLASTn tool (Camacho et al., 2009) against the NCBI database ([www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)) to identify the species of bacterial strains.

## Phytotoxicity Test

Seed germination tests were used to assess the phytotoxicity of the three selected strains. Cucumber seeds (*Cucumis sativus* L. cv Chinese Long) were surface sterilized by washing with 3% (v/v) sodium hypochlorite and then with sterilized distilled water. One hundred seeds were incubated in a bacterial suspension of each PGPR strains ( $10^8$  cfu mL<sup>-1</sup>) for 4 h at 28°C. Control seeds were incubated in sterile Nutrient Broth (Oxoid). The germination was carried out for 5 days in the dark at 22°C, placing the seeds between two layers of filter paper moistened with 0.5 mM  $CaSO_4$  (Stefan et al., 2008). The results were expressed as the ratio of the number of germinated seeds to the total number of tested seeds.

## Persistence Assay in Soil

The PGPR strains were made competent chemically (Cohen et al., 1972) and labeled with the Green Fluorescent Protein (*gfp*) by *in vitro* transformation (Zhao et al., 2013) using the pGLO<sup>TM</sup> plasmid (BIO-RAD). The transformants were selected on Nutrient Broth (Oxoid) supplemented with 0.5% (w/v) L-arabinose and 0.01% (w/v) ampicillin and preserved at -80°C in 20% (v/v) glycerol. The stability of plasmid maintenance was evaluated by growing the transformed clones for 40 days under non-selective conditions at 28°C. Every 3 days the presence of the plasmid was ascertained by plating aliquots of the clones on selective and differential media (Nutrient Broth (Oxoid) supplemented with 0.5% (w/v) L-arabinose and 0.01% (w/v) ampicillin), then the liquid culture was centrifuged and the growth medium renewed. Having verified the long-term stability of the plasmid (40 days), the tagged strains were used in soil microcosms by inoculating fresh soil with  $10^8$  cfu g<sup>-1</sup>DW

incubated for 30 days at 28°C. To evaluate and quantify the presence of the tagged strains, the bacterial communities were desorbed from soil by sonication and bacteria were isolated and counted by the standard dilution plating technique on Nutrient Agar (Oxoid) supplemented with 0.5% (w/v) L-arabinose and 0.01% (w/v) ampicillin after 24 h from inoculation and hereafter every 7 days. Three independent replicates were performed for each sampling time, in order not to alter the soil-microorganism system through sampling of soil aliquots.

## Trial 1: Pot Experiment in Climatic Chamber

Cucumber seeds (*Cucumis sativus* L. cv Chinese Long), previously surface sterilized as before described, were germinated for 5 days in the dark at 22°C onto moist filter paper in 0.5 mM  $CaSO_4$  solution. Homogenous seedlings were then transferred in pots contained 70 g of a growth medium consisting of soil, sterilized peat and sterilized perlite at the ratio of 60:30:10. The soil was a sandy loam, classified as a Haplic Calcisol (FAO, 1998) collected from the South of Italy and had the following chemical-physical characteristic:  $pH_{H_2O}$  7.4,  $C_{org}$  7.0 g kg<sup>-1</sup>,  $N_{tot}$  0.8 g kg<sup>-1</sup>, P 11.7 mg kg<sup>-1</sup>, and K 104 mg kg<sup>-1</sup> (Sofa et al., 2010).

The experiment was performed in a climatic chamber with the following conditions: 18/6 h light/dark, 22°C and 44  $\mu$ mol m<sup>-2</sup> s<sup>-1</sup> light intensity. The pots were inoculated with 7 mL of broth culture of each bacteria (BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*) at  $10^8$  cfu mL<sup>-1</sup> in the day of the transplant and after 15 days. The control pots were inoculated with the same amounts of sterile Nutrient Broth. The experiment was performed in six replicates in a complete randomized block design. After 30 days of cultivation, physiological parameters, such as photosynthetic rate, transpiration rate and stomatal conductance, were measured using the Li-6400 XT Portable Photosynthesis System (Licor Inc. Lincoln, NE, USA). Plant height (cm), root length (cm), above ground and root biomass (g), above ground and root water content (%), and the leaf chlorophyll content were recorded (Hiscox and Israelstam, 1979). To quantify the content of macro (P, S, K, Ca) and micro-nutrients (Fe, Zn, Mn, Ni, Cu, Br, Sr) of roots and leaves, the Total Reflection X-Ray Fluorescence Spectrometer (TXRF, Bruker S2 PICOFOX) was used.

## Trial 2: Pot Experiment in Tunnel

On the basis of the results obtained in Trial 1, the best performing PGPR was used for further inoculation studies, together with a commercial fertilizer. Cucumber seeds (*Cucumis sativus* L. cv Chinese Long) were surface sterilized and germinated as described above. Homogenous seedlings were then transferred in 19 cm diameter pots, containing about 2 kg of soil, sterilized peat and sterilized perlite mixture at the ratio of 60:30:10 and arranged in a complete randomized block with six replicates for each treatment. Four treatments were performed: inoculation (Inoculated), half-strength fertigation (0.5x Fert Not-Inoculated), inoculation supplemented with half-strength fertigation (0.5x Fert Inoculated), and full-strength fertigation (Fert). The fertigation treatments were carried out using the commercial fertilizer Green-GO<sup>®</sup> 12:8:24 (N:P:K) + 10 CaO

(Biolchim) (**Supplementary Table 1**) at the recommended rate of 5–15 Kg 1,000 m<sup>-2</sup>. In our study each pot received 0.06 g of fertilizer in 100 mL tap water, twice a week. The inoculum was performed once per week the day after the fertigation using 100 mL of 10<sup>8</sup> cfu mL<sup>-1</sup> of TFD26 *P. koreensis* suspension. The fruits, collected during the cultivation, were freeze-dried using Laborota 4001 efficient (Heidolph) and preserved at -20°C for further analysis. After 60 days of cultivation the morphological and physiological assessments, as described for Trial 1, were carried out. In addition, C and N content of root and shoots were evaluated using elemental analyzer Flash EA 1112 and microbial biomass content of soil was determined by the standard chloroform fumigation extraction method (Brookes, 1995).

## Nutraceuticals and Elemental Analyses of Fruits

Freeze-dried cucumber fruits samples were homogenized, and 100 mg of cucumber powder were extracted with 1 mL methanol (HPLC grade, Merck, Darmstadt, Germany). The mixtures were subjected to sonication for 30 min at 4°C and, afterwards, they were centrifuged at 14,000 xg for 30 min at 0°C; the supernatants were then collected and filtered through a 0.2 µm nylon filter, as previously described (Valentinuzzi et al., 2015).

Organic acids and sugars were separated simultaneously by HPLC equipped with a cation exchange column Aminex 87-H column (300 × 7.8 mm, 9 µm, Bio-Rad) using an isocratic elution with 10 mM H<sub>2</sub>SO<sub>4</sub> as carrier solution at a flow rate of 0.6 mL min<sup>-1</sup>. Organic acids were detected at 210 nm using a Waters 2998 photodiode array detector (Waters Spa, Italy), whilst sugars were detected by a Waters 2414 refractive index detector (Waters Spa, Italy).

Lyophilized and ground cucumber tissues samples were mineralized with ultra-pure 68% HNO<sub>3</sub> (Carlo Erba) using a Single Reaction Chamber Microwave (SRC, UltraWAVE, Milestone Inc, Shelton, CT, USA). Mineralized samples were diluted with distilled water, filtered, and elements concentration determined by inductively-coupled plasma - optical emission spectrometry – ICP-OES (Arcos Ametek, Spectro, Germany), using tomato leaves (SRM 1573a) and spinach leaves (SRM 1547) as external certified reference material, as previously described (Pii et al., 2015a).

## Statistical Analyses

The results are reported as mean ± standard error (SE) of six independent biological replicates. The significance of differences among means was calculated by One-way ANOVA with *post-hoc* Tukey HSD with  $\alpha = 0.05$  using R software (version 3.6.0). The Principal Component Analysis (PCA) has been carried out by applying the *prcomp* function of the R software package ggfortify (Tang et al., 2016). The following R packages were used for data visualization and statistical analyses: ggplot2 v.3.2.0 (Wickham, 2016), Agricolae v.1.3-1 (de Mendiburu, 2019), and ggfortify (Tang et al., 2016).

## RESULTS

### Strain Identification

The analyses of 16S rDNA sequences allowed to establish the species of BFD160, TFD26, and BFS112 clones as *E. asburiae*, *P. koreensis*, and *P. linii*, respectively (**Table 1**).

### Phytotoxicity Test

The cucumber seeds incubated with the selected PGPR showed an inhibition of the germination rate by about 11, 15, and 9% for BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, respectively. However, these values resulted not statistically significant, when compared with the control seeds incubated in the sterile media (**Supplementary Table 2**).

### Persistence Assay in Soil

The three clones, labeled using the method of plasmid transformation reported by Zhao et al. (2013), had maximum growth after 18 h of incubation at 28°C, similarly to the wild type strains. The stability assay of plasmid demonstrated that *gfp*-tagged strains completely maintained their marker genes up to 40 days, in absence of the selective medium, as all the colonies for each of the three strains grew on the selective and differential plates and appeared fluorescent under UV light at each assay up to 40 days of incubation (data not shown).

The numbers of PGPR cfu in the soil were counted after 24 h from the inoculation and then every week for 30 days on selective and differential medium. The results of PGPR persistence test showed that the number of bacteria in soil decreased gradually during the experiment until their complete absence. In detail, results showed that the initial density of 10<sup>8</sup> cfu g<sup>-1</sup>DW of PGPR decreased to 10<sup>4</sup> cfu g<sup>-1</sup>DW after 15 days and to 10<sup>3</sup> cfu g<sup>-1</sup>DW after 20 days with a trend perfectly comparable among all strains (data not shown). Consequently, the PGPR strains were repeatedly inoculated, depending on the duration and the complexity of the trials.

## Trial 1

### Effects of PGPR on Roots and Shoots of Plants Grown in Climatic Chamber

#### Growth parameters

Plant height, root length, above ground and root biomass, above ground and root water content of cucumber plants grown in inoculated soil microcosms were positively affected by the treatment, when compared to the control plants (**Supplementary Table 2**). In particular, the inoculation with TFD26 *P. koreensis* and BFS112 *P. linii* significantly enhanced the water content either of the above ground and of the root (**Supplementary Table 2**). Likewise, the chlorophyll a and b content resulted higher in the leaves of the plants grown in soil microcosms inoculated with the same strains (**Supplementary Table 2**). On the contrary, the effects of BFD160 *E. asburiae* were variable. Plants grown in soil microcosms treated with this PGPR exhibited an increment in plant height (+48, +27, and +17% with BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, respectively), water content (+2% with all the inoculants), and biomass (+50, +17, and +27%

**TABLE 1** | Identification of bacterial strains used in the inoculation experiments on the base of 16S rDNA sequencing.

Genus	Species <sup>a</sup>	Strain	GeneBank accession number	Origin	References
<i>Enterobacter</i>	<i>asburiae</i> (99.26%)	BFD160	KX290147	Italy	Scagliola et al., 2016
<i>Pseudomonas</i>	<i>koreensis</i> (99.73%)	TFD26	KX290158	Italy	Scagliola et al., 2016
<i>Pseudomonas</i>	<i>linii</i> (99.31%)	BFS112	KX290180	Italy	Scagliola et al., 2016

<sup>a</sup> The species were identified in this study; percentage of similarity in brackets.

with BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, respectively), but showed slight decrease of leaf chlorophyll content. Interestingly, the same clone significantly reduced the root length of about 54% (**Supplementary Table 2**).

### Physiological parameters

All the physiological activities were markedly enhanced by the employment of the three PGPR. In particular, the photosynthetic rate was incremented 1.7, 2.4, and 2.0 times in the plants grown in soil microcosms inoculated with BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, respectively, as compared with the non-treated plants. Furthermore, the treatment with TFD26 *P. koreensis* had a significant positive effect on the transpiration rate and stomatal conductance, too, which were increased 3.2 and 3.4 times, respectively (**Supplementary Table 2**).

### Micro and macronutrient content

The data of the micro and macronutrient content are summarized in **Supplementary Table 3**. The most considerable result was the positive effect determined by the treatment of the soil microcosms with TFD26 *P. koreensis*, which increased the content of about all elements, either in leaves or in roots, with particular emphasis for Fe, whose concentration was enhanced by about 37.5 and 54% in leaves and in roots, respectively.

## Trial 2

### Effects of PGPR on Roots and Shoots of Plants Grown in Tunnel

#### Growth parameters

The chemical fertilization at both doses, with and without the inoculum, decreased the water content of roots; no statistically significant variation among treatments was observed in shoots (**Figure 1**). Shoots length and thickness were not significantly affected by fertilization and/or inoculum, too (**Supplementary Table 4**).

#### Plant physiological parameters and fruit production

Among the physiological parameters investigated, only the chlorophyll a content was significantly affected by the treatments, being the values of plants under half dose of chemical fertilizer implemented by the inoculum significantly higher than those with under half dose without inoculum and comparable to the values of plants under the commercial dose of fertirrigation. On the other hand, it is worth noting that the simple inoculum with PGPR was not enough to guarantee a comparable content of chlorophyll a (**Figure 2**). Other parameters such as chlorophyll b content, photosynthetic capacity, stomatal conductance, and transpiration capacity did not statistically change among plants

under two doses of fertilizers and with or without the inoculum (**Supplementary Table 4**). In addition, the different fertilization practices did not show any effects on the fruits production trait, which was evaluated as the number of fruits (data not shown), fruit weight and fruit length (**Supplementary Table 4**).

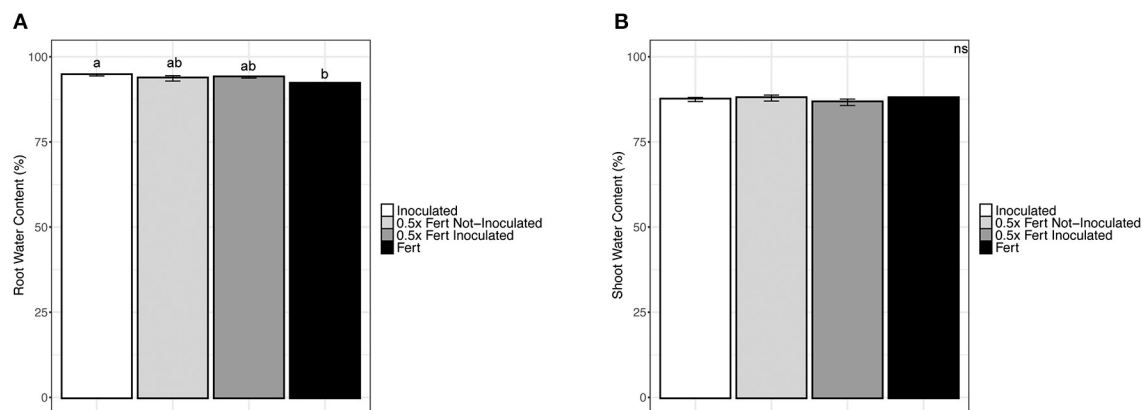
#### Soil microbial biomass

Data reported in **Figure 3** clearly indicate that the chemical fertilization negatively affects the soil microbial biomass that is, quite obviously, increased by the inoculum. Interestingly, the use of half dose of fertilizer in combination with the inoculum allows to reach almost the same high values without subtracting the important mineral nutrients to the plants.

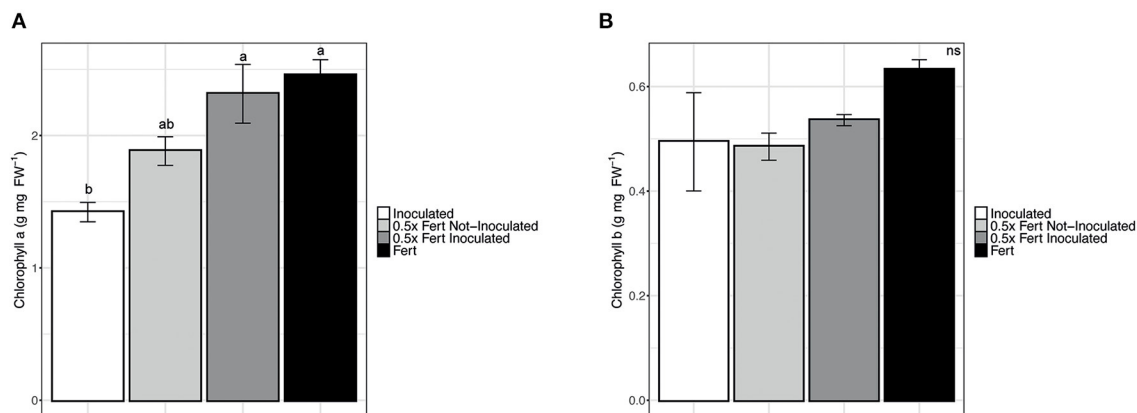
## Ionic Profiling of Roots, Shoots, and Fruits

The ionic profile of roots, shoots, and fruits has been analyzed in order to unravel whether the different agronomic practices imposed might have affected the uptake and allocation of mineral nutrients in cucumber plants. The Principal Components Analysis carried out on the root ionome (**Figure 4A**) generated a model, in which the sum of Principal Component 1 (PC1) and PC2 accounted for 82% of the total variance. The scatter plot reported in **Figure 4A** showed the clustering of samples according with the different treatments imposed; the distribution along the PC1 was mainly influenced in the positive direction by the concentration of micronutrients, namely Fe, manganese (Mn), and zinc (Zn). Indeed, the concentrations of these elements were significantly higher in both 0.5x Fert Inoculated and 0.5x Fert Not-Inoculated samples, as compared to both Inoculated and Fert samples (**Supplementary Table 5**). On the other hand, the distribution of samples along the PC2 showed a separate clustering of Inoculated and Fert samples, being this distribution mainly driven by copper (Cu) in the positive direction and sulfur (S) in the negative one (**Figure 4A**). As predicted by PCA, the concentration of S was significantly higher in the Inoculated samples as compared to the Fert ones (**Supplementary Table 5**), whereas, unexpectedly, the concentration of Cu just showed an increasing trend in the Fert samples, yet not statistically different from that detected in the Inoculated roots (**Supplementary Table 5**).

At shoot level, the multivariate analyses produced a model, whose PC1 and PC2 described ~79.5% of the total variance (**Figure 4B**). As already observed in roots, the samples separated according with the treatments imposed, forming four independent clusters. The distribution along the PC1 (56.5% of the total variance) was predominantly determined by the



**FIGURE 1 |** Water content in cucumber plants. **(A)** Water content in roots of cucumber plants harvested after 60 days of cultivation in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). **(B)** Water content in shoots of cucumber plants harvested after 60 days of cultivation in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). Data are reported as means  $\pm$  SE. Different letters indicate significant differences according to one-way ANOVA with Tukey *post-hoc* tests ( $p < 0.05$ ). Letters were omitted when no significant differences were found.



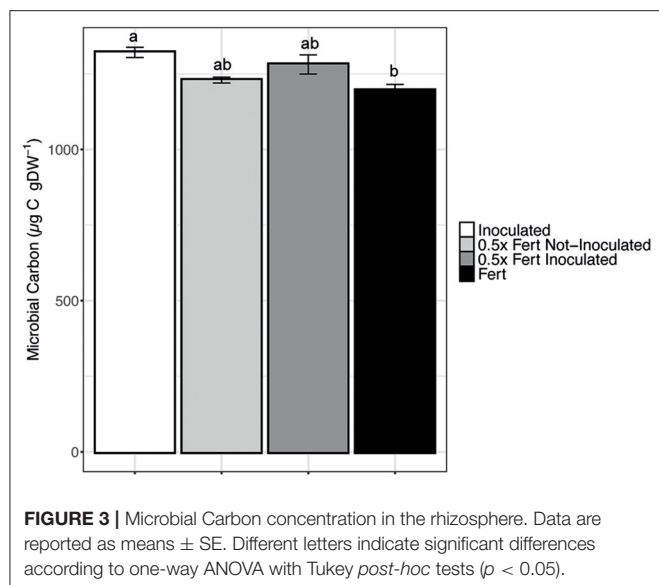
**FIGURE 2 |** Chlorophylls concentration in cucumber leaves. **(A)** Chlorophyll a concentration in leaves of cucumber plants harvested after 60 days of cultivation in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). **(B)** Chlorophyll b concentration in leaves of cucumber plants harvested after 60 days of cultivation in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). Data are reported as means  $\pm$  SE. Different letters indicate significant differences according to one-way ANOVA with Tukey *post-hoc* tests ( $p < 0.05$ ). Letters were omitted when no significant differences were found.

concentration of both micro- (i.e., Cu, Mn, and Fe) and macronutrients [i.e., phosphorus (P), and calcium (Ca)]; in fact, Fe, Cu, and Ca displayed a significantly higher concentration in 0.5x Fert Inoculated and Fert samples as compared to the other two treatments. On the other hand, Mn and P were more concentrated in 0.5x Fert Inoculated plants as compared to the other plants analyzed (**Supplementary Table 6**). The PC2 (23% of the total variance) highlighted the separation between Inoculated and 0.5x Fert Not-Inoculated plants (**Figure 4B**), being this distribution mainly driven by S in the positive direction and by potassium (K) and Zn in the negative one. Indeed, the 0.5x Fert Not-Inoculated showed the highest concentration of S as compared to the other samples, whilst the concentrations of K and Zn were significantly higher in the shoot of Inoculated plants

as compared to those detected in 0.5x Fert Not-Inoculated plants (**Supplementary Table 6**).

The scatter plot obtained from the fruit ionome PCA accounted for ~77% of the total dataset variance and highlighted a clear separation along PC1 of the Inoculated samples respect to the others (**Figure 5**). This clustering is mainly determined by the concentrations of Cu, Fe, molybdenum (Mo), and S, which, in fact, resulted significantly higher in cucumbers produced by inoculated plants (**Supplementary Table 7**). On the other hand, the other samples, namely 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated and Fert, displayed a separation along PC2 that was mainly driven by Mg in the positive direction and by Ca, Zn, and sodium (Na) in the negative one (**Figure 5**). The concentration of Mg resulted in fact higher in 0.5x Fert Not-Inoculated samples





with respect to both 0.5x Fert Inoculated and Fert samples (Supplementary Table 7). On the other hand, Ca and Zn were highly significantly accumulated in both 0.5x Fert Inoculated and Fert samples compared to 0.5x Fert Not-Inoculated, whilst Na displayed the highest concentration in 0.5x Fert Inoculated (Supplementary Table 7).

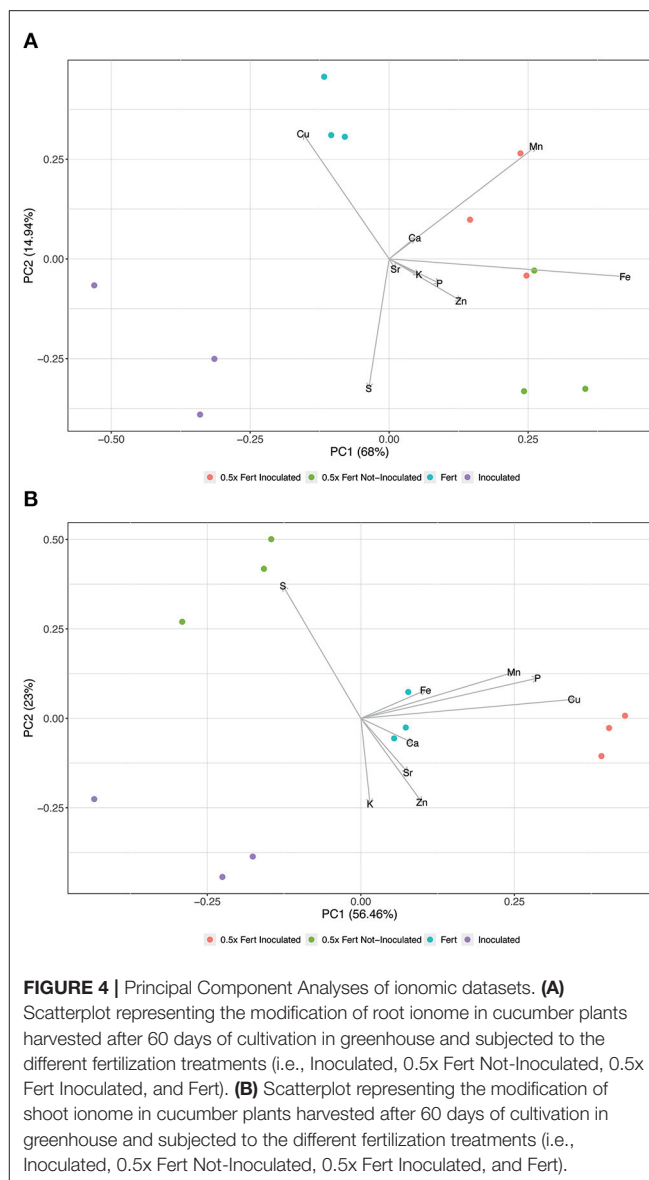
### Nutraceuticals Content

In order to assess whether the different fertilization practices have affected the content of nutraceutical compounds in cucumber fruits, a targeted metabolomic approach has been undertaken. The quantification of antioxidant molecules, as for instance flavonoids and flavonols, did not reveal any significant variation according with the treatment (Supplementary Table 8). Cucumber fruits sugar content was determined by HPLC and the results showed that both glucose and fructose did not display significant alterations depending on the fertilization practices (Supplementary Table 8). On the other hand, sucrose was significantly accumulated in fruits of Fert plants, whereas the lowest concentrations were observed in cucumber fruits produced by Inoculated and 0.5x Fert Not-Inoculated plants (Figure 6A).

The analysis of organic acids (OA) content highlighted that only citrate, among the other OA detected (i.e., malate,  $\alpha$ -ketoglutarate, fumarate, and succinate), displayed a variation in the concentration depending on the treatment imposed (Figure 6B and Supplementary Table 8). In particular, fruits produced by Inoculated and 0.5x Fert Not-Inoculated plants had citrate concentrations that were almost doubled as compared to those detected in 0.5x Fert Inoculated and Fert samples (Figure 6B).

## DISCUSSION

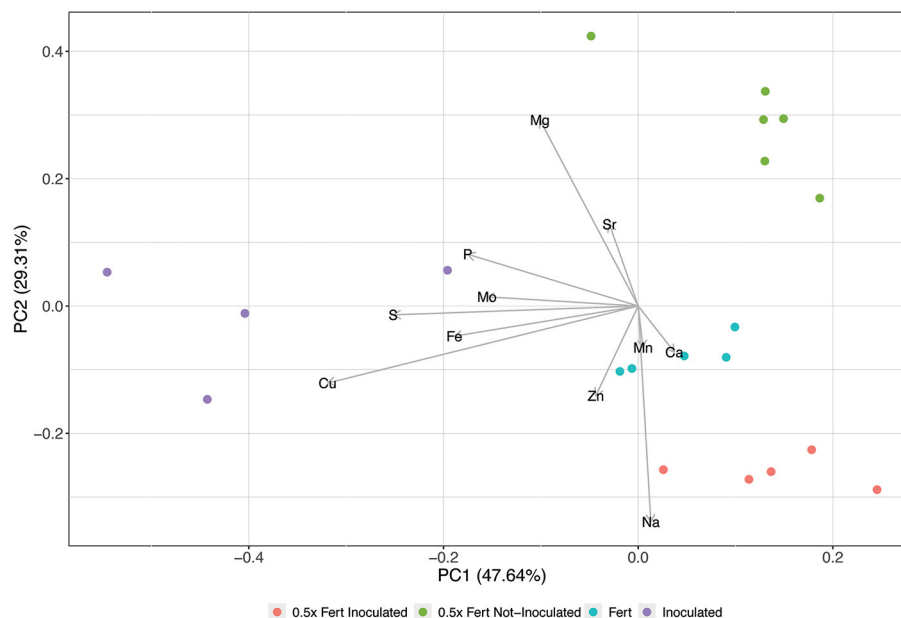
Plant Growth Promoting Bacteria (PGPR), inhabiting rhizosphere and colonizing the surface of roots, play beneficial



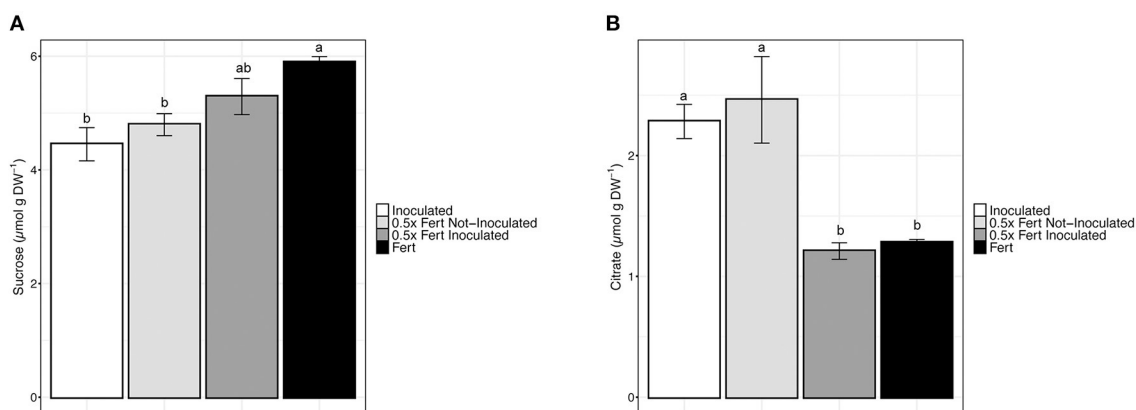
roles that directly or indirectly affect plant health. Thus, PGPR offer a sustainable alternative to conventional agricultural practices, stimulating plant growth and considerably helping to limit the input of fertilizers necessary in growing substrates (Bashan, 1998).

In the present investigation, three different PGPR, BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, isolated and characterized for their PGP activities (phosphates solubilization, production of siderophores, and IAA) in a previous study (Scagliola et al., 2016), were tested in a controlled soil-plant system (climatic chamber, 30 days plant growth) to select only one strain (TFD26 *P. koreensis*) with the best performances, to be further used in a tunnel experiment with a longer cultivation period, up to fruits production.

The effect of the three PGPR on the germination processes has been evaluated *in vitro* and the results showed a slightly decrease



**FIGURE 5 |** Principal Component Analyses of fruit ionome signature. Scatterplot representing the modification of cucumber fruit ionome harvested from plants cultivated for 60 days in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert).



**FIGURE 6 |** Nutraceuticals content in cucumber fruits. **(A)** Concentration of sucrose in cucumber fruit harvested from plants cultivated for 60 days in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). **(B)** Concentration of citrate in cucumber fruit harvested from plants cultivated for 60 days in greenhouse and subjected to the different fertilization treatments (i.e., Inoculated, 0.5x Fert Not-Inoculated, 0.5x Fert Inoculated, and Fert). Data are reported as means  $\pm$  SE. Different letters indicate significant differences according to one-way ANOVA with Tukey *post-hoc* tests ( $p < 0.05$ ). Letters were omitted when no significant differences were found.

in the germination rate, yet not statistically significant, likely ascribable to the competition for nutrient resources between the cucumber seeds and the microorganisms (Stefan et al., 2008). These observations allowed to conclude that the strains hereby used do not exert toxic actions on seed germination. Furthermore, as suggested by Zhou et al. (2013), the observed effects could disappear during the seedling's development.

To facilitate the monitoring of PGPR and determine their survival in agricultural soil experiments, the strains, BFD160 *E. asburiae*, TFD26 *P. koreensis*, and BFS112 *P. linii*, were tagged

with the *gfp* and liquid broth cultivation was carried out to assess the effect of the marker on the growth and on the labeling stability. The data indicated that the growth of the *gfp*-tagged PGPR, and likely the whole metabolism, were not affected by the labeling, obtained through the introgression of the recombinant plasmid. Indeed, the plasmid was maintained at least until 40 days of cultivation. On the other hand, the inoculation of agricultural soil with the labeled bacterial strains resulted in a gradual decrease of BFD160<sup>gfp</sup> *E. asburiae*, TFD26<sup>gfp</sup> *P. koreensis*, and BFS112<sup>gfp</sup> *P. linii*, most likely because of the competition with

autochthonous microorganisms. In fact, it is well-known that the bacterial species decline after the inoculation in soil, mainly because of the competition with native communities and the hostility of either biotic or abiotic interactions (van Veen et al., 1997), whilst the survival of inocula is higher in the rhizosphere than in the bulk soil (Fischer et al., 2010). It is noteworthy that the declining trend was the same for BFD160<sup>gfp</sup> *E. asburiae*, TFD26<sup>gfp</sup> *P. koreensis*, and BFS112<sup>gfp</sup> *P. linii*, independently of the genera they belong to. These observations were fundamental for the setting up of the experimental plan for trial 2, in which repeated inoculations were carried out weekly, in order to maintain the bacterial density at a constant level during the 60 days of cultivation.

As expected, the inoculation of the two strains belonging to *Pseudomonas* spp. had positive effects on growth and physiological parameters. It is well-known that some strains of *Pseudomonas* spp. have different features that positively affect plant health, making them well suited for PGPR (Saharan and Nehra, 2011). In particular, according to the growth chamber trial, the plants grown in soil treated with TFD26 *P. koreensis* exhibited the most evident improvements. As previously reported (Scagliola et al., 2016), TFD26 *P. koreensis* was able to secrete low amounts of IAA, a phytohormone, which in low concentration (from  $10^{-10}$  to  $10^{-9}$  M) enhances the root extension (Murphy, 2006), fostering plant mineral uptake and, indirectly, plant development (Spaepen et al., 2008). Consistently, the PGPR traits of *P. koreensis* have been also recently demonstrated with the isolate S150 through *in vitro* tests on *Arabidopsis thaliana* plants (Gu et al., 2020). In addition, our findings showed that the presence in soil of TFD26 *P. koreensis* increased the plant content of several nutrients in either leaves or roots, such as Fe, Ca, Mn, Ni, Zn. Besides, the inoculation possibly improved the translocation of S and K, whose amounts resulted higher in leaves than in roots, when compared to the control. Indeed, related *P. koreensis* strains were recently shown to feature relevant K solubilizing ability in soil, thus increasing the K bioavailability to plants (Reyes-Castillo et al., 2019; Adhikari et al., 2020). Very interestingly, the Fe content was significantly increased, and this effect might be correlated to the capacity of the same strain to produce siderophores, organic compounds which increase the Fe bioavailability (Scagliola et al., 2016). Furthermore, among the three strains used in the growth chamber test, the inoculum of TFD26 *P. koreensis* was responsible for a relevant increase of all physiological parameters assessed (photosynthetic rate, transpiration rate, and stomatal conductance). Accordingly, it was used to determine its effects on growth and physiological parameters, as well as on fruit quality, when used in combination or in alternative to a chemical fertilization management (Trial 2).

It has been clearly demonstrated that the mineral elements concentration in the tissues of agricultural plants can be influenced by the growing conditions, as for instance the chemical and physical characteristics of the growth substrate, the fertilization practice, and the inoculation with PGPR. These alterations in the ionic signature sometimes give rise to the increase in the concentration of essential and/or non-essential mineral elements, which can result in health-promoting effects

for consumers (Tomasi et al., 2009; Pii et al., 2015a; Astolfi et al., 2018).

At leaf level, the treatment with the commercial fertilizer caused the highest accumulation of Ca, whose structural and biochemical (e.g., in the signaling process) role in both plants and animals is well-demonstrated (Marschner, 2012). Interestingly, the inoculated samples, independently of the treatment with the fertilizers (i.e., Inoculated and 0.5x Fert Inoculated), presented a Ca concentration that was comparable with the Fert samples. At fruit level, the 0.5x Fert Inoculated samples also showed the highest concentration of Ca, comparable to the values measured in fruits produced by Fert plants. A higher Ca concentration in the edible part of agricultural product might have a particular relevance to human health promotion, since the lack of this macronutrient has been epidemiologically related to several diseases (Sharma et al., 2017). The treatment with bacteria also caused a higher accumulation of S in the root tissues, albeit the concentration of S in the shoots of the same plants was lower as compared to the not-inoculated counterparts (i.e., Fert and 0.5x Fert Not-Inoculated). Indeed, the higher accumulation in the leaves of not-inoculated samples, regardless of the fertilization regime, might suggest a possible ongoing stress response. In fact, S has been demonstrated to be involved in several metabolic pathways that lead to the production of different metabolites, like hydrogen sulfide, cysteine, methionine and glutathione, that are usually produced to cope with both biotic and abiotic stressors (Abdalla and Mühling, 2019; Kopriva et al., 2019). Nevertheless, the higher S concentration detected in cucumber fruits produced by inoculated plants might represent a positive nutraceutical feature, since it might hint the higher accumulation of bioactive phytochemicals related with the scavenging action of oxidative stress (González-Morales et al., 2017). Concerning micronutrients (i.e., Fe, Mn, and Zn), the inoculation with the PGPR showed positive effects on the concentrations at the root level only when plants were also supplemented with half strength fertilizer. The same trend was also shown at leaf level. Indeed, the promoting effect of TFD26 *P. koreensis* inoculation on the Fe acquisition mechanisms (i.e., Fe chelate reductase activity) in cucumber plants has already been demonstrated (Scagliola et al., 2016). Consistently, Murgese et al. (2020) demonstrated that the inoculation of *C. melo* L. plants with a mixed microbial community, also featuring TFD26 *P. koreensis*, induced the upregulation of genes (i.e., *CmFRO* and *CmIRT1*) involved in the reduction and uptake of  $\text{Fe}^{2+}$  in dicot plants. In this context, the enhanced activity of Fe uptake system upon PGPR inoculation (Pii et al., 2016b) could also account for the higher concentration of other bivalent cations in roots, namely Mn and Zn, observed at root level. In fact, Fe, in its reduced form, is transported across the plasma membrane of root cells through the IRT1-like protein (Connolly et al., 2003) which is also able to catalyze the transmembrane fluxes of Mn, Zn, Cu, and Cd (Korshunova et al., 1999). On the other hand, consistently with the results obtained in PGPR-inoculated strawberries (Pii et al., 2018), the fruits produced by inoculated plants showed the highest concentration of micronutrients, like Cu, Fe, and Mo. These results might suggest a more efficient root-shoot translocation as compared to the other samples considered, thus resulting

in a micronutrient fortification of cucumber fruits. Beside the concentration of mineral elements, the inoculation of cucumber plants with the sole TFD26 *P. koreensis* resulted in the lowest concentration of sucrose and the highest concentration of citrate in fruits. These results were not in agreement with the increase in the sugar content and the decrease of detected titratable acidity, for instance, in cherry tomatoes and strawberries after the inoculation with PGPR (Pii et al., 2018; Aini et al., 2019). This evidence further highlights that plants response to PGPR inoculation can be highly specific and depending on the two interacting partners and the soil characteristics.

In general, the results of the second trial, performed in conditions closer to the field scale including the fruit production step, are in accordance with those achieved in very controlled short-term growth (Trial 1). In some cases, the physiological and biochemical determinations in the presence of the inoculum are just comparable and not significantly higher than those measured in controls, very likely because a shorter trial makes the effects more evident. It is also worth noting that the first trial did not plan a fertilization treatment, just comparing plant watering, with or without the inoculum, a condition that is really far from the real agronomic practices. Overall, our results indicate that the inoculum, when added to a limited amount of chemical fertilizer, albeit still in partially controlled conditions, allows the plant to reach at least the same performances as in presence of the commercial dose of the fertilizer. This can highlight that bioinoculants might be considered as a valid complement of chemical fertilizers for a more sustainable agricultural scenario; nonetheless, in order to obtain more comprehensive knowledge about the possible activities of the

investigated microbial strain in realistic conditions, field trials will be required.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

MS, SC, CC, and YP: experimental design and manuscript writing and critical revision. MS, FV, TM, and YP: experiments execution and data collection. MS and YP: data analyses and visualization. MS, TM, SC, CC, and YP: data interpretation. CC, TM, and YP: financial support. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.622169/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# A Bacterial Consortium Interacts With Different Varieties of Maize, Promotes the Plant Growth, and Reduces the Application of Chemical Fertilizer Under Field Conditions

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The success of beneficial bacteria in improving the crop growth and yield depends on an adequate plant-bacteria interaction. In this work, the capability of *Azospirillum brasilense* Sp7, *Pseudomonas putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A to interact with six maize varieties was evaluated by both single-bacterium application and consortium application. The bacterial consortium efficiently colonized the rhizosphere of the autochthonous yellow and H48 hybrid varieties. Bacterial colonization by the consortium was higher than under single-bacterium colonization. The two maize varieties assayed under greenhouse conditions showed increased plant growth compared to the control. The effect of consortium inoculation plus 50% fertilization was compared with the 100% nitrogen fertilization under field conditions using the autochthonous yellow maize. Inoculation with the consortium plus 50% urea produced a similar grain yield compared to 100% urea fertilization. However, a biomass decrease was observed in plants inoculated with the consortium plus 50% urea compared to the other treatments. Furthermore, the safety of these bacteria was evaluated in a rat model after oral administration. Animals did not present any negative effects, after bacterial administration. In conclusion, the bacterial consortium offers a safety alternative that can reduce chemical fertilization by half while producing the same crop yield obtained with 100% fertilization. Decreased chemical fertilization could avoid contamination and reduce the cost in agricultural practices.

**Keywords:** consortium, bacterial safety, urea reduction, crop yield, field experiments

## INTRODUCTION

The study of the interaction of plant growth-promoting rhizobacteria (PGPR) with plants of agricultural interest has led to the development of biofertilizers or bio-inoculants (Kumar et al., 2007; Zahid et al., 2015). These rhizobacteria have the ability to increase plant growth and yield (Naveed et al., 2008). Furthermore, the rhizobacteria can protect plants from phytopathogens (Wang et al., 2012; Singh et al., 2014), increase plant tolerance to high concentrations of heavy metals (Hassan et al., 2014; Ramírez et al., 2019, 2020), and perform bioremediation (Glick, 2003; Sheng et al., 2012).

The rhizobacteria with the capability to promote the growth of plants include several species such as *Pseudomonas fluorescens* and *P. aeruginosa* for maize (Adjanohoun et al., 2011); *Paenibacillus lentimorbus* and *Bacillus amyloliquefaciens* for rice (Bisht and Chauhan, 2020); *Rhizobium pisi* and *Pseudomonas monteilii* for bean (Sánchez et al., 2014); and *Bacillus subtilis* and *Pseudomonas fluorescens* for *Capsicum annuum* (Sundaramoorthy et al., 2012). Furthermore, some of those bacteria perform control of fungal diseases produced by *Fusarium solani* (Sundaramoorthy et al., 2012).

In the last two decades, the synergy of two or more than two PGPRs has been investigated after they are simultaneously inoculated in the same plant (Lally et al., 2017; Mpanga et al., 2019). The application of consortia can increase the production and growth of maize and cucumber plants compared to the inoculation of individual bacteria (Ehteshami et al., 2007; Wang et al., 2012). Co-inoculation of *Rhizobium* sp. and *Pseudomonas* sp. in *Phaseolus vulgaris* increased nodulation and growth parameters, compared to single inoculation (Sánchez et al., 2014). Chickpea inoculated with a consortium formulated with *Rhizobium* sp. and *Mesorhizobium ciceri* also increased plant production and plant growth (Shahzad et al., 2014). The study of soybeans and common beans co-inoculated with *Bradyrhizobium japonicum* and *Azospirillum brasilense* revealed an increment in the yield (Hungria et al., 2013). Regarding to microbial consortia applied to maize plants, the inoculation of *A. brasilense* and *Bacillus subtilis* improved phosphorus uptake, plant development, and corn grain yield (Pereira et al., 2020). Similarly, the consortium of *Trichoderma harzianum* OMG16 and *B. amyloliquefaciens* FZB42 increased the shoot dry matter and grain yield in maize (Mpanga et al., 2019).

In addition, the inoculation of plants with a PGPR consortium improved plant tolerance to abiotic stresses such as water deficit (Ehteshami et al., 2007; Wang et al., 2012; Shahzad et al., 2014), the tolerance to high salt concentration (Ahmad et al., 2013), and the high concentrations of heavy metals (Sheng et al., 2012; Hassan et al., 2014). Decreased application of chemical fertilization together with a good crop yield has been reported in plants inoculated with a bacterial consortium (Da Costa et al., 2013; Shahzad et al., 2013).

Traditional agricultural practices are based on the application of chemical fertilization to obtain high crop yields (Marks et al., 2013). However, the ecological costs generated by the massive application of chemical fertilizers are high, such as the contamination of agricultural soils, water, and the air with

significant production of greenhouse gases (Marks et al., 2013; Coskun et al., 2017) and health damage to farmers (Hansen and Donohoe, 2003). Therefore, current agricultural practices require the adoption of new alternatives allowing high yields to be obtained with decreases in the pollutants generated by nitrogen fertilization (Hungria et al., 2013; Vacheron et al., 2013). In this context, *P. vulgaris* inoculated with selected microbial consortia showed an increment in fruit production with respect to the control, when plants were treated with 75% of chemical fertilization; similar grain yield and plant growth were obtained in treatments added with 100% of chemical fertilization (Chauhan and Bagyaraj, 2015). The co-inoculation of *A. brasilense* and *B. subtilis* in sugarcane (variety RB92579) growing in a field a 75% reduction in phosphorus fertilization increased the dry matter content, total phosphorus accumulation, and the production of stems by 38% (Rosa et al., 2020).

The beneficial effects on plants (plant growth and yield) inoculated with bacterial formulations (individual strains or in a consortium) could be affected by several factors, such as the bacterial genotype used for the formulation and the plant variety. These factors have been reported to play a role in sugarcane (Muñoz-Rojas and Caballero-Mellado, 2003; De Oliveira et al., 2006), corn (Mrkovački et al., 2016), tomato (Vaikuntapu et al., 2014), and bean (Sánchez et al., 2014). The analysis of several studies suggests that three key steps are required to achieve the beneficial effect of bacteria on plants: attraction of PGPR to a host plant, root colonization, and functional associative symbiosis (Droge et al., 2012). Therefore, those steps must also be evaluated for consortium formulations (Molina-Romero et al., 2017).

Furthermore, PGPR used to formulate individual inoculants or consortia must be harmless, and they should be safe to human and animal health (Vílchez et al., 2015). The literature suggests the use of rhizobacteria classified as non-pathogenic (BSL-1), to design beneficial bacterial formulations for field application. In the case of BSL-2 microorganisms (pathogens and opportunistic pathogens), it is recommended to carry out experiment under strict containment and regulatory practices. However, applications of these microorganisms are not recommended (Keswani et al., 2019).

In our previous work (Molina-Romero et al., 2017), bacterial antagonism assays were performed among 20 strains using the double layer agar plate method. Using data on compatibility and desiccation resistance, a consortium formulated with four plant growth-promoting rhizobacteria (*A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178A) was designed. The compatibility of the selected strains for the bacterial consortium was verified under different growing conditions. All of strains were able to coexist and adapt under different environmental conditions. In addition, the strains of the consortium were experimentally evaluated for their capability to stimulate the growth of maize and their PGPR characteristics (Molina-Romero et al., 2017). *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF-178A, and the consortium displayed a high production of siderophores; *P. putida* KT2440, *Acinetobacter* sp. EMM02, and the consortium showed the highest phosphate solubilisation capacity; and *A. brasilense* Sp7



and *Acinetobacter* sp. EMM02 presented the highest production of total indole compounds (Molina-Romero et al., 2017). Those observed characteristics were in line with other studies (Gamalero et al., 2004; Gulati et al., 2009; Planchamp et al., 2014; Rojas-Tapias et al., 2014; Lin et al., 2018; Bharwad and Rajkumar, 2020).

This consortium showed a beneficial effect on autochthonous blue maize. The benefits remained after subjecting the bacterial consortium to desiccation stress prior to the plant inoculation (Molina-Romero et al., 2017). However, this consortium has not been explored in other maize varieties or under field conditions.

This research aimed to evaluate the capability of *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A (alone or in a consortium) to interact and stimulate the plant growth of 6 maize varieties, under greenhouse conditions. We selected the most promising maize variety for field application. This variety was again inoculated with the consortium and its capability to promote the plant growth was tested under field conditions, using different treatments of urea fertilization (50 and 100% of the recommended N dose). In addition, the effects of the oral administration of all PGPR used to formulate the consortium were evaluated in adult male Long Evans rats.

## MATERIALS AND METHODS

### Germination Rate

Fifty seeds of different maize varieties were inoculated using a bacterial mixture formulated as described previously (Molina-Romero et al., 2017). The maize varieties used were two autochthonous (yellow and red from the Huejotzingo region) and four hybrids (1463, 1069, 888, and H48), and hybrid varieties were coated with the carboxin-thiram fungicide (Abati et al., 2014; Haghaniifar et al., 2018) by providers from the municipality of Huejotzingo. Inoculation was performed during 1 h by submerging seeds in the bacterial suspension containing  $10^7$  Colony Forming Units (CFU)/mL of *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178. After inoculation, seeds were placed on sterile filter paper moistened with sterile water deposited in a sterile petri dish and placed at 20°C for 48–72 h for germination. For the control treatment of each corn variety, seeds were submerged in distilled water only.

### Adherence and Colonization

The cell suspension was prepared as described by Molina-Romero et al. (2017). Inoculation of autochthonous and hybrid seeds (coated with fungicide) was carried out by submerging them in the bacterial suspensions for 1 h. The bacterial numbers of the suspension were determined in the order of  $10^6$  to  $10^7$  CFU/mL by using the Massive Seal Drop Plate (MSDP) method (Corral-Lugo et al., 2012), selection media were used for each strain, determining the population for the individual strains and the bacterial consortium.

Fifty seeds of each variety (autochthonous or hybrid) were inoculated for 1 h with the respective bacterial suspension (single strains or the consortium). Inoculated seeds were sown in 50 mL

conical tubes with 15 g of sterile vermiculite. Five milliliters of sterile MS liquid and 2 mL of water was added to each tube (Murashige and Skoog, 1962). All tubes were placed in a greenhouse with 16 h of light and a temperature of 30°C during the day and 8 h of darkness and a temperature of 25°C during the night.

Six inoculated seeds were extracted within 24 h of sowing and the bacterial number adhered to seeds was determined by the MSDP method. For this, seeds were placed in 50 mL conical tubes with 3 mL of sterile water for 1.5 h. Selective media for each bacterial strain and the bacterial consortium were used for quantification (Molina-Romero et al., 2017). Plates were incubated for 24 to 48 h at 30°C. The other inoculated seeds (with an individual bacterium or in a consortium) sown in sterile vermiculite were kept under greenhouse conditions for 21 days. Plants were watered with distilled water every 4 days. At 21 days post inoculation (dpi), six plants of each treatment were used to determine rhizosphere colonization (Rodríguez-Andrade et al., 2015). The root of each plant was placed in 50 mL conical tubes with 10 mL of sterile water for 1.5 h. To determine the bacterial population extracted from the roots, the MSDP technique was used with each selection media for each bacterium; the incubation conditions of the plates were 24 to 48 h at 30°C (Rodríguez-Andrade et al., 2015; Molina-Romero et al., 2017). The vermiculite strongly adhered to the root was removed and its dry weight determined to calculate the parameter Colony-Forming Units / gram of Vermiculite (CFU/gV) (Morales-García et al., 2011; Rodríguez-Andrade et al., 2015; Molina-Romero et al., 2017; Pazos-Rojas et al., 2019).

Some isolated bacteria were selected to confirm bacterial identity. To do it, the 16S rDNA gene was amplified. The 1.5 kb amplifications were digested with the *MspI* enzyme; subsequently, the electrophoretic separation was performed to observe the characteristic restriction pattern of the PGPR inoculated in each plant (Molina-Romero et al., 2017).

### Animal Experiments

Adult male Long Evans rats (with an average weight of 250 to 270 g) were obtained from the Benemérita Universidad Autónoma de Puebla, México. The animals were housed in experimental cages; food and tap water were free to demand. Environmental conditions were maintained at a temperature of  $22^\circ\text{C} \pm 2^\circ\text{C}$  and 60% relative humidity, with a light-dark cycle of 12 h. The protocol in this study was carried out according to the official Mexican standard NOM-062-ZOO-1999.

The treatments established to evaluate the PGPR-rat interaction were five (bacterial consortium and the four independent strains: *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178). For the control treatment, animals were not inoculated. Four animals were inoculated for each treatment ( $n = 4$  rats).

Rats were dewormed and vitaminized before inoculation. A cell suspension of each strain and consortium was formulated with a population of  $1 \times 10^7$  CFU/mL, according to Molina-Romero et al. (2017). The administration of 1 mL of cell suspension was orally introduced with a cannula, after the sedation of the animal with phenobarbital (0.7 mL/270 g weight).

Animals of the control treatment were administered with 1 mL of physiological solution. Both inoculated and control rats were observed twice, every day, over a period of 30 days. Two independent experiments were established. The parameters evaluated in the rats were the alteration of motor activity, weight loss, diarrhea, and the presence of lethargy. In the presence of the aforementioned parameters and other symptoms that compromise the life of the animal, the slaughter would take place.

At the end of the evaluation time of the symptoms of bacterial infection (1 month), the rats were sedated and sacrificed by decapitation. The extraction of the meninges, small intestine, and a blood sample from the heart, under aseptic conditions, was performed. Organs and samples were homogenized with a physiological solution for subsequent seeding in the selection media designed for each PGPR (Molina-Romero et al., 2017). The incubation conditions of the selection media inoculated with the samples from the rats of each treatment were 48 h and 30°C. The procedures described in this study were based on the Standards for the use and care of laboratory animals of the Mexican Council of Animal Care and as indicated in NOM-062-ZOO-1999, which are also approved by the Animals and Ethics Committee of the BUAP. All effort was made to minimize the number of animals used and to ensure minimal pain and discomfort.

## Determination of Growth Promotion Parameters Under Greenhouse

Fifty seeds were inoculated for each treatment and were sown in pots containing 650 g of sterile vermiculite. Irrigation for each pot was done with 500 milliliters of sterile MS nutrient solution (Murashige and Skoog, 1962) and 200 mL of sterile water. The greenhouse cultivation conditions were 16 h of light at 30°C during the day and 8 h of darkened at 22°C during the night. Seedlings were developed up to 45 dpi with regular irrigation with distilled water.

At 45 days after inoculation (dpi) plants were removed from the vermiculite and washed with water, and the excess water was dried with absorbent paper. A tape measure and Vernier were used to measure the height of the plant and the stem diameter, respectively. The fresh weight of the seedlings was determined with the analytical balance. Subsequently, the samples were dried in the oven at 75°C until they reached a constant weight. This procedure was also carried out to determine the growth parameters in the field experiment.

## Field Experiment

### Localization of the Field Experiment

The field experiment was carried out in the summer of 2015. The experiment was carried out in the municipality of Huejotzingo, Puebla México, located between 19° 06' and 19° 16' in north latitude; 98° 20' and 98° 38' in west longitude meridians. The climate is temperate sub-humid with rains in summer, with an average maximum temperature of 16°C and a minimum of 2°C. The average annual precipitation is 900 to 1,100 mm in the territory. Physicochemical analysis of a soil sample from the experimental place was determined as sandy clay soil, according to standard methods (Shahzad et al., 2014).

## Treatments and Experimental Design

Four treatments were established in the field: (1) Bacterial consortium treatment; seeds inoculated with the consortium alone, without urea application in the field. (2) Urea treatment; seeds without bacterial inoculation with a complete dose of urea fertilization (100%) in the field. (3) 50% urea treatment plus consortium; seeds inoculated with the consortium and half dose of urea fertilization applied in the field. (4) Control treatment, non-inoculated seeds without urea application in the field.

The seeds (from the bacterial consortium and 50% urea plus consortium treatments) were inoculated as previously described in the adherence and colonization section. The seeds of the control treatment and 100% urea were immersed in sterile distilled water for 1 h to discard the effect of water. The seeds were sown manually in plots (0.8 m wide by 152 m long, with 8 replicates for each treatment) with a distance from plant to plant of 80 cm.

Nitrogen chemical fertilization was applied using the commercial urea formula (46-0-0) in only two treatments: 100% urea (69 kg of N/ha) and 50% urea (34.5 kg of N/ha) plus consortium treatments. For the irrigation in the temporary field, a water channel was used.

The design employed was randomized complete block design (RCBD) with four treatments and 8 replicates, the experimental area was divided into 973 m<sup>2</sup> for each treatment. The plot was separated by 1.0 m, with small furrows of approximately 1.5 m to prevent surface mixing with the bacteria and fertilizer used in each treatment. This is because of the strong rains that are common in the summer season.

## Recording of Growth Parameters and Production

The corn growth period was carried out in the traditional period season from March to June 2016. Growth parameters (plant height, root dry weight, root length, and plant diameter) were evaluated using 20 plants of each treatment, through the design of random blocks. The plant growth parameters were recorded for 80 days after sowing as previously described in the methodology of the greenhouse experiment.

Biomass and production were recorded after the maturity of the crop, 190 days after sowing, the corn was harvested and the cobs were separated, later they were weighed on a balance; the data were converted to Kg/ha, following the unit method.

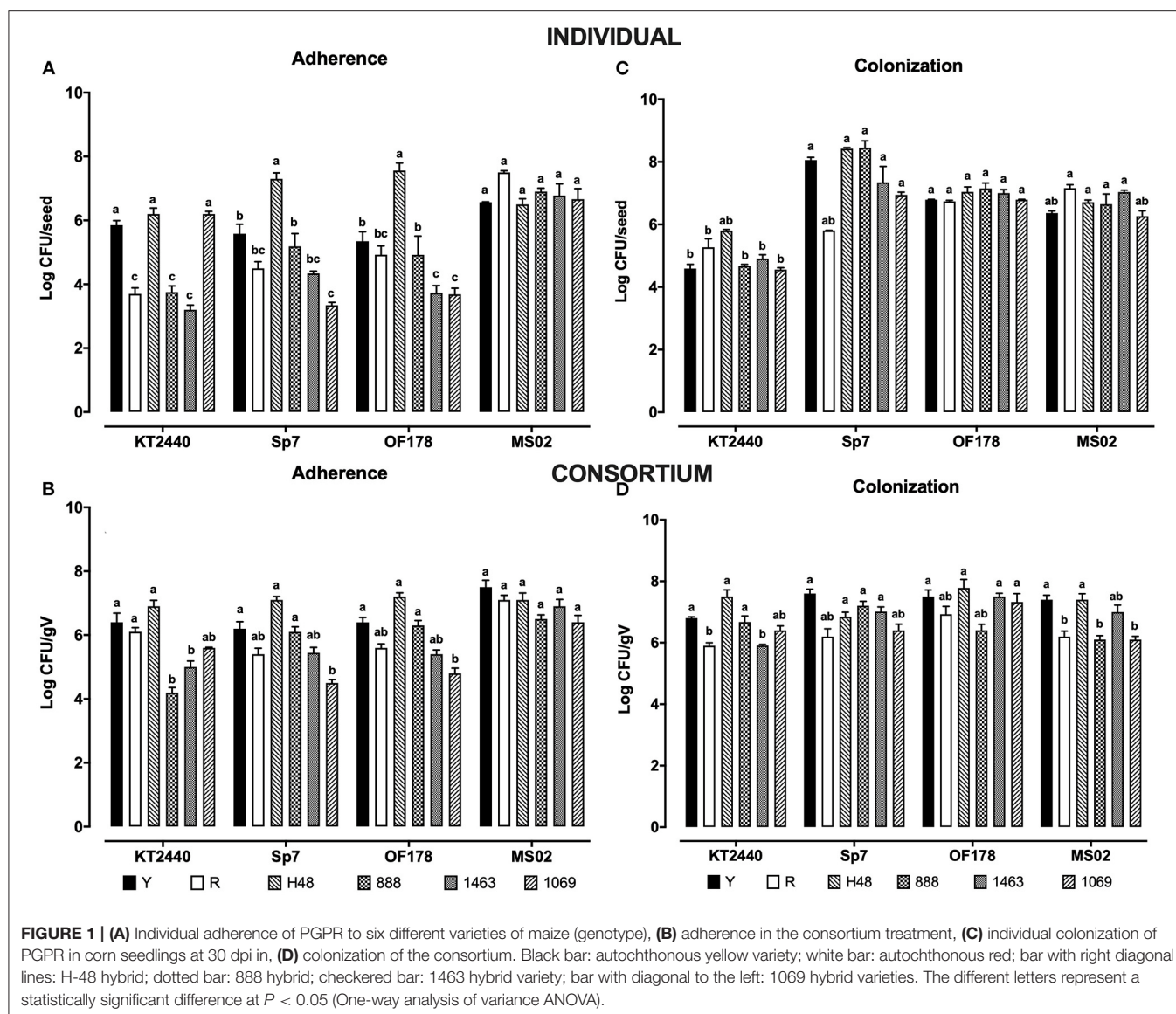
## Statistical Analysis

Data on plants (adherence, colonization, and growth parameters) were subjected to a one-way analysis of variance (ANOVA). Significant differences between average were obtained with Duncan multiple range test at  $P \leq 0.05$ , using SigmaPlot 12 (Handel Scientific Software).

## RESULTS

### Germination of Maize Inoculated With a Bacterial Consortium

The percentage of germination in both yellow and red autochthonous maize varieties with and without bacterial



inoculation was of 100%. All hybrid varieties showed a germination percentage of 100% when they were inoculated with the bacterial consortium. In contrast, the non-inoculated seeds of 1069 and 1463 hybrids varieties presented 96% germination. The H48 and 888 hybrids presented 98 and 100% of germination, respectively.

## Adherence

The four strains (alone or in a consortium) had the ability to adhere to the six maize seed varieties under *in vitro* conditions. The individual adhesion of *Acinetobacter* sp. MS02 presented a similar trend in all varieties explored, on the order of  $10^6$ - $10^7$  CFU/seed (Figure 1A). The adhesion of this strain increased one order of magnitude, in the autochthonous yellow and the hybrid H48 varieties, when it was inoculated in a consortium (Figure 1B). However, in the hybrids varieties (888, 1463, and 1069) and the red autochthonous variety, adhesion

maintained the same tendency when the bacteria were inoculated individually or in a consortium.

*A. brasilense* Sp7 and *Sphingomonas* sp. OF178 had a similar adhesion capability in most varieties explored. They adhered at high numbers ( $10^5$ - $10^7$  CFU/seed) to the yellow autochthonous variety, and to the hybrid varieties H48 and 888. However, they adhered at low numbers ( $10^3$ - $10^4$  CFU/seed) to the red autochthonous variety and the hybrid varieties 1069 and 1463 (Figure 1A).

The adhesion of *P. putida* KT2440 to the autochthonous red variety, and the 888 and 1463 hybrids was lower, with bacterial numbers on the order of  $10^3$  CFU/seed. However, KT2440 showed a high adhesion in the H48, 1069, and yellow autochthonous varieties ( $10^5$ - $10^6$  CFU/seed).

Interestingly, *A. brasilense* Sp7, *P. putida* KT2440, and *Sphingomonas* sp. OF178 inoculated in a consortium, showed an increased adhesion (one or two orders of magnitude) (Figure 1B)



compared to their individual adhesion in hybrid varieties 888, 1463, and 1069, and the autochthonous yellow and red varieties (**Figure 1B**). For the H48 hybrid, adhesion of the bacterial strains remained similar under individual or consortium inoculation ( $10^7$  CFU/seed).

## Rhizosphere Colonization

*Sphingomonas* sp. OF178 and *Acinetobacter* sp. MS02 showed a similar rhizosphere colonization in the six corn varieties,  $10^6$ – $10^7$  CFU/gV and  $10^5$  to  $10^6$  CFU/gV, respectively (**Figure 1C**).

In the yellow autochthonous variety, and the H48 and 888 hybrids, *A. brasilense* Sp7 presented a higher individual colonization ( $10^8$  CFU/gV) (**Figure 1C**) than was observed in the red autochthonous variety and 1463 and 1069 hybrids ( $10^6$  CFU/gV).

The low individual colonization of *P. putida* KT2440 ( $10^4$  to  $10^5$  CFU/gV) was observed in the autochthonous and hybrid varieties (**Figure 1C**).

The colonization of *A. brasilense* Sp7, *Sphingomonas* sp. OF178, and *Acinetobacter* sp. MS02 strains in the consortium showed a rhizosphere colonization ranging from  $10^6$  to  $10^7$  CFU/gV, for all tested varieties (**Figure 1D**). In several cases, bacterial colonization in the consortium was increased. The rhizosphere colonization of *P. putida* KT2440 increased in the consortium inoculation treatments, in most of maize varieties evaluated, with the exception of the autochthonous red variety, in which the colonization remained similar to individual rhizosphere colonization (**Figure 1D**).

## Interaction Tests of PGPR With the Rat Animal Model

The effect of PGPR inoculation on the rat model was evaluated. The rats inoculated with *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, *Sphingomonas* sp. OF178A, or the bacterial consortium did not show any changes in their motor activity, and there was not weight loss or lethargy observed from the time of inoculation until 30 days after inoculation (**Table 1**). Therefore, rats inoculated with the bacterial consortium or individual treatments did not exhibit any alterations, indicating that there was no damage to their health. Interestingly, bacteria were undetectable, in blood, meninges, and small intestine samples from rats treated with the bacterial consortium or individual strains (**Table 1**).

## Effect of Consortium Inoculation on Growth Promotion in the H48 Hybrid and Yellow Autochthonous Varieties Under Greenhouse Conditions

The bacterial consortium showed an adequate capacity to adhere to and colonize the seeds of the H48 hybrid and yellow autochthonous varieties. The adherence was  $10^6$  to  $10^7$  CFU/seed, and colonization rate was  $10^6$  to  $10^7$  CFU/gV in the two varieties. Likewise, when the seeds of these two varieties were inoculated with the four PGPR strains individually, they presented similar adherence and colonization.

Based on these results, the H48 hybrid and autochthonous yellow varieties were inoculated with the bacterial consortium to evaluate whether the consortium promotes plant growth under greenhouse conditions.

The dry weight of the aerial parts of the H48 hybrid plants inoculated with the bacterial consortium was 7.23 g ( $\pm 0.81$ ), and it was 5.62 g ( $\pm 0.58$ ) for control plants. The difference was significant statistically ( $p \leq 0.05$ ) (**Figure 2E**). The dry weight of the aerial part of the autochthonous yellow variety inoculated with the consortium ( $5.43 \pm 0.38$ ) was significantly higher than that of the control plants 4.08 g ( $\pm 0.55$ ) (**Figure 2A**).

The root dry weight of both varieties inoculated with the consortium was significantly higher (5.33 g for H48 and 3.04 g for indigenous yellow) than that of the control plants (3.82 and 1.87 g, respectively) (**Figures 2B,F**).

Likewise, the H48 hybrid and indigenous yellow varieties inoculated with the consortium surpassed the control treatment in the parameters of height and diameter, with a significant difference (**Figures 2C,D,G,H**).

## Autochthonous Yellow Variety Under Field Conditions

The native yellow maize was the variety showing the most stimulation after the inoculation of the bacterial consortium, under greenhouse conditions. Also, this variety is of great interest to farmers in the region Huejotzingo region of Puebla, because they prefer to cultivate indigenous corn varieties. Therefore, we decided to evaluate the effect of bacterial inoculation on the autochthonous yellow variety under field conditions.

The results of the field experiment showed that treatments with bacterial inoculation increased the plant height, dry root weight, and root length of the yellow variety compared to the control plants.

Plants subjected to the 50% urea plus consortium treatment and the plants fertilized with 100% urea presented the higher values of plant height, root dry weight, root length, and plant diameter. In general, parameters of growth between these two treatments were not significantly different (**Table 2A**).

The height, root length, and dry weight of the plants inoculated with the consortium compared to the controls showed significantly higher values: 204.62 and 184.63 cm of plant height, 36.28 and 33.38 cm root length; and 7.33 and 6.11 g of dry weight, respectively (**Table 2A**). However, the diameter of the plants inoculated with the bacterial consortium was similar to the values recorded for the control plants, without any statistically significant differences (**Table 2A**).

The plants treated with 100% urea and the control plants showed the highest biomass production under field conditions. In contrast, the treatments resulting in the lowest biomass production included the plants inoculated with the bacterial consortium alone and plants treated with 50% urea plus the consortium (4.74 and 4.59 t/ha, respectively) (**Table 2B**).

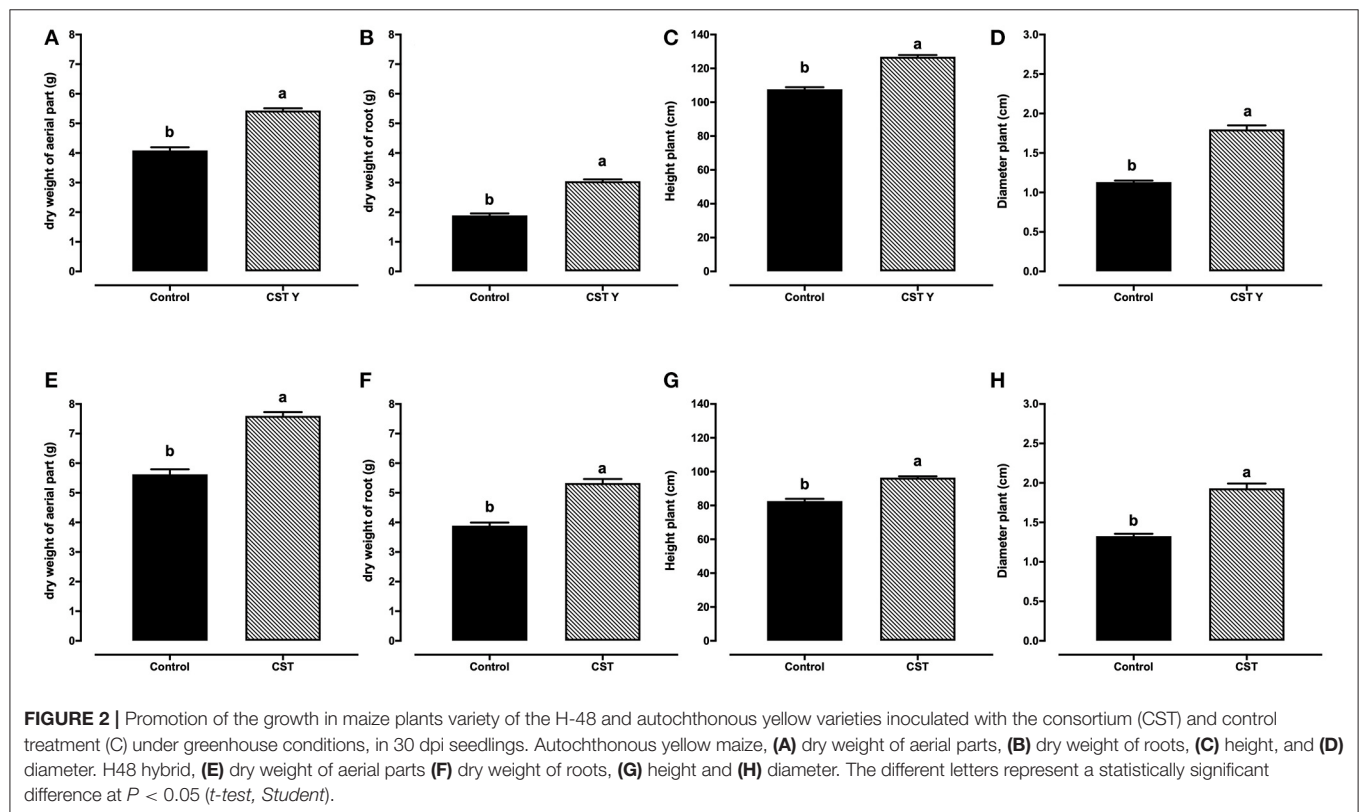
The 50% urea plus consortium treatment resulted in the highest grain yield, followed by the 100% urea treatment (5353.50 and 4914.50 kg/ha, respectively). In contrast, the bacterial consortium treatment resulted in the third highest in grain



**TABLE 1** | Effects of PGPR on the rat animal model.

Treatment	Motor activity	Lethargy	Weight loss of the animal	Animal sacrifice	PGPR isolation, after oral inoculation		
					Blood	Meninges	Small intestine
Control	N	A	N	A	NI	NI	NI
<i>A. brasilense</i> Sp7	N	A	N	A	NI	NI	NI
<i>P. putida</i> KT2440	N	A	N	A	NI	NI	NI
<i>Acinetobacter</i> sp. EMM02	N	A	N	A	NI	NI	NI
<i>Sphingomonas</i> sp. OF178A	N	A	N	A	NI	NI	NI
Consortium	N	A	N	A	NI	NI	NI

N, normal; A, absent; NI, not isolated. The control treatment was not PGPR-inoculated. Four animals were inoculated for each treatment, individual and consortium ( $n = 4$ ). Data were obtained from two independent experiments.



production; and the lowest production was presented by the control (4549.3 and 3916.0 kg/ha, respectively) (Table 2B).

An increase of 1437.5 kg/ha was obtained with the 50% urea plus consortium treatment compared to the control (Table 2B).

## DISCUSSION

Bacterial establishment in the rhizosphere is a crucial step to obtain the beneficial effect of PGPR on the host plant. Therefore, an adequate adhesion and colonization must occur to obtain the desired effects (Drogue et al., 2012; Shahzad et al., 2013). Individual and consortium bacterial adhesion to maize for *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A was variable among the maize

seed varieties explored in this work, suggesting differences in bacterium-seed affinity. It is important to highlight that the adhesion was improved when bacterial strains were applied in the consortium to all corn varieties used and was in agreement with our previous observations in the blue autochthonous variety (Molina-Romero et al., 2017).

A correlation between colonization capacity and the ability to stimulate plants has been reported (Moradtalab et al., 2020). For example, a colonization on the order of  $10^7$  CFU/g root dry weight stimulates the plant biomass of the forage maize (Piromyou et al., 2011). In our work, the colonization following individual and bacterial consortium inoculation in the 888, 1069, 1463, and red-autochthonous varieties was less efficient compared to that in the H48 and autochthonous yellow

**TABLE 2 |** Effect of the consortium inoculation on the growth of autochthonous yellow maize under field conditions using different levels of urea fertilization.

<b>(A)</b>				
<b>Treatment</b>	<b>Plant Height (cm)</b>	<b>Dry root weight (g)</b>	<b>Root Length (cm)</b>	<b>Plant diameter (cm)</b>
Control	184.63 ± 6.64 c	6.11 ± 0.51 c	33.38 ± 2.35 c	8.55 ± 0.64 b
Consortium	204.62 ± 13.17 b	7.33 ± 0.74 b	36.28 ± 2.19 b	9.55 ± 0.73 ab
Urea 100%	221.50 ± 17.07 a	7.23 ± 0.60 b	40.28 ± 2.39 a	10.32 ± 0.89 a
Urea 50% + consortium	228.84 ± 11.24 a	8.30 ± 0.69 a	42.52 ± 2.94 a	10.44 ± 0.69 a

<b>(B)</b>		
<b>Treatment</b>	<b>Total biomass t/ha</b>	<b>Grain production t/ha</b>
Control	5.10 ± 2.4 a	3.91 ± 0.26 c
Consortium	4.74 ± 1.6 ab	4.54 ± 0.24 b
Urea 100%	5.15 ± 3.9 a	4.91 ± 0.25 ab
Urea 50% + consortium	4.59 ± 1.9 b	5.35 ± 0.15 a

**(A)** Treatments: consortium (consortium alone), 100% urea (fertilization with full dose of urea), urea 50% + consortium (inoculation of the consortium plus fertilization with half the dose of urea). The autochthonous yellow maize variety was used for field experiments, and evaluation was performed at 80 days after sowing. Data represent the means and SD of 20 replicates, analyzed with one-way ANOVA followed by a Tukey-test ( $p \leq 0.05$ ). **(B)** total biomass per hectare at the time of harvest (190 days after planting); Total grain production per hectare. Data represent the means and SD of eight replicates. Different letters in each column indicate significant differences. The biomass and grain production data were analyzed with the Duncan multiple range test at  $p \leq 0.05$ .

varieties. However, the inoculation of the bacterial consortium improved the bacterial colonization capacity compared to the individual treatments. However, a correlation between adequate colonization and the growth promotion of the plants of the H48 hybrid and autochthonous yellow varieties was observed.

Bacterial colonization depends on the plant variety. The colonization of a consortium formulated with *Gluconacetobacter diazotrophicus*, *Herbaspirillum seropedicae*, *Herbaspirillum rubrisubalbicans*, *Azospirillum amazonense*, and *Burkholderia tropica* was found to differ in two varieties of sugar cane (SP70-1143 and SP 813250) (De Oliveira et al., 2006). The colonization capability of other consortia (*Pseudomonas striata* and *Piriformospora indica*) is also plant-dependent in maize varieties or mung beans varieties (Singh et al., 2009). In the present study, we also observed the influence of the maize variety on the bacterial colonization.

Few studies have evaluated the microbiological safety of PGPR before field application. Bacterial strains used to improve plant growth must be safe for human health and the environment (Kampers et al., 2019; Visnovsky et al., 2020). An example in which bacterial innocuity was explored is provided by the work reported by Chávez-Ramírez et al. (2020) in *Paenibacillus polymyxa* (NMA1017), whose safety was tested on tobacco leaves and the *Galleria mellonella* larvae, reinforcing its safe application as a biocontrol agent (Chávez-Ramírez et al., 2020). In the present work, we used a rat model to explore whether the bacterial strains provoke adverse effects on the animals after the oral administration of 1 mL containing  $10^7$  CFU/mL. The absence of signs of disease and the lack of bacteria in the blood, meninges, and small intestines of the animals inoculated with the bacterial consortium or individual strains, suggested that the bacterial strains were innocuous to the rats and they could be safe for human health when applied to the crops.

It is important to verify the safety of PGPR before they are used as biofertilizers, especially if they are closely related to

pathogenic bacterial strains. For example, *Bacillus* sp. (RZ2MS9) and *Burkholderia ambifaria* (RZ2MS16) present a potential risk due to their taxonomic proximity to pathogenic groups (Batista et al., 2018; Ferreira et al., 2019), especially when a formulation includes bacteria considered to be opportunistic pathogens, such as *P. aeruginosa* (Lavakush et al., 2014). By conducting this verification, we can ensure that the application in the field does not represent a health risk to people who are in contact with the formulation when it is applied to plants.

Regarding the plant growth promotion under greenhouse conditions, the autochthonous yellow variety and the H48 hybrid inoculated with the bacterial consortium showed significant increases in height, diameter, the dry weight of the aerial part and the roots compared to the control. The benefit to growth parameters was probably due to the ability of the bacteria of the consortium to produce indoles, solubilise phosphates, and produce siderophores (Molina-Romero et al., 2017). These beneficial effects agree with the previous observations made in blue maize inoculated with this same consortium (Molina-Romero et al., 2017). Similarly, an increase in root dry weight was recorded in jolly hybrid maize, when it was inoculated with a *Bacillus subtilis* FZB24 and *B. subtilis* GB03 consortium (Myresiotis et al., 2015). In contrast, a consortium of *Azospirillum lipoferum*, *P. fluorescens*, *P. putida*, and chitosan did not increase the height, diameter, or number of leaves per plant but significantly increased the dry weight of the roots and the aerial parts of the maize under greenhouse conditions (Agbodjato et al., 2016). The co-inoculation of maize with *A. brasilense* and *B. subtilis* has also shown greater benefits than individual inoculation (Pereira et al., 2020).

In the present fieldwork, the inoculation of the bacterial consortium in the autochthonous yellow variety produced a beneficial effect on the plants, increasing the height, dry root weight, and root length of the plant and yield. However, the total biomass and the diameter of the plants inoculated with

the consortium were similar to the control. These growth-promotion results showed a trend similar to the trend reported in a field study indicating that the maize inoculation with a consortium with *Azotobacter chroococcum* and *A. liporefum* resulted in increments in shoot and seed dry weight, plant height, and yield compared to the individual inoculation of PGPR and the control (Biari et al., 2008).

In this study, nitrogen fertilization at 100% and the treatment with the consortium plus 50% urea resulted in the greatest increments in height, diameter, dry root weight, and grain production in comparison to non-inoculated plants. These results showed that the consortium stimulates the growth of autochthonous maize when a half dose of mineral nitrogen used in traditional Mexican agricultural practices is added, which generated results similar to the application of a complete nitrogen dose.

Similar trends have been reported in other studies. For example, the rice inoculation with a consortium (combined *Pseudomonas* culture, *A. chroococcum*, and *A. brasilense*) plus the application of 50% mineral phosphorus resulted in results similar to the complete dose of phosphorus plus the consortium (Lavakush et al., 2014). Additionally, the hybrid maize (33M15) has been inoculated with *Pseudomonas thivervalensis* (STF3) (Shahzad et al., 2013), the hybrid corn (786) has been inoculated with *P. fluorescens* biotype G (N3) (Naveed et al., 2008), and safflower has been inoculated with *Azospirillum* sp. and *Azotobacter* sp. (Nosheen et al., 2016). In sunflower, the highest grain production, oil, and protein content were observed in association with the consortium (*Azotobacter* sp. and *Azospirillum* sp.) plus 50% nitrogen fertilization (Naseri and Mirzaei, 2010).

Most of the studies in which plants were inoculated with PGPR or consortia have reported an increase in yield and biomass (Ehteshami et al., 2007; Kumar et al., 2007; Shahzad et al., 2013). In this work a higher grain yield was observed following the application of the consortium plus 50% urea while the biomass production was lower compared to the control, consortium inoculation alone, or 100% urea treatment. Most likely, during the interaction with bacteria, nutrients in the plant are directed to grain formation instead of other plant structures (Okuno et al., 2014), which should be clarified in the future. The results observed in maize inoculated with the consortium and treated a half of the dose of nitrogen fertilization contrasts with the direct correlation established between nutrition and higher grain production, particularly when abundant mineral nitrogen is available (Rozier et al., 2017; Bisht and Chauhan, 2020).

Plants interact with efficient indole-producing and phosphate solubilizing bacteria under low-nutrient conditions. However, in a moderate nutrient scheme, plants selectively associate with bacteria with a higher capacity for phosphate solubilization (Da Costa et al., 2013; Pii et al., 2019; Bisht and Chauhan, 2020). As observed in this work, under treatment with 50% urea plus the consortium, we registered a higher promotion of plant growth and grain production due to the beneficial effect of the consortium. This effect could be due to a high phosphate solubilization capability and indole production among the strains of the consortium (Molina-Romero et al., 2017). However, it is necessary to do more work addressing this topic, perhaps using

mutants defective in these mechanisms to verify their roles in the interaction.

Under conditions of abundant nutrients, this selective interaction with bacteria is abolished (Da Costa et al., 2013; Di Salvo et al., 2018), showing that fertilization affects the PGPR-plant interaction. More work is required to define the role of bacterial mixtures on plant inoculation under field conditions. However, the eco-friendly technologies for increasing crop yields are promising (Morales-García et al., 2019).

The application of consortia in agricultural practice is offered as an alternative to implementing sustainable agriculture practices (García De Salamone et al., 2010), due to the reduction of chemical fertilization without compromising the grain yield (Tilman et al., 2002). Only 50% of nitrogen fertilizer applied into the field is absorbed by plants; the remaining half is lost to the environment in the form of ammonia, nitrate, and nitrous oxide, contributing to the pollution of soil, water, and air and to climate change (Coskun et al., 2017; Schröder et al., 2018). Increasing the application of mineral nitrogen fertilizer in rice, corn, wheat, and barley can increase methane and nitrous oxide emissions from agriculture, which directly impacts global warming, due to the characteristics of methane and nitrous oxide as greenhouse gases (Bodelier et al., 2000; Good and Beatty, 2011).

The bacterial consortium studied in this work presents the characteristics of biofertilizer: inducing efficient interaction with the plant, and ecological nature, and contributions to lowering the costs of maize production due to the reduced of mineral nitrogen requirement. Consequently, a decrease in diminution of pollution caused by nitrogen fertilization occurs. Furthermore, the consortium is safe to handle when applied under field conditions.

## CONCLUSION

This research presents the potential of a second-generation consortium formulated with *A. brasilense* Sp7, *P. putida* KT2440, *Acinetobacter* sp. EMM02, and *Sphingomonas* sp. OF178A. The bacterial strains of the consortium are compatible, resistant to desiccation, and efficient for field applications. Since the bacteria of this consortium interact efficiently with the autochthonous yellow maize variety, they trigger a beneficial effect on the grain yield. In addition, this bacterial consortium offers an alternative allowing the efficient use of half the recommended amount of nitrogen fertilizer. The use of the consortium allows a 50% reduction in mineral nitrogen application and generates important benefits for agricultural practices such as lower costs to the producer and a significant decrease in environmental pollution. Based on the results of the animal model, we suggest that the bacterial consortium is a safe formulation for use and manipulation under field conditions and does not cause health problems to animals.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The experiments with animals were carried out in strict accordance with the Mexican Law of Animal Treatment and Protection Guidelines. The animal study was reviewed and approved by Committee of Benemerita Universidad Autonoma de Puebla.

## AUTHOR CONTRIBUTIONS

BV, YM-G, and JM-R: data curation. DM-R and YM-G: funding acquisition. BV, DM-R, and JM-R: research. SJ-S, CO-G, and DM-R: methodology. SJ-S and CO-G: resources. BV, AB, and DM-R: software. JM-R: supervision. AB, YM-G, and DM-R: writing—original draft. DM-R,

AB, and JM-R: writing—review and editing. All authors have read and agree to the published version of the manuscript.

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# Rhizobia as a Source of Plant Growth-Promoting Molecules: Potential Applications and Possible Operational Mechanisms

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The symbiotic interaction between rhizobia and legumes that leads to nodule formation is a complex chemical conversation involving plant release of *nod*-gene inducing signal molecules and bacterial secretion of lipo-chito-oligosaccharide nodulation factors. During this process, the rhizobia and their legume hosts can synthesize and release various phytohormones, such as IAA, lumichrome, riboflavin, lipo-chito-oligosaccharide Nod factors, rhizobitoxine, gibberellins, jasmonates, brassinosteroids, ethylene, cytokinins and the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase that can directly or indirectly stimulate plant growth. Whereas these attributes may promote plant adaptation to various edapho-climatic stresses including the limitations in nutrient elements required for plant growth promotion, tapping their full potential requires understanding of the mechanisms involved in their action. In this regard, several N<sub>2</sub>-fixing rhizobia have been cited for plant growth promotion by solubilizing soil-bound P in the rhizosphere via the synthesis of gluconic acid under the control of pyrroloquinoline quinone (PQQ) genes, just as others are known for the synthesis and release of siderophores for enhanced Fe nutrition in plants, the chelation of heavy metals in the reclamation of contaminated soils, and as biocontrol agents against diseases. Some of these metabolites can enhance plant growth via the suppression of the deleterious effects of other antagonistic molecules, as exemplified by the reduction in the deleterious effect of ethylene by ACC deaminase synthesized by rhizobia. Although symbiotic rhizobia are capable of triggering biological outcomes with direct and indirect effects on plant mineral nutrition, insect pest and disease resistance, a greater understanding of the mechanisms involved remains a challenge in tapping the maximum benefits of the molecules involved. Rather than the effects of individual rhizobial or plant metabolites however, a deeper understanding of their synergistic interactions may be useful in alleviating the effects of multiple plant stress factors for increased growth and productivity.

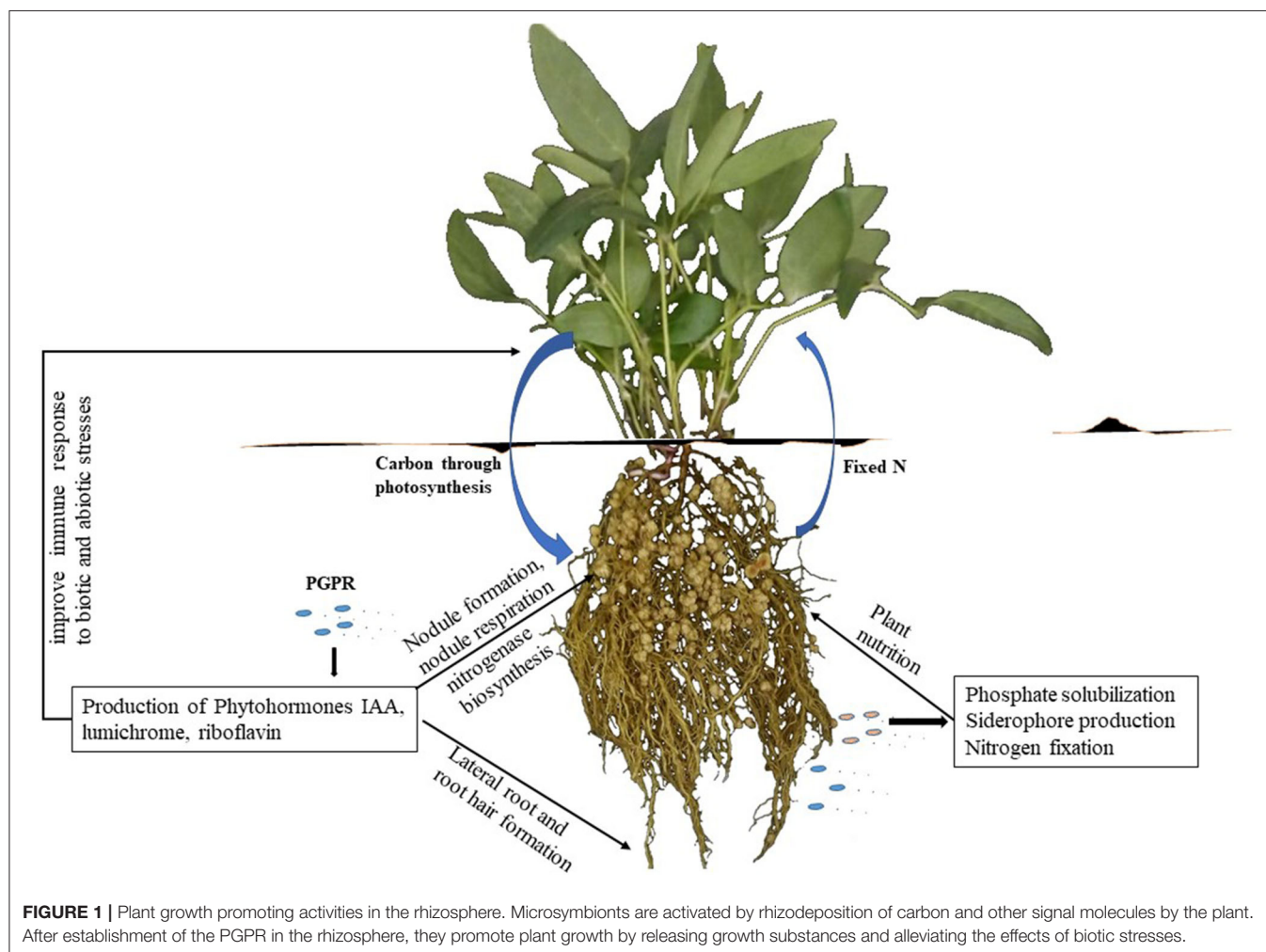
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## INTRODUCTION

Nitrogen is an essential component of all amino acids and nucleic acids, thus making it an important plant nutrient element. Although the atmosphere consists of 78.1%  $N_2$  gas, plants cannot use it unless it is converted into a usable form (Ferguson et al., 2010). Biological  $N_2$  fixation (BNF) is a free source of N that can be exploited by resource-poor farmers for increased crop yields (Giller and Cadisch, 1995), making it one of the most important microbiological processes on earth; globally,  $\sim 33\text{--}46$  Tg of N year<sup>-1</sup> is contributed by the legume-rhizobia symbiosis (Herridge, 2008). So far, 21 bacterial genera have been identified as nodule-forming microsymbionts (Wang et al., 2019a). These microsymbionts are distributed among major bacterial genera of the alpha-proteobacteria such as *Rhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Mesorhizobium*, *Ensifer* (*Sinorhizobium*), *Neorhizobium*, *Pararhizobium*, and *Allorhizobium*, collectively termed “rhizobia”, which can form symbiotic associations with diverse legumes. Similarly, species of the beta-rhizobia *Cupriavidus*, *Paraburkholderia*, and *Trinickia* can also form symbiotic relationships with members of the

*Papilionoideae* and *Caesalpinioideae* (LPWG, 2017; Sprent et al., 2017).

The formation of root nodules in symbiotic legumes involves a complex molecular signaling between the legume host and the rhizobial microsymbiont (Oldroyd et al., 2011). At the onset of nodule organogenesis, the rhizodeposition of flavonoid compounds from legume seed coats or root exudates induce the synthesis of NodD proteins which activate the transcription of genes needed to produce rhizobial lipo-chito oligosaccharide Nod factors (Andrews and Andrews, 2017). Upon perception of Nod factors by the plant, nodule organogenesis commences via the curling of the root hair tips leading to formation of an infection thread, mitotic division of root cortical cells, and consequently the formation of a nodule primordium (Oldroyd et al., 2011). Rhizobia penetrate the cortical cells via the infection thread and are eventually released into the nodule primordium housed in host-derived cells known as symbiosomes (Okubo et al., 2012). Rhizobia undergo differentiation into  $N_2$ -fixing bacteroids which carry out the conversion of atmospheric  $N_2$  into ammonia in a reaction catalyzed by the nitrogenase enzyme (Udvardi and Kahn, 1992). Whereas many rhizobia invade roots





via the infection thread, others utilize either “crack entry” or root epidermal cells as points of infection (González-Sama et al., 2004; Ardley et al., 2013; Bianco, 2014). When N<sub>2</sub> fixation commences, the bacteroids in root nodules supply the host legume with fixed N while receiving C compounds from host photosynthesis (Udvardi and Kahn, 1992).

Besides the provision of symbiotic N, some rhizobia also exhibit physiologically desirable traits such as the production of plant growth-promoting phytohormones, which include indole-3-acetic acids (IAA), cytokinins, gibberellins, riboflavin, lumichrome, Nod factors, etc (Table 1, Figure 1), all of which play diverse roles in enhancing plant growth and productivity (Dakora and Phillips, 2002; Berg, 2009). Thus, rhizobia confer several advantages on plants in addition to the provision of N from symbiotic interactions. The beneficial effects of rhizobia are mediated by the production of diverse

metabolites and enzymes that are directly or indirectly elicited by rhizobia and plants during nodule formation (Table 1). Of the plant growth-promoting molecules, the secretion of IAA, 1-aminocyclopropane-1-carboxylate (ACC) deaminase, lumichrome, riboflavin and protons for phosphate solubilization have been suggested as important PGPR mechanisms underlying plant growth promotion (Figure 1) (Li et al., 2000; Matiru and Dakora, 2005; Gravel et al., 2007; Bal et al., 2013; Dakora et al., 2015). ACC deaminase is an enzyme that can decrease deleterious amounts of ethylene in higher plants, leading to increased productivity. Lumichrome and riboflavin are novel molecules from rhizobial exudates that are known to stimulate plant growth. On the other hand, IAA is an important member of the auxin family that is responsible for controlling plant physiological processes, including cell enlargement and division, tissue differentiation and light/gravity

**TABLE 1 |** Rhizobial metabolites and their functional roles in plant growth and development.

Rhizobial metabolite	Functional roles	References
Lipo-chito oligosaccharides (Nod factors)	*Stimulates seed germination Stimulates lateral root formation Induces the expression of flavonoid genes Regulates phytohormone biosynthesis and homeostasis in legumes Promotes root colonization by arbuscular mycorrhizal fungi Causes cell division and embryogenesis	Dakora, 2003; Prithiviraj et al., 2003; Kidaj et al., 2012
Riboflavin	*Increased bacterial root colonization and nodule occupancy Primed plant defense response Serves as vitamins for plants and bacteria Plant growth promotion	Yang et al., 2002; Dakora, 2003; Zhang et al., 2009; Angulo et al., 2013; Dakora et al., 2015
Lumichrome	Induces plant tolerance to drought through stomatal control Stimulates seedling growth Stimulates root CO <sub>2</sub> production Promotes early tillering in sorghum plants	Dakora, 2003; Dakora et al., 2015
Nitrogenase-linked H <sub>2</sub>	Increases soil microbial population and carbon deposition	Dong and Layzell, 2001; Dakora, 2003
Indole-3-acetic acid (IAA)	*Promotes nodulation *Promotes root growth and plant development	Pii et al., 2007; Camerini et al., 2008; Spaepen et al., 2008; Stajković et al., 2011; Zúñiga et al., 2013
Cytokinin	Regulates nodule organogenesis Increases plant growth	Sturtevant and Taller, 1989; Ping and Bolland, 2004; Giron et al., 2013; Gauthier-Coles et al., 2019
Gibberellin	Promotes nodulation in a dose-dependent manner Promotes seed germination and plant development	Bottini et al., 2004; Ferguson et al., 2005; Foo et al., 2016; McGuinness et al., 2019
Ethylene	Involved in plant defense response to pathogens Promotes plant growth	Dubois et al., 2018
Siderophore	Mobilizes of Fe for plant uptake Increases nodulation and N <sub>2</sub> fixation Improves plant adaptation to pathogen and stress *Increased plant growth	Duhan et al., 1998; Arora et al., 2001; Katiyar and Goel, 2004; Rungin et al., 2012; Hao et al., 2014; Lebrazi and Fikri-Benbrahim, 2018
1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase)	*Improves nodulation and plant growth via the regulation of ethylene production	Ma et al., 2003; Glick et al., 2007; Barnawal et al., 2014; Nascimento et al., 2019
Rhizobitoxine	Improves nodulation and plant growth via the regulation of ethylene production	Ma et al., 2003
Jasmonates	Has role in mycorrhizal and the legume-rhizobium symbiosis. Implicated in the autoregulation of nodulation in legumes. Improved plant response to drought stress	Hause and Schaarschmidt, 2009; Hettenhausen et al., 2015; Wang et al., 2019b
Brassinosteroids	*Regulates nodule number, probably through the regulation of ethylene production. Reported for plant growth promotion	Ferguson et al., 2005; Bartwal et al., 2013; Foo et al., 2016; McGuinness et al., 2019

NB: Superscript (\*) before functional roles indicate experiments in which mutants for a particular trait were compared with wild-type, with one strain being negative for the trait. All other experiments either involved exogenous application of signal molecules, with or without gene expression studies.

responses (Teale et al., 2006; Shokri and Emtiazi, 2010) (**Table 1**).

Plant growth promoting rhizobacteria (PGPR) comprise a diverse group of bacteria that present growth benefits to plants through several mechanisms. The rhizosphere soil of plants tends to contain PGPR that are capable of releasing protons to solubilize soil-bound phosphorus for plant use, and are usually referred to as phosphorus-solubilizing bacteria (PSB) (Chaiarn and Lumyong, 2009). The detailed mechanisms by which microsymbionts alter plant growth have remained elusive. Nevertheless, efforts at identifying highly effective rhizobial strains that combine adaptation to their environment with plant-growth promoting traits could improve plant growth and inoculation response under field conditions. This review addresses the roles played by the secretion of siderophore, IAA, ACC deaminase, lumichrome, riboflavin and protons for P solubilization in plant growth promotion, especially during the legume-rhizobia symbiosis. The secretion of these molecules by non-rhizobial plant growth promoting bacteria leading to increased plant development is also discussed.

## RHIZOBIAL SYMBIONTS AS PROMOTERS OF LEGUME PLANT GROWTH

Legumes that harbor efficient rhizobia in their root or stem nodules often meet their N requirements from N<sub>2</sub> fixation (Belane et al., 2011; Mohale et al., 2014). It is this symbiotic trait that gives legumes a superior survival advantage over their non-legume counterparts in N-depleted soils where other plant growth requirements are optimal. Nodulation and N<sub>2</sub> fixation in legumes are an interactive process which involve the action of rhizobial Nod factors; and during the process, some rhizobia may produce phytohormones such as IAA, gibberellic acid and cytokinins which present plant growth promoting effects (Bottini et al., 1989; Hayashi et al., 2014) (**Table 1**). Aside serving as signal molecules in the early stages of nodule formation, Nod factors isolated from *Rhizobium leguminosarum* were also found to increase seed germination, nodulation and plant growth in pea and vetch, as well as pod yield in pea (Kidaj et al., 2012). Similarly, Nod factors from *Bradyrhizobium japonicum* strain 532C increased germination and early plant growth in soybean and other non-leguminous crops; moreover, the parent culture of *Bradyrhizobium japonicum* strain 532C, but not its mutant deficient in Nod factor synthesis, was also found to induce similar growth effects on plants (Prithiviraj et al., 2003). These observations clearly stress the multiple roles of rhizobial Nod factors as both signal molecules for nodulation and plant growth promoters in diverse crops. On the other hand, an IAA overproducing mutant of *Sinorhizobium meliloti* increased nodulation in *Medicago* sp. when compared to the parent strain, a report that stresses the involvement of this auxin in nodule formation aside its role in plant growth enhancement (Pii et al., 2007). Rhizobia can also increase nutrient availability in the root zone, especially N and P (Chebotar et al., 2001; Argaw, 2012), produce molecules that inhibit pathogens (Tavares et al., 2018), as well as alter rhizosphere chemistry involving

the regulation of ethylene levels (Nascimento et al., 2018). Moreover, aside the growth promoting effects of cytokinin from either plant or bacterial origin, they are also involved in the nodulation process of legumes as well as plant response to pathogens (Giron et al., 2013; Gauthier-Coles et al., 2019). In pea plants, deficiencies in gibberellins and brassinosteroids were associated with impaired nodulation, pointing to their significant role in the symbiotic process and subsequent plant growth (Ferguson et al., 2005; Foo et al., 2016). The significant roles of the legume-rhizobia symbiosis in enhancing plant growth and productivity has been demonstrated in several legumes including soybean (*Glycine max*), mung bean (*Vigna radiata*), chickpea (*Cicer arietinum*), common bean (*Phaseolus vulgaris*), cowpea (*Vigna unguiculata*), Bambara groundnut (*Vigna subterranea*) and Kersting's groundnut (Dashti et al., 1998; Shaharouna et al., 2006; Chihaoui et al., 2015; Gyogluu et al., 2018; Mohammed et al., 2018; Abdiev et al., 2019; Dabo et al., 2019; Ibny et al., 2019). However, a major limitation to tapping the maximum benefit of the symbiosis is related to its susceptibility to diverse environmental stress factors. For example, even when rhizobial strains exhibit high N<sub>2</sub>-fixing efficiency, their wider utilization in the field can be unpredictable, further hampering their adoption (Ulzen et al., 2016). Thus, tapping the maximum benefit of rhizobia for improved plant performance will require selection and/or engineering strains with multiple adaptive and plant growth promoting traits.

## RHIZOBIAL PRODUCTION OF IAA AND N<sub>2</sub> FIXATION IN LEGUMES

Indole acetic acid (IAA) is an important member of the auxin family responsible for controlling plant physiological processes, including cell division and enlargement, tissue differentiation, and light/gravity response (Teale et al., 2006; Shokri and Emtiazi, 2010). Rhizobia together with other bacteria, fungi and algae are producers of auxins, especially IAA (Shokri and Emtiazi, 2010), a common by-product of L-tryptophan metabolism in several microorganisms, including rhizobia (Datta and Basu, 2000; Ghosh and Basu, 2006; Mandal et al., 2007). The level of IAA production can vary among rhizobial isolates; and endogenous phenolic acids such as protocatechuic acid, 4-hydroxybenzaldehyde and p-coumaric acid in root nodules could increase IAA synthesis by rhizobial isolates from *Vigna mungo* (Mandal et al., 2009). Microbes and plants can mutualistically benefit from each other, with the microbes releasing plant growth-promoting substances in return for photosynthate from the plant. For example, the N-fixed by bacteroids in root nodules is transported to the aerial parts of plants in exchange for the carbon compounds from photosynthesis (Udvardi and Kahn, 1992; Kaiser et al., 2015).

IAA production by cowpea-nodulating rhizobia from South Africa and Mozambique differed, with about 80% of the rhizobial symbionts producing from 0.64 to 56.46 µg/ml of IAA (Dabo et al., 2019). Similar variations in the levels of IAA synthesis were also reported for rhizobial symbionts of Bambara groundnut in South African and Malian soils (Ibny et al., 2019). However,

there is so far, no assessment of the relationship between rhizobial secretion of IAA and plant growth and/or grain yield. Nevertheless, Ali et al. (2008) showed that exogenous supply of IAA increased nitrogenase activity and leghemoglobin concentration in root nodules just as an engineered high IAA producing rhizobial strain showed significantly higher nitrogenase activity than the parent strain (Defez et al., 2019). Outside the legume symbiosis, bacterial production of IAA also presents growth promoting effects on non-legumes. In wheat for instance, root growth was reduced in plants treated with mutants of *Azospirillum brasilense* exhibiting low IAA synthesis than the wild-type (Spaepen et al., 2008). Moreover, whereas IAA synthesis is known for its plant-growth promoting effects, the mechanism seems to involve its degradation by bacteria; and was earlier demonstrated in *Arabidopsis thaliana* where an IAA degradation mutant of *Burkholderia phytofirmans* PsJN failed to increase root elongation when compared to the wild-type in the presence of exogenous IAA supply (Zúñiga et al., 2013).

IAA production by rhizobia is controlled by specific genes, and the overproduction of this auxin has been reported in several bacterial mutants, including that of *Ensifer* (*Sinorhizobium*) *meliloti* RD64 when compared to its wild type (Defez et al., 2019). The *fixJ* gene which works as a regulator to switch on the nitrogen fixation genes was highly expressed at 42 days of inoculation in the mutant of *Ensifer* (*Sinorhizobium*) *meliloti* RD64 leading to abundant FixJ protein which is regulated by *nifA*, *fixK1*, and *fixK* genes (Defez et al., 2019; Alemneh et al., 2020). Additionally, the IAA-overproducing *Ensifer meliloti* RD64 also showed an enhanced expression of the *fixNOQP1,2* operon genes (which code for haem-copper cbb3-type oxidases at 40 days after inoculation), with an upregulation of *gltA*, *icd*, and *sucA* genes (responsible for TCA cycle enzymes) in the bacteroids (Defez et al., 2019). The inoculation of soybean with IAA-producing bacteria also showed upregulation of the *otsA* gene, which encodes for trehalose 6-phosphate synthase, and plays a major role in nodule formation, nodule respiration and nitrogenase biosynthesis (Suárez et al., 2008; Bargaz et al., 2013). Moreover, IAA-producing rhizobia have also been shown to significantly increase free amino acids such as valine, alanine, aspartic acid and glutamic acids in host plants as well as photosynthetic products supplied to bacteroids in root nodules (Tsikou et al., 2013; Erice et al., 2014; Defez et al., 2019). These findings together suggest that IAA promotes an increase in nitrogen fixation via the upregulation of the genes involved in carbon transport to N<sub>2</sub>-fixing bacteroids (Fisher, 1994; Defez et al., 2019).

## BACTERIAL SECRETION OF SIDEROPHORES ENHANCES FE NUTRITION, PROMOTES PHYTOREMEDIATION AND CONTROLS PLANT PATHOGENS

Beyond the reduction of N<sub>2</sub> to NH<sub>3</sub> for direct use by plants in the synthesis of macromolecules such as chlorophyll, Rubisco

and nucleic acids, some rhizobial strains are known to improve the availability and uptake of iron via siderophore production. Iron is one of the essential elements required by plants for growth and is a component of critical macromolecules such as leghaemoglobin and nitrogenase, both required for the N<sub>2</sub> fixation process (Paudyal et al., 2007). As a result, a deficiency in Fe limits the efficiency of N<sub>2</sub> fixation through a reduction in nodule development and nitrogenase activity (Duhan et al., 1998; Stajković et al., 2011). Fe exists in soils as the divalent (Fe<sup>2+</sup>) and trivalent (Fe<sup>3+</sup>) cations, and its availability for plant uptake is governed by several factors including soil pH and the levels of other soil nutrients (Rajkumar et al., 2010). Whereas siderophores are known to have diverse chemical structures, most bacteria produce the catecholate-type while fungi together with some bacteria produce the hydroxamate-type siderophores (Carson et al., 2000; Schalk et al., 2011; Grobelak and Hiller, 2017). For example, bacteria isolated from the rhizosphere of *Arabidopsis thaliana* were shown to produce both catecholate and hydroxamate siderophores, which subsequently improved plant growth and phytoremediation by decreasing metal toxicity to plants (Grobelak and Hiller, 2017). Siderophores have a high affinity for Fe<sup>3+</sup> where soil Fe is low and can reduce it to Fe<sup>2+</sup> for uptake and utilization by legumes (Gopalakrishnan et al., 2015; Lebrazi and Fikri-Benbrahim, 2018). Identifying siderophore-producing rhizobia is therefore important for increased plant growth and crop yields, especially of nodulated legumes. Several species of rhizobia have been identified as siderophore producers with benefits beyond Fe<sup>3+</sup> sequestration. Many of these rhizobia have been found to differ markedly in their secretion of siderophores, with consequences on host plant nodulation (Carson et al., 1992). For example, increased siderophore production by *Rhizobium* and *Bradyrhizobium* strains that nodulated pigeon pea revealed increases in nodule mass, shoot N and Fe content (Duhan et al., 1998). The co-inoculation of common bean with *Rhizobium phaseoli* and a siderophore-producing *Pseudomonas* sp. LG also resulted in greater N and P accumulation (Stajković et al., 2011). Although the mechanisms of growth promotion remain complex and elusive, several studies involving mutants also point to the plant growth promoting traits of siderophores. For example, a mutant of *Pseudomonas fluorescence* with greater capacity for siderophore synthesis was found to increase plant growth in mung bean over the wild-type counterpart (Katiyar and Goel, 2004). Similarly, a wild-type endophytic *Streptomyces* sp. GMKU 3100 markedly improved root and shoot growth in mung bean and rice when compared to its siderophore-deficient mutant (Rungin et al., 2012). To tap the benefits of this trait, siderophore-producing microsymbionts such as *Sinorhizobium meliloti*, *Rhizobium leguminosarum*, *Rhizobium leguminosarum* bv. *trifoli*, and *Bradyrhizobium japonicum* have been identified as potential strains for use as Fe-based biofertilizers for increased crop yields (Guerinot, 1991). In addition to enhancing Fe nutrition and promoting plant growth, siderophores have also been implicated in plant adaptation to high concentrations of heavy metals in agricultural soils. Apparently, bacterial siderophores can chelate heavy metals such as Al, Cd, Cu, Pb and Zn to form complexes that are not toxic to plants (Braud

et al., 2009; Schalk et al., 2011). Rhizobial siderophores are therefore important in the reclamation of contaminated soils for agricultural use. Although the legume-rhizobia symbiosis is sensitive to Al toxicity, siderophores can bind with Al ions and alleviate their toxic effect (Jaiswal et al., 2018), thus enhancing phytoremediation. So far, however, little is known about the chemistry of siderophores' interaction with heavy metals. Similarly, little attempt has been made to identify legume-rhizobia symbioses with efficiency in reclaiming contaminated soils associated with mining.

The genetics of siderophore production is rather complex. However, some genes involved in siderophore formation have been characterized. Rhizobia need iron for their own use in growth and symbiotic functioning, just as many proteins involved in the N<sub>2</sub> fixation process require iron for the synthesis of hemoglobin needed for transporting O<sub>2</sub> to respiring bacteroids. There are different types of siderophores. Rhizobia have the ability to produce a catechol siderophore to acquire iron under iron-poor conditions in the soil (Datta and Chakrabarty, 2014). The gene for rhizobactin 1021, a hydroxamate siderophore, was expressed under iron stress in *Sinorhizobium meliloti* (Lynch et al., 2001). Six genes (*rhbABCDEF*) needed in the biosynthesis of the siderophore have been identified and are located on an operon that is repressed under iron-replete conditions, while the gene *rhtA* encodes the outer membrane receptor protein for rhizobactin 1021 siderophore (Lynch et al., 2001). The transcription of both *rhbABCDEF* and *rhtA* genes is regulated by the product of the eighth gene in the cluster, namely *rhxA*, which has the characteristics of an AraC-type transcriptional activator (Lynch et al., 2001). According to McRose et al. (2018), some bacteria use quorum sensing (QS) to regulate siderophore production, including its concentration and ability to promote Fe uptake. In *B. japonicum* strain 61A152, a simple molecule such as citric acid can act as a siderophore (Guerinot et al., 1990), a finding confirmed by Siqueira et al. (2014) who showed that *B. japonicum* (Bj CPAC15), *B. diazoefficiens* strains Bd CPAC7, Bd USDA 110<sup>T</sup> and Bj USDA 6<sup>T</sup> all had three genes encoding citrate synthase enzyme. However, they also found genes related to the biosynthesis of catecholate siderophores in strains Bj CPAC 15 and Bd CPAC 7. So far, however, siderophores produced by rhizobia have been identified in only *S. meliloti*, *B. japonicum*, and *B. diazoefficiens* (Lynch et al., 2001; Siqueira et al., 2014). With the many new symbionts continuously being reported, it is important to also identify their siderophores, as a necessary step for their use as Fe biofertilizers. Furthermore, more studies are needed to ascertain the Fe-releasing efficiency of the different types of siderophores.

## THE ROLE OF LUMICHROME AND RIBOFLAVIN IN PLANT GROWTH PROMOTION

Microorganisms such as rhizobia are crucial in the promotion of plant growth, as they can synthesize and release phytohormones that alter the rhizosphere chemistry in favor of plant growth

(Table 1). Rhizobial metabolites such as riboflavin and lumichrome are reportedly involved in chemical cross-talks leading to plant growth promotion, as well as improved immune response to biotic and abiotic stresses (Dakora et al., 2015; Kanazawa et al., 2020). As a result, soil microbes such as rhizobia and non-rhizobial bacteria have been used as inoculants because of these additional benefits. Exogenous application of lumichrome to both monocots and dicots yielded varied results, pointing to a dose-dependent beneficial outcome which is likely to be influenced by the type of plant species and the environment (Matiru and Dakora, 2005). Thus, any anticipated use of bacterial metabolites as plant growth promoters in cropping systems would require a better understanding of their operational mechanisms. Riboflavin released by rhizobia into the rhizosphere can promote microsymbiont colonization of root hairs, leading to greater and more effective nodulation and N<sub>2</sub> fixation. For example, mutants of *Sinorhizobium meliloti* containing additional copies of the *ribBA* genes were found to exhibit increased synthesis of riboflavin leading to greater root colonization in *Medicago sativa* (Yang et al., 2002). Conversely, a *Rhizobium leguminosarum* *ribN* mutant exhibited reduced nodule occupancy when compared to the wild-type, further pointing to the vital role of riboflavin in the establishment of the legume-rhizobia symbiosis (Angulo et al., 2013). Lumichrome, the degradation by-product of riboflavin, is also reported to promote plant growth through improved symbiotic and photosynthetic functioning in legumes (Table 1; Phillips et al., 1999; Matiru and Dakora, 2005). Although the genetic basis for the involvement of lumichrome on stomatal function is scanty, the study by Matiru and Dakora (2005) reported increases in leaf stomatal conductance and transpiration rates when compared to untreated plants, with rhizobial inoculation producing similar responses. Similarly, lumichrome treatment was reported to increase photosynthetic rates in corn and soybean when compared to untreated plants (Khan et al., 2008). At the molecular level, lumichrome can induce the expression of genes involved in cell differentiation and cell expansion leading to increased plant biomass accumulation (Pholo et al., 2018). Given their roles at different stages of the legume-rhizobia symbiosis, riboflavin and lumichrome released by rhizobia can maximize plant growth and increase crop productivity via several alterations in the plant's physiology, including enhanced symbiosis and photosynthetic functioning.

The genetic basis of plant growth promotion by lumichrome (chemically defined as 7,8 dimethylalloxazine) stems from its ability to induce the expression of genes responsible for cell growth and mitotic division, and appears to coordinate cell division and proliferation in developing leaves. Lumichrome is also reported to increase CO<sub>2</sub> concentration in the rhizosphere (Phillips et al., 1999) which is needed for the growth of N<sub>2</sub>-fixing rhizobia and mycorrhizal fungi (Maier et al., 1979). Pholo et al. (2018) found that the enhancement of mitotic *CYCD*<sub>3.3</sub>, *CYCA*<sub>1.1</sub>, *SP1L*<sub>3</sub>, *RSW*<sub>7</sub>, and *PDF*<sub>1</sub> transcripts in lumichrome-treated *Arabidopsis thaliana* plants resulted in high plant biomass from cell differentiation and cell expansion. Moreover, lumichrome also increased starch accumulation in soybean and tomato by increasing glyceraldehyde 3-phosphate



denhydrogenase (GAPDH) transcripts and NAD-dependent enzyme activity (Gouws et al., 2012). Additionally, lumichrome can also reduce the levels of gene expression associated with ethylene metabolism such as *Acc oxydase 1 (ACO1)*, and a C<sub>2</sub>H<sub>2</sub> zinc finger protein, leading to a minimal effect of ethylene on plant growth. However, Pholo et al. (2018) found a synergistic ethylene-auxin cross-talk via a reciprocal over-expression of *ACO1* and *SAUR54* in which ethylene activated the auxin biosynthetic pathway and regulated *Arabidopsis* growth, in addition to suppressing the negative effects of methyl jasmonate (MeJa) on chlorophyll loss and decreases in Rubisco and photosynthesis (Pholo et al., 2018). For example, treating *Arabidopsis thaliana* or soybean with Methyl jasmonate caused a decrease in leaf photosynthetic rates due to impaired chlorophyll production (Jung, 2004; Anjum et al., 2011). Whereas, pre-incubation of *Bradyrhizobium japonicum* with jasmonates prior to inoculation led to enhanced nodulation and N<sub>2</sub> fixation in soybean (Mabood and Smith, 2005), the possible indirect inhibitory effect of jasmonate on symbiosis via reduced photosynthetic functioning remains to be determined.

## ACC DEAMINASE-PRODUCING RHIZOBIAL BACTERIA

ACC (1-aminocyclopropane-1-carboxylic acid) deaminase is a known precursor of ethylene (Penrose and Glick, 2003) and is a plant growth-promoting enzyme (Table 1) that uses pyridoxal 5-phosphate (PLP) as substrate (Honma and Shimomura, 1978). ACC deaminase enzyme localized in the cytoplasm, is encoded by *acdS* gene (Honma and Shimomura, 1978; Jacobson et al., 1994). In *Mesorhizobium loti*, this *acdS* gene is located in the symbiotic island and is regulated by the *NifA2* gene (Nukui et al., 2006). When ACC produced by plants is exuded into the rhizosphere, rhizobacteria that express ACC deaminase activity take up the ACC and degrade it within the bacterial cytoplasm (Glick et al., 1998; Penrose and Glick, 2003). This enzyme is constitutively expressed during nodule initiation, and under stressed conditions in plant roots (Ligero et al., 1986; Spaink, 1997; Saleem et al., 2007). The plant exuded ACC is often used by bacteria as an N source; however, ACC deaminase can also degrade ACC to lower ethylene levels, especially in plants exposed to biotic and abiotic stresses, as well as during nodule initiation by legumes and rhizobia (Glick et al., 1998). Earlier studies have shown the harmful effect of ethylene in inhibiting nodule formation through interfering with root hair deformation, infection thread elongation into the inner cortex, calcium spiking and the proliferation of rhizobia in legumes such as *Pisum sativum*, *Trifolium repens*, and *Medicago sativa* (Goodlass and Smith, 1979; Peters and Crist-Estes, 1989; Lee and LaRue, 1992; Oldroyd et al., 2001; Tamimi and Timko, 2003; Lohar et al., 2009). However, co-inoculation of either *Rhizobium tropici* CIAT899 or *Cupriavidus taiwanensis* STM894 with the wild-type ACC deaminase-producing *Pseudomonas fluorescens* YsS6 was found to improve nodulation and plant growth in *Phaseolus vulgaris* and *Mimosa pudica*, respectively, when compared to its mutant defective in ACC deaminase

production (Nascimento et al., 2019). Ma et al. (2004) also found that an ACC deaminase-producing *Sinorhizobium meliloti* mutant elicited 40% more nodulation in *Medicago sativa* than the parent strain. Thus, the mechanism by which ACC deaminase-producing bacteria increase plant nodulation seems to involve the regulation of both ACC oxidase activity and ethylene synthesis, as observed in pea plants treated with ACC deaminase-producing *Arthrobacter protophormiae* (Barnawal et al., 2014). Furthermore, a *S. meliloti* strain harboring the *acdS* gene and co-inoculated with *P. putida* UW4 increased nodule number in *Medicago lupulina* when compared to inoculation with the wild type (Kong et al., 2015). The important plant growth promoting traits of these bacteria-produced molecules points to the need to bioprospect for rhizobial strains that possess such traits for increased symbiotic performance, plant growth promotion and increased grain yield.

The *PvACS* gene in *Phaseolus vulgaris* encodes ACC synthase which causes the formation of ethylene responsible for nodule senescence (Nascimento et al., 2018; Serova et al., 2018). The *PvGS(n-1)*, a gene controlling the transcription of glutamine synthetase and commonly found in senescent nodules, is needed for ammonium assimilation in legumes (Hungria and Kaschuk, 2014; da Silva et al., 2019). However, the expression of this gene was found to enhance nitrogenase activity and leghaemoglobin concentration, leading to delayed senescence which increased ammonia assimilation and N<sub>2</sub> fixation (Lara et al., 1983; Alemneh et al., 2020). Another enzyme, Uricase II, is needed in the metabolism of N-fixed for export as ureides from root nodules to shoots and tends to decline in senescent nodules (Papadopolou et al., 1995; Capote-Mainez and Sánchez, 1997) as found for leghemoglobin, which is a physiological marker for nodule senescence. ACC deaminase-producing rhizobia can stimulate nodule formation and function and thus increase the amount of N<sub>2</sub>-fixed needed for plant growth.

## RHIZOBIA-AIDED ACQUISITION OF PHOSPHORUS BY NODULATED LEGUMES

To increase crop yields for meeting global food security would require major nutrient inputs, especially N and P. Plants take up P in the form of phosphate that comes from the 83% of the world's P reserves occurring as rock phosphate in only Morocco, China, South Africa and the USA (Vaccari, 2009). The world's vast 5.7 billion hectares of agricultural land are generally deficient in P that must be added as input to increase crop productivity (Mouazen and Kuang, 2016). In Africa, most smallholder farmers grow their crops with insufficient or no P input. However, plant growth-promoting bacteria and fungi can influence plant development directly or indirectly by facilitating the supply and uptake of mineral nutrients from the soil. Solubilization of unavailable soil P compounds by symbiotic rhizobia in the rhizosphere is one strategy for enhancing P availability for plant growth and yield (Marra et al., 2012). Although P is an important macronutrient for plant development, about 95 to 99% of the soil P occurs in insoluble form which is not useable by plants (Vassilev et al., 2001). Some rhizobial bacteria are however

capable of solubilizing unavailable soil P for plant uptake and growth. Aside plants, bacteroids in root nodules also require P for their metabolism; and the high-affinity phosphate transporter PstSCAB is known to promote the efficiency of the *Sinorhizobium fredii*-soybean symbiosis (Hu et al., 2018), just as the uptake of inorganic P leading to enhanced N<sub>2</sub> fixation during P starvation is facilitated by the *pho*CDET genes which encode the ABC-type transport system in *Sinorhizobium meliloti* (Bardin et al., 1996; Yuan et al., 2006). Thus, given the vital roles of P in both plant and bacterial metabolism, screening for P-solubilizing traits in N<sub>2</sub>-fixing rhizobia can be a cheaper and useful strategy to ameliorate the negative effects of soil P stress on plants for improved crop yields and food security.

Immobilization of P can occur in inorganic and organic forms (Rodríguez and Fraga, 1999). During microbial P transformation, the carboxyl and hydroxyl ion containing organic acids release their protons and bind to cations (Bergkemper et al., 2016), thus lowering the pH (Richardson and Simpson, 2011). The acidification caused by microbial activity can result in the release of P ions by substitution with H<sup>+</sup> (Rodríguez and Fraga, 1999; Richardson and Simpson, 2011). In low-P soils, rhizobia can solubilize soil-bound P in the rhizosphere through acidification by synthesizing gluconic acid under the control of pyrroloquinoline quinone (PQQ) genes (Yadav et al., 2020). Of all the organic acids, gluconic acid is most potent in P solubilization and the oxidation of glucose to gluconic acid by rhizobia is an important step in the solubilization of soil P (Richardson et al., 2011). Gene *gcd* in rhizobia encodes quinoprotein glucose dehydrogenase (PQQGDH), which is involved in the release of organic anions to solubilize inorganic P (Rodríguez et al., 2000). In addition to organic acids, inorganic acids and mycorrhizal fungi in soil can also enhance phosphorus solubilization (Alori et al., 2017). Whereas rhizobia are largely known for their N<sub>2</sub>-fixing traits, mycorrhizal fungi are particularly known for their role in the acquisition of phosphorus and other nutrients required by plants (Bolan, 1991). A synergistic interaction was observed when faba bean was treated with *Rhizobium leguminosarum* bv. *viciae* and arbuscular mycorrhizal fungi which resulted in increased nodulation and nitrogenase activity probably through enhanced P acquisition by the mycorrhizal partner (Abd-Alla et al., 2014).

Rhizobia and other microbes harbour genes such as *phoD* and *phoA* which encode alkaline phosphatase, the *appA* gene which encodes phytase, and the *phn* gene which encodes C-P lyase enzymes that can convert soil organic-P into available P for plant uptake (Rodríguez et al., 2006). The organic P however occupies about 30–50% of the total soil P pool in forms such as inositol phosphate (soil phytate), phosphomonoesters, phosphodiester, phospholipids, nucleic acids, and phosphotriesters (Rodríguez and Fraga, 1999). The activity of microbial P-enzymes on the huge P reservoir in soils largely accounts for P supply to terrestrial plants. P assimilation under P-poor soil conditions is usually achieved using high-affinity P transporters, in contrast to P-rich soils where low-affinity P transporters are involved (Hsieh and Wanner, 2010). The expression of genes *phoU*, *phoR*, and *phoB* in soil microbes largely regulates the P-starvation response in cropping systems for increased use of external P sources (Eder

et al., 1996). However, information is lacking from a systems approach that links P status of the soil to plant and bacterial gene expression for P-enzyme formation/release for increased P availability, uptake and utilization. For example, what are the cues for low-P sensing in soil by plants and microbes, and what is the timeframe required for biological processes such gene expression to occur? And what are the regulatory mechanisms underlying the build-up of P pool in response to its demand by plants?

## ROLE OF SYMBIOTIC RHIZOBIA IN PLANT DEFENSE

The successful induction of root nodules and their subsequent colonization by rhizobia require the production of rhizobial Nod factors that allow for their recognition by the host legume (Via et al., 2016). Aside their growth promoting effects, rhizobia have been implicated in processes leading to induced systemic resistance (ISR) in host plants which is governed by complex mechanisms; for example, inoculating common bean with *Rhizobium etli* led to enhanced resistance to infection by *Pseudomonas syringae* pv. *phaseolicola* via the accumulation of reactive oxygen species, increased callose production and the activation of defense related genes (Díaz-Valle and Alvarez-Venegas, 2019). The response of host plants to pathogen infection can include a molecular cross-talk between salicylic acid and jasmonates, both of which play key roles in the activation of plant defense related genes (Pieterse et al., 2012). Upon attack by insects for instance, intricate processes which include the synthesis of salicylic acid and jasmonates occur leading to the activation of genes responsible for plant defense (Hettenhausen et al., 2015; Wang et al., 2019b). Moreover, siderophores produced by symbiotic rhizobia and other microbes do not only enhance Fe nutrition for healthy plant growth and grain yield in legumes, but also serve as biocontrol agents against pathogens (Table 1). For example, siderophores produced by *Sinorhizobium meliloti* were shown to suppress *Macrophomina phaseolina*, the causal agent of charcoal rot in groundnut (Arora et al., 2001). Similarly, co-inoculation of groundnut with *Rhizobium* and *Trichoderma harzianum* successfully inhibited infection by *Sclerotium rolfsii*, the fungal pathogen that causes stem rot disease (Ganesan et al., 2007). A *Rhizobium* species was also found to protect soybean from root rot caused by *Phytophthora megasperma*, while a *Sinorhizobium* sp. inhibited plant infection by *Fusarium oxysporum* (Deshwal et al., 2003). Rhizobitoxine-producing strains of *Bradyrhizobium japonicum* were also able to successfully block infection of soybean by *Macrophomina phaseolina*, the causal pathogen of charcoal rot (Deshwal et al., 2003).

In addition to their roles as signal molecules in the legume-rhizobia symbiosis, rhizobial metabolites such as riboflavin and lumichrome, as well as vitamins which include thiamine, biotin, niacin and ascorbic acid have been implicated in legume plant defense against pathogens (Mehboob et al., 2009; Palacios et al., 2014). For instance, spraying riboflavin on tobacco and *Arabidopsis* caused resistance to *Peronospora parasitica*,

*Pseudomonas syringae* pv. tomato, the Tobacco mosaic virus and *Alternaria alternata* (Dong and Beer, 2000), possibly due to the expression of pathogenesis-related genes which induced systemic acquired resistance to the pathogens. In *Arabidopsis*, riboflavin was similarly found to induce the priming of plant defense response toward infection by *Pseudomonas syringae* pv. tomato and was linked to the expression of genes involved in plant defense responses (Zhang et al., 2009). Moreover, *Mesorhizobium loti* induced the expression of the Phenylalanine Ammonia lyase (*LjPAL1*) gene responsible for the synthesis of salicylic acid, and consequently altered the response of *Lotus japonicum* to infection by *Pseudomonas syringae* (Chen et al., 2017). Legumes are also reported to protect themselves against pathogens using isoflavonoids, phytoalexins and phytoanticipins (Dakora and Phillips, 1996). It is therefore possible that the health of a legume plant is dependent on a molecular cross-talk involving several defense molecules such as isoflavones, riboflavin, thiamine and other yet unknown molecules (Subramanian et al., 2004; Ahn et al., 2005; Yadav et al., 2020). In *Arabidopsis* for example, infection with *Sclerotinia sclerotium* increased the expression of the *IFS1* gene that codes for isoflavone synthase and highlights the involvement of isoflavones as plant defense molecules (Subramanian et al., 2004). Also, a thiamine treatment led to the accumulation of hydrogen peroxide ( $H_2O_2$ ) and a build-up of lignin in roots of rice following infection with the root-knot nematode (*Meloidogyne graminicola*); this was associated with the increased transcription of the *OsPAL1* and *OsC4H* genes involved in the phenylpropanoid pathway (Huang et al., 2016). Clearly, plants have diverse ways of overcoming biotic and abiotic stress within their environments through the synthesis of novel molecules. For example, following the infection of *Arabidopsis* by *Pieris rapae*, one branch of the jasmonate signaling pathway regulated by the *MYC2* gene was expressed (Santino et al., 2013). Clearly, a lot remains to be unraveled regarding the complex chemical cross-talks involved in plant adaptation to disease infection and pathogen attack.

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## CONCLUSIONS

Plant growth and productivity is dependent on multiple factors, which include mineral nutrition, resistance to insect pests and diseases. Fortunately, symbiotic rhizobia are capable of triggering biological pathways that cause outcomes with direct and indirect effects on plant growth promotion and protection. Studying the interlinkages of outcomes from the legume-rhizobia symbioses has the potential to identify microsymbionts for use as inoculants due to the multiplicity of functions that they elicit. For example, rhizobia can be identified that (i) produce greater symbiotic N for host plant growth and productivity (ii) elicit the synthesis of host-plant compounds for defense and increased plant growth, (iii) produce environmental cues that regulate stomatal function and (iv) emit vitamins as growth factors for plant defense and increased growth/productivity. Whereas, some of the outcomes triggered by rhizobia may be tied to their symbiotic interactions with legumes, the effects of some of the signal molecules produced often extends to non-legumes, thus indicating a wider distribution of these traits among diverse bacterial genera.

## AUTHOR CONTRIBUTIONS

SJ and MM drafted the manuscript. FI produced the photo used in **Figure 1**. The photo was shot by her from her glasshouse studies. Some of the ideas in the manuscript are also from her Master's thesis. FD conceived the idea, edited, and approved the final version of the paper. All authors contributed to the article and approved the submitted version.

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# Rhizobacteria From Root Nodules of an Indigenous Legume Enhance Salinity Stress Tolerance in Soybean

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Soybean is the most widely grown legume worldwide, but it is a glycophyte and salinity stress can decrease its yield potential up to 50%. Plant growth promoting rhizobacteria (PGPR) are known to enhance growth and induce tolerance to abiotic stresses including salinity. The aim of this study was to isolate such PGPR from the root nodules of *Amphicarpaea bracteata*, a North American relative of soybean. Isolated strains were identified, and 15 strains were screened for potential utilization as PGPR of soybean through a series of greenhouse trials. Four isolates that greatly improved shoot and root growth were further selected and screened under a range of salt concentrations. Two of the most promising strains, *Rhizobium* sp. SL42 and *Hydrogenophaga* sp. SL48 were ascertained to exert the greatest beneficial effects on soybean growth and salinity tolerance. They were co-inoculated with *Bradyrhizobium japonicum* 532C (*Bj*) and the plants were grown up to the harvest stage. The treatment of *Bj*+SL42 resulted in higher shoot biomass than the control, 18% at the vegetative stage, 16% at flowering, 7.5% at pod-filling, and 4.6% at harvest and seed weight was increased by 4.3% under salt stress ( $EC_e = 7.4$  ds/m). Grain yield was raised under optimal conditions by 7.4 and 8.1% with treatments *Bj*+SL48 and *Bj*+SL42+SL48, respectively. Nitrogen assimilation and shoot  $K^+/Na^+$  ratio were also higher in the co-inoculation treatments. This study suggested that inoculation with bacteria from an indigenous legume can induce stress tolerance, improve growth and yield to support sustainability, and encourage ecological adaptability of soybean.

**Keywords:** co-inoculation, PGPR, stress tolerance, salinity, soybean, native legume

## INTRODUCTION

Salinity is a major threat to agricultural sustainability and undermining the crop productivity on arable lands worldwide of which more than 50% is predicted to be affected by 2050 (Ashraf, 1994). It is anticipated that climate changes may hasten the aridisation of the Great Plains of North America during this century, leading to deficits in groundwater level and increased salinization of soil and water resources (Florinsky et al., 2009). The Canadian Prairies are susceptible to salinity, due to their soil type, moisture loss, high evapotranspiration rates and mineral salts in groundwater (Wiebe et al., 2007). The temporal fluctuation of soil salinity in this region is controlled by groundwater depth, which in turn is related to annual precipitation (Florinsky et al., 2009). Excessive use of road salts during winter may risk contamination of water

sources and groundwater table in regions above 40°N (EnvironmentCanada, 2001). Soil salinity is also a pressing problem in many states of the USA on both irrigated and rainfed agricultural lands (NRCS, 2002). Salinity stress is mainly caused by uptake of NaCl, the dominant salt in nature, which creates both osmotic and ionic imbalances in plants. These lead to physiological dysfunctions that inhibit plant growth and development, thereby declining crop yield (Munns and Tester, 2008). Soil salinization has caused an estimated annual loss of \$257 million CAD to Canadian farmers in 1998. Even though salinization risk has been lowered in the Prairies through better land-soil-water management practices, it persists to be a localized issue (AAFC, 2020).

Soybean [*Glycine max* (L.) Merrill] is an agriculturally important grain legume and oilseed crop worldwide. Due to the abundant protein (36%) and oil (19%) reserves in its seed, soybean has found uses as food for human consumption, animal feed, edible oil and industrial products (Thoenes, 2004). Soybean is capable of fixing atmospheric nitrogen through its symbiotic association with species of *Bradyrhizobium* (*B. japonicum*, *B. elkani*, *B. liaoningense*, and *B. yuanmingense*) in root nodules. Hence, it is an ideal rotation crop with corn, wheat, cotton and other arable crops, to increase soil nitrogen content and reduce production costs. Cultivation of soybean has gained significance in North America after world war II (Cloutier, 2017). Now, Canada and the USA are prominent global suppliers and consumers with the USA ranking first (60% of soybean trade). Soybean production has been steadily on the rise in Canada during recent years (**Supplementary Table 1**) and cultivation has expanded in the southern regions, bolstered by the introduction of early-maturing varieties (Dorff, 2007).

However, sub-optimal growing conditions are met with environmental challenges other than just low temperatures. Soybean is basically a short-day plant (development is influenced by daylength), relatively resistant to temperature fluctuations (more extreme temperatures affect flowering and pod-setting), grown in a wide range of soils (except very sandy), sensitive to waterlogging and moderately tolerant to drought and salinity (FAO, 2002). According to the FAO crop database, “yield decrease due to soil salinity is: 0% at EC<sub>e</sub> 5 mmhos/cm, 10% at 5.5, 25% at 6.2, 50% at 7.5 and 100% at EC<sub>e</sub> 10 mmhos/cm.” Salinity stress may cause physiological and biochemical disorders in soybean that inhibit seed germination and plant growth, aggravate leaf chlorosis and bleaching, decrease biomass accumulation, restrain nodulation and nitrogen fixation, and reduce yield and seed quality (Phang et al., 2008). Salinity has significantly reduced the germination percentage, plant height and shoot dry weight of 45 day-old plants of three soybean cultivars. There was also an increase in sodium and chloride levels in the leaf tissues (Essa, 2002). In soybean cv. Williams, seedling growth declined to 5% at 220 mmolal NaCl and no growth was recorded at 330 mmolal NaCl (Hosseini et al., 2002). Association mapping of soybean seed germination revealed 1,142 single nucleotide polymorphisms associated with salt tolerance. Salinity tolerance is influenced by numerous genetic and environmental factors and a complex trait, such that molecular breeding for salt-tolerant soybean cultivars has been challenging (Kan et al., 2015).

When commercial cultivation of soybean began in North America (early twentieth century), seeds were inoculated with the *Bradyrhizobium* strains capable of nodulating soybean to facilitate nitrogen fixation because they were not existing in the soil. Thus, populations of bradyrhizobia have become established in soils that had no prior soybean cropping history as a result of selective enrichment over the years by the host plant (Weaver et al., 1972). Subsequently, there has been research interest in exploring the symbiotic associations of native legumes and their relationships with soybean. *Amphicarpaea bracteata* (American hog peanut) is an annual plant of the family Leguminosae, native to eastern North America, found in a variety of partially shaded, wet habitats (Parker, 1994). *A. bracteata* is closely related to soybean, confirmed by molecular studies and both genera are classified in the subtribe Glycininae of the tribe Phaseoleae (Zhu et al., 1995). Symbiotic specificity and nodule formation with rhizobia strains are genetically controlled by nodulation restriction alleles in the host legume (Devine et al., 1990; Wilkinson et al., 1996). The inoculation of soybean plants with 10 *Bradyrhizobium* strains from *A. bracteata* resulted in nodule formation but no nitrogen fixation (Marr et al., 1997). Micro-evolution was observed within *Bradyrhizobium* populations from the soils of soybean field sites in eastern Canada and the isolated strains were clustered with isolates from the native legumes (Tang et al., 2012). In a later study by Bromfield et al. (2017), inoculation of soybean with root-zone soils of native legumes including *A. bracteata* resulted in nodulation. Upon isolation, bacteria of the *Bradyrhizobium* genus and closely related taxa were inoculated onto soybean, and some of the bacteria containing *nodC* and *nifH* gene sequences effectively fixed nitrogen, while the others were ineffective.

Symbiotic association with rhizobia has been the primary focus of plant-microbe interaction research on legumes, and more particularly soybean, but there are also other beneficial plant growth promoting rhizobacteria (PGPR) associated with them. Endophytic bacteria were isolated from soybean nodules and co-inoculation of *Bacillus subtilis* NEB4, *B. subtilis* NEB5 and *B. thuringiensis* NEB17 with *B. japonicum* increased soybean growth and plant dry weight (Bai et al., 2002a). PGPR influence plant growth through direct and indirect mechanisms such as nitrogen fixation, nutrient assimilation, and secretion of exopolysaccharides and signaling molecules (Hynes et al., 2008; Adesemoye and Kloepper, 2009). Distinct genera of PGPR have been known to act as elicitors of induced systemic tolerance to abiotic stress (Yang et al., 2009). Many studies have reported on the beneficial role of PGPR co-inoculated with *Bradyrhizobium* on growth, yield and stress tolerance of soybean. Co-inoculation with *Serratia proteamaculans* 1–102 and *S. liquefaciens* 2–68 increased plant dry weight and nodule number in soybean under sub-optimal root-zone temperatures in a soil-less media (Bai et al., 2002b). In a field study, seed co-inoculation with *Azospirillum brasilense* increased soybean yield by 14.1% (Hungria et al., 2013). Co-inoculation with *Pseudomonas putida* TSAU1 improved plant growth, root architecture, nitrogen and phosphorous content of soybean under salt stress in a hydroponic experiment (Egamberdieva et al., 2017).

Diverse PGPR may be associated within the nodules of *A. bracteata* and they may confer better adaptation of soybean plants to the soil and environmental conditions prevailing in Canada and benefit co-inoculation with *B. japonicum* for nitrogen fixation. The current study had two objectives. First, bacteria isolated from the root nodules of *A. bracteata* were screened for their ability to enhance plant growth and salt stress tolerance of soybean by evaluating seed germination and growth parameters in a greenhouse environment. Successive screening was then performed at a range of salt concentrations to determine the threshold salinity tolerance of soybean, inoculated with selected isolates. Second, two of the most promising bacteria were co-inoculated with *Bradyrhizobium japonicum* 532C, to validate their role as PGPR able to induce salinity tolerance, improve nutrient assimilation and increase growth and yield of soybean plants.

## MATERIALS AND METHODS

### Isolation of Bacteria From Root Nodules

Plants of *Amphicarpa bracteata* were collected along the shore of Lac St. Louis on the Macdonald Campus of McGill University, located in Sainte-Anne-de-Bellevue, Quebec, Canada. The nodules present on the roots of *A. bracteata* were relatively smaller and fewer than those of cultivated soybean plants (**Supplementary Figure 1**). The nodules were separated from the roots, washed and surface sterilized using 70% (v/v) ethanol for two min. They were crushed using micro pestles and the suspension was serially diluted in sterile water. The dilutions (from  $10^{-2}$  to  $10^{-7}$ ) were plated on Kings B and yeast extract mannitol (YEM) agar plates. The plates were incubated at 25 °C for 24–96 h. Single colonies of bacteria (excluding mold or actinomycetes) that were morphologically different from one another were re-isolated on new agar plates to obtain pure colonies (**Supplementary Figure 2**). The individual colonies of 15 isolates were grown in liquid broth for culture maintenance and stored in glycerol stocks at  $-80^{\circ}\text{C}$ .

### Preparation of Bacterial Culture

The bacteria were grown in Kings B or YEM broth for 48 h, incubated at 25°C and 150 rpm. The cultures were harvested by centrifugation at  $5,000 \times g$  for 10 min, room temperature (Awel™ MF 48-R, NuAire, USA) and the supernatant was discarded. The pellet was suspended in 10 mM  $\text{MgSO}_4$  and the optical density was adjusted to 0.1 at  $A_{600\text{nm}}$  (Ultraspec 4300 pro UV/Visible Spectrophotometer, Biochrom). The prepared suspension was used in downstream experiments.

### Identification of Nodule Bacteria

The identification of the isolated bacteria was done by Sanger di-deoxy nucleotide sequencing (Genome Quebec, Montreal, Canada) of the 16S rRNA gene. Briefly, the samples were diluted 1:10 with water and the PCR mix was prepared with Taq DNA polymerase (Roche FastStart High Fidelity PCR system 2500 U), 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), and 1492R (3'-TACGGYTACCTTGTTACGACTT-5') primers and run in the PCR cycler (Eppendorf Mastercycler®

ProS) for 40 cycles. The amplified product was sequenced on Applied Biosystems™ 3730XL DNA Analyzer platform. The assembled sequences (in FASTA format) were queried for similarity using the BLAST tool to find reference prokaryotic type strains (<https://blast.ncbi.nlm.nih.gov/>). Based on the score and percent identity, the isolated strains were classified into specific genus and species and the assembled sequences were then submitted to GenBank, NCBI (<https://submit.ncbi.nlm.nih.gov/subs/genbank/>). A phylogenetic analysis was performed with EMBL-EBI webservices API tools: multiple sequence alignment was generated using the interface for Clustal Omega program (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The output was used to generate a phylogenetic tree with the Simple Phylogeny tool ([https://www.ebi.ac.uk/Tools/phylogeny/simple\\_phylogeny/](https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/)) using the ClustalW2 program. The phylogeny tree was constructed using iTOL (Interactive Tree of Life <https://itol.embl.de/>) interface (Letunic and Bork, 2019) with the Phylogenetic tree file.

### Screening for Salinity Tolerance of the Isolates

The isolates were tested for their tolerance capacity of salt stress at 100, 250, and 500 mM NaCl solution. The initial culture was adjusted to 0.01 OD and added to the growth media with added salt in a 96-well plate. The plate was incubated in Cytation5™ reader (BioTek Instruments Inc.,) at 25 °C and the growth curve was measured at  $A_{600\text{nm}}$  for up to 48 h. The isolates were also screened for PGP activities including biofilm formation, nitrogen fixation, phosphorous solubilization, production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, indole-3-acetic acid (IAA) and siderophores following standard protocols (Jensen et al., 1960; Schwyn and Neilands, 1987; Bric et al., 1991; Penrose and Glick, 2003; O'Toole, 2011; Goswami et al., 2014).

### Screening of Bacterial Isolates for Inducing Salinity Tolerance in Soybean Seed Germination Assay I

Soybean seeds (Absolute RR) were soaked in the bacterial cell suspension (at a rate of 100  $\mu\text{L}$  per seed) or 10 mM  $\text{MgSO}_4$  (control) for 30 min. The seeds were then placed on Petri dishes (10 seeds per plate) lined with P8 filter paper containing 5 mL of sterile water or 100 mM NaCl solution ( $\text{EC} = 9.8 \text{ ds m}^{-1}$ ). The plates were sealed with parafilm, incubated at 25 °C in the dark inside a growth chamber and germination was counted at 24, 36, and 48 h periods.

### Greenhouse Trial I

Seeds were bacterized with the inoculum at a rate of 500  $\mu\text{L}$  per seed. Bacterized and control seeds (5 seeds per pot) were placed in 15.25 cm (diameter) pots filled with vermiculite (Perlite Canada Inc.) treated with 300 mL water or 100 mM NaCl solution ( $\text{EC}_e = 5 \text{ ds m}^{-1}$ ). The pots were placed in a greenhouse room maintained at  $25 \pm 2^{\circ}\text{C}$  and 50% relative humidity (**Supplementary Figure 3**). Seedling emergence was counted on 7<sup>th</sup> and 8<sup>th</sup> DAP (days after planting) and the plants were thinned to one seedling per pot. The plants were irrigated with 50 mL water thrice a week and fertilized with

$\frac{1}{2}$  strength Hoagland's solution once a week and sampled at 28<sup>th</sup> DAP. Plant growth variables including plant height, leaf area, shoot dry weight, and root dry weight were measured. Roots were scanned (EPSON Expression 11000XL) and analyzed using WinRHIZO™ (Regent Instruments Inc.) image analysis platform to measure root volume, length, and surface area.

## Seed Germination Assay II

Based on the previous experiments, four bacterial isolates were selected and tested further for their ability to induce salt stress tolerance in soybean. A seed germination experiment as described above was conducted at different levels of salinity (0, 100, 125, 150, 175, and 200 mM NaCl solution) and at two cell densities ( $1 \times 10^8$  and  $1 \times 10^{10}$  cfu mL<sup>-1</sup>).

## Greenhouse Trial II

A pot experiment with the different salinity levels and four bacterial isolates at  $1 \times 10^8$  cfu mL<sup>-1</sup> was set in a greenhouse to test the salinity tolerance threshold of soybean. A procedure similar to that described above was followed and the plants were sampled at 28<sup>th</sup> DAP. All experiments were repeated twice with six replications for each treatment.

## Plant Growth and Development of Soybean Under Salt Stress

Two bacterial isolates were selected and co-inoculated with *Bradyrhizobium japonicum* 532C (all strains at  $1 \times 10^8$  cfu mL<sup>-1</sup>) on soybean seeds (seed bacterization). The seeds were then placed in 25.5 cm (diameter) pots filled with vermiculite and each pot received 1 L water or 150 mM NaCl solution ( $EC_e = 7.4$  ds m<sup>-1</sup>). The pots were placed in a greenhouse room maintained at  $25 \pm 2^\circ\text{C}$  and 50% relative humidity. Irrigation was set at 50 mL (+25 mL, if light intensity during the day was >1,000 lux) per pot per day during the vegetative stage and increased to 100 mL (+25 mL) during flowering and pod-filling stages and then reduced to 75 mL (+25 mL) during the harvest stage. The plants were given 1 g of water-soluble fertilizer in 1 L water (6-11-31, Hydroponic, Plant Prod, Canada) and 2 g of triple superphosphate per pot, at 2 weeks after seeding and then regularly at every growth stage after sampling the previous growth stage. The plants were sampled at mid-vegetative (~30 DAP), mid-flowering (~60 DAP), mid-pod-filling (~90 DAP) and harvest (~110 DAP) stages, and growth variables were measured (Supplementary Figures 4 and 5). The experiment was repeated twice with 12 replications for each treatment. Dried tissue samples were ground for elemental analysis, measured as mg g<sup>-1</sup> dry weight of the plant tissue. N and P were measured on a flow injection analyzer (FIA) (Lachat QuickChem 8000, Hach® USA) and K, Ca and Na were measured after dilutions and appropriate modifier addition on an atomic absorption spectrophotometer (AAS) (Varian 220FS). Seed composition was analyzed at SGS Agrifood laboratories, SGS Canada Inc., Guelph, Canada. Nodules were collected from soybean plants after harvest; bacteria were grown on YEM agar plates similar to the isolation

procedure described above and colonies were observed after 48 h of incubation.

## Statistical Analysis

Data were analyzed using a generalized linear mixed model that was performed using the GLIMMIX procedure in SAS (v 9.4, SAS Inc., Cary, NC). The SAS PROC GLIMMIX models were “mixed” due to the inclusion of fixed (treatment, salinity, and treatment  $\times$  salinity) and random (RANDOM Rep) effects. The normal distribution was not assumed for the response (i.e., the observed variables) and therefore the models were “generalized.” Distributions were specified using the “DIST =” option in the MODEL statement and selected from the exponential family of distributions based on model fit statistics, that is, the Bayesian Information Criterion (BIC) that is part of the PROC GLIMMIX output (IC = Q was specified in the PROC GLIMMIX statement). Variance homogeneity was not assumed, and the structure of variance heterogeneity was specified using a “RANDOM \_RESIDUAL\_/GROUP =” statement and selected based on the BIC. Multiple comparisons were adjusted according to Scheffé's method (i.e., the ADJUST = SCHEFFE option in the LSMEANS statement). Effect slice tables were produced using SLICE and SLICEDIFF in the LSMEANS statement. The data for soybean growth and yield were broken into subsets based on the observed stages of plant development, and data from the respective subsets were analyzed separately using PROC GLIMMIX.

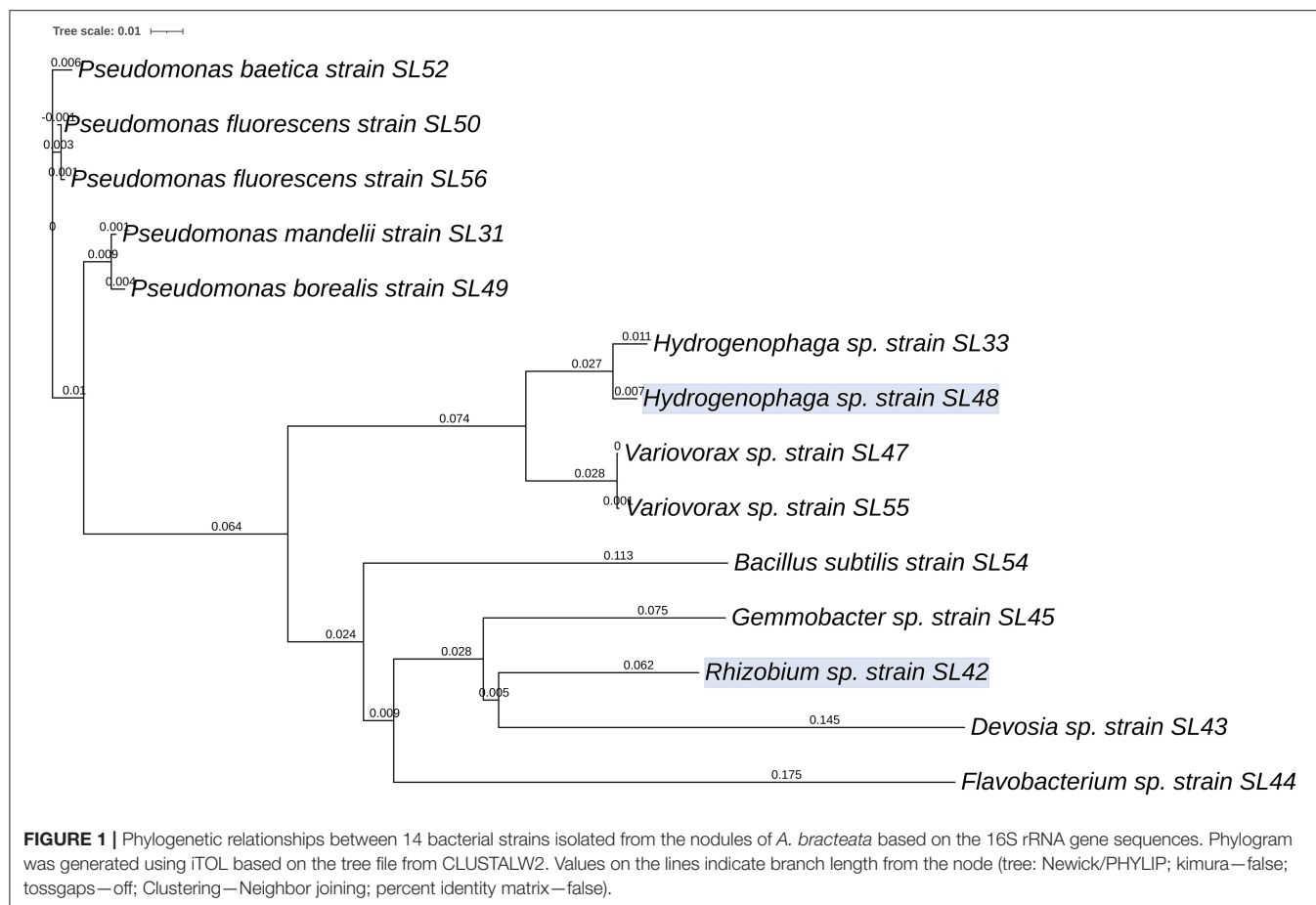
## RESULTS

### Nodule Bacteria of *Amphicarpaea bracteata*

Bacterial colonies from *A. bracteata* nodules were obtained from  $10^{-3}$ ,  $10^{-4}$ , and  $10^{-5}$  dilutions. Since *Bradyrhizobium* takes nearly a week to grow on YEM plates, colonies that grew on the agar were selected after 24 to 96 h incubations. These non-*Bradyrhizobium* colonies of endophytic bacteria were re-isolated and 15 such pure cultures were grown and maintained on Kings B and YEM. One of the strains, SL45, was difficult to culture further and not used in downstream experiments. Of these putative PGPR isolates, there were at least 10 morphologically distinct colonies and they were arbitrarily labeled for identification purposes.

The bacteria identified using partial sequencing of the 16S rRNA gene showed the presence of diverse genera thriving in the nodules of *A. bracteata* (Supplementary Table 2) that could be isolated successfully. Five strains were belonging to *Pseudomonas*, two belonging to each of the genera *Hydrogenophaga* and *Variovorax*. The isolates SL36 and SL53 could not be identified using Sanger sequencing because of poor quality PCR product. One *Rhizobium* species was isolated, which is presumed to be one of the associated symbionts of *A. bracteata* for biological nitrogen fixation. A neighbor-joining phylogenetic tree file was constructed using the 16S rRNA gene sequences (Figure 1). The phylogenetic tree revealed that *Hydrogenophaga* and *Variovorax* genera are in the same cluster whereas the *Bacillus subtilis*, *Gemmobacter sp.*, *Flavobacterium sp.*, *Rhizobium sp.*, and *Devosia*





*sp.*, are in the subsequent nodes of divergence, distant from one another.

The isolates differed from each other in their growth patterns when grown under salt conditions (**Supplementary Figure 6**). There was a gradual decrease in growth of strain SL31 with increasing salt concentrations, but it still reached ~1 OD in 500 mM NaCl at 48 h, which is the highest level of growth among all the isolates. Steady growth was observed in strain SL42 up to 250 mM NaCl, but growth was almost negligible at 500 mM NaCl. The salt concentration of 100 mM NaCl increased the growth of strains SL47 and SL48 when compared to 0 mM NaCl, but growth decreased at higher salt concentrations. Growth declined for strain SL52 but progressed for strain SL53 with increasing salt concentrations. Growth was either reduced or inhibited under salt for the other isolates and markedly lower than the isolates mentioned above. Many of the isolates exhibited PGPR characteristics of ACC deaminase and IAA production (**Table 1** and **Supplementary Figure 7**). *Rhizobium* *sp.* SL42 and *Hydrogenophaga* *sp.* SL48 showed a strong affinity for nitrogen fixation, ACC deaminase activity and biofilm formation. Moreover, *Hydrogenophaga* *sp.* SL48 also exhibited profuse IAA synthesis from L-tryptophan.

## Isolated Bacteria Induce Salinity Tolerance in Soybean

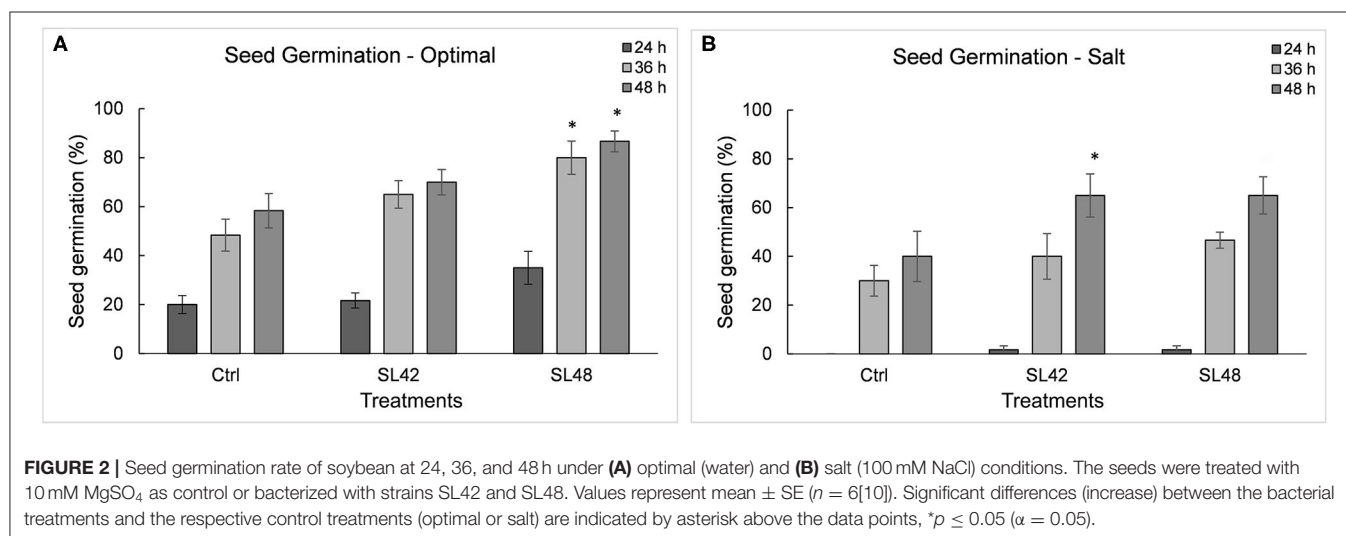
Seed germination was counted when radicle emergence was observed (**Supplementary Figure 8**). There were significant differences between optimal and salt stress conditions at various time points and also among treatments ( $P = 0.002$ ). Under optimal conditions, inoculation with strains SL43, SL47, SL48, and SL49 had significantly increased ( $P = 0.0001$ ) germination rates at 36 and 48 h (~80 %) compared to the control treatment (60%). Under 100 mM NaCl, the germination rate was negligible at 24 h with 0% for control treatment and <5% for the isolates. Germination rate at 36 h was higher ( $P = 0.004$ ) for the treatments SL42, SL47, SL48, SL49, and SL53 (~40%) than the control (30%). There was a greater increase ( $P = 0.0031$ ) in germination rate at 48 h for treatments SL42 and SL48 (65%) than the control (40%) (**Figure 2**) and treatments with other isolates SL36, SL43, SL47, SL49, SL52, SL53, and SL55 were also higher (50–55%) (**Supplementary Figure 9**).

Seedling emergence under optimal conditions was not significantly higher for treatments when compared to the control (**Supplementary Figure 10**). However, under salt stress of 100 mM NaCl emergence rate was significantly increased ( $P = 0.0002$ ) for all but SL31 of the bacterial treatments at 8<sup>th</sup>

**TABLE 1** | PGPR characteristics of the isolated strains characterized using biochemical assays.

	Strain	N—fixation	P—solubilization	IAA	ACC deaminase	Biofilm
1.	SL31	—	—	—	++	—
2.	SL33	—	—	+	—	++
3.	SL36	++	—	—	++	+++
4.	SL42	+++	—	+	++	++++
5.	SL43	—	—	—	—	—
6.	SL44	—	—	—	—	—
7.	SL47	—	—	+++	+++	++++
8.	SL48	+++	—	++++	++	+++
9.	SL49	—	—	+++	++	—
10.	SL50	—	—	++	+++	—
11.	SL52	—	—	+	+++	—
12.	SL53	+++	—	+	++	++
13.	SL54	+++	—	—	++	+
14.	SL55	—	—	++	+++	—
15.	SL56	—	—	++	+++	—

Qualitative assessment: — indicates absence of the trait, + indicates presence of the trait, and additional + indicates the intensity of the trait exhibited by the isolates.

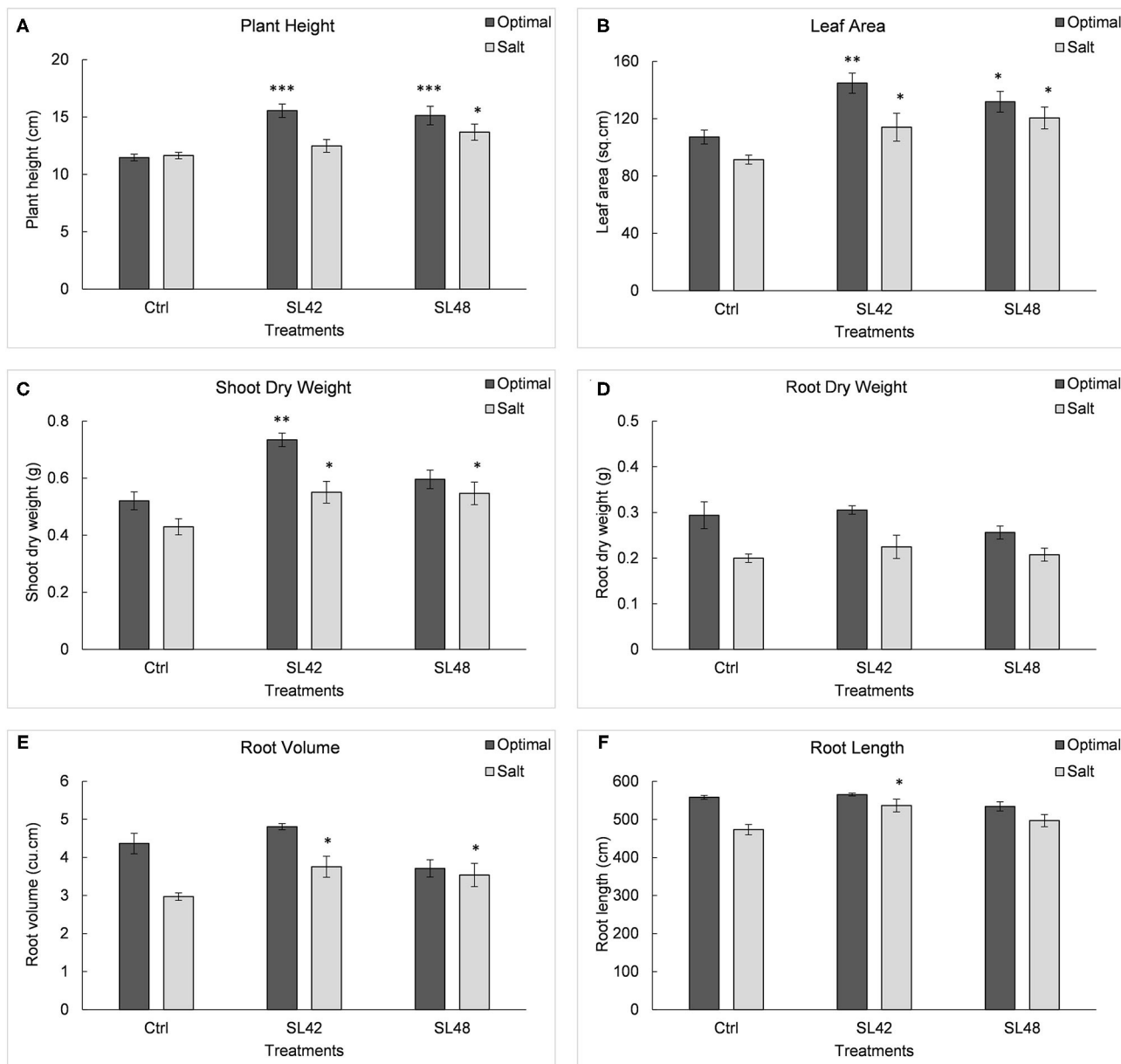


DAP. Growth variables of soybean plants were measured at 28<sup>th</sup> DAP (**Supplementary Figure 11**). Significant increases ( $P < 0.0001$ ) were observed in plant height for treatments SL42, SL43, SL47, SL48, and SL49 compared to the control under optimal conditions. Treatments with SL47 and SL48 showed significant increases ( $P < 0.0001$ ) under salt stress and slight increases in plant height were also observed for treatments SL42 and SL49. Leaf area was significantly higher ( $P < 0.0001$ ) for treatments with SL42, SL47, SL48, and SL49 than the control under both optimal and salt stress conditions. A parallel outcome was observed in shoot biomass, with treatments SL42, SL43, SL47, SL49, and SL50 showing significant increases ( $P < 0.0001$ ) under optimal conditions and treatments SL42, SL44, SL47, SL48, SL49, and SL55 showing significant increases ( $P < 0.0001$ ) under salt stress. Root dry weight was significantly higher for treatments SL36 and SL43 under optimal conditions ( $P < 0.0001$ ) and for

the treatments SL33, SL36, SL43, SL50, SL55, and SL56 under salt stress ( $P = 0.0004$ ). However, root volume was significantly increased ( $P = 0.0003$ ) for treatments SL31, SL33, SL36, SL42, SL48, SL49, SL50, SL55, and SL56 compared to the control treatment under salt stress. Results of the most beneficial strains, SL42 and SL48 are shown in **Figure 3**. Yet only treatments SL42 and SL50 showed significant increases ( $P = 0.04$ ) in root length, and SL31 and SL42 showed significant increases ( $P = 0.01$ ) in root surface area under salt stress (**Supplementary Figure 12**).

## Soybean Growth Under Different Salt Concentrations

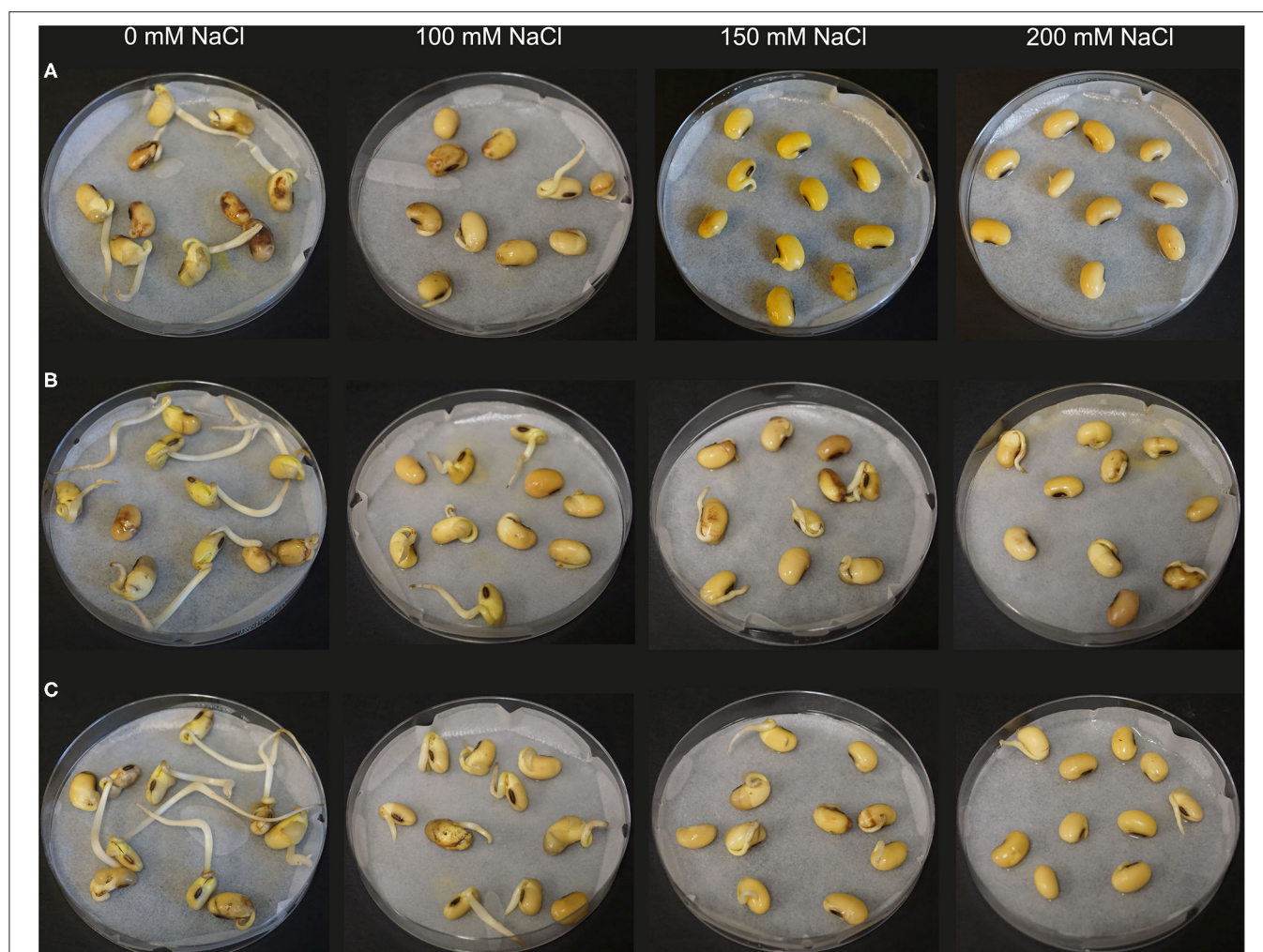
Based on the results of the first screening, four isolates *Rhizobium* sp. SL42, *Hydrogenophaga* sp. SL48, *Pseudomonas borealis* SL49, and *Variovorax* sp. SL55 were selected for the next trial. Seed germination of soybean was differentially affected under a range



**FIGURE 3 |** Growth variables of soybean, (A) Plant height, (B) Leaf area index, (C) Shoot dry weight, (D) Root dry weight, (E) Root volume, and (F) Root length measured at 28<sup>th</sup> DAP under optimal (water) and salt (100 mM NaCl) conditions. The seeds were treated with 10 mM MgSO<sub>4</sub> as control or bacterized with strains SL42 and SL48. Values represent mean ± SE (*n* = 6). Significant differences (increase) between the bacterial treatments and the respective control treatments (optimal or salt) are indicated by asterisk above the data points, \**p* ≤ 0.05, \*\**p* ≤ 0.001, \*\*\**p* ≤ 0.0001 ( $\alpha$  = 0.05).

of salt concentrations (Figure 4). All bacterial treatments resulted in increases over the control treatment. The germination rate was 65% for SL42, 80% for SL48, 60% for SL49, and 70% for SL55 at 0 mM NaCl after 72 h compared to the 45% germination rate in control treatment. At 100 and 125 mM NaCl, the germination rate was around 60% for the bacterial treatments and 42% for the control treatment. The germination rate at  $1 \times 10^8$  cfu mL<sup>-1</sup> was 58% for SL42, 55% for SL48, 60% for SL49, and 43% for SL55 at 150 mM, while the control

reached about 40%. The germination rate was considerably lower at higher salt concentrations of 175 and 200 mM NaCl. For SL55, no significant increase in germination rate was observed except at 175 mM NaCl (Supplementary Figures 13 and 14). The germination rates for the two inoculums,  $1 \times 10^8$  and  $1 \times 10^{10}$  cfu mL<sup>-1</sup> were mostly parallel to each other but slight variations were present in a few cases and  $1 \times 10^8$  cfu mL<sup>-1</sup> was selected as the inoculum density for successive experiments.



**FIGURE 4 |** Seed germination of soybean at 72 h under increasing salt concentrations (0, 100, 150, and 200 mM NaCl). The seeds were treated with **(A)** 10 mM  $MgSO_4$  as control or bacterized with strains **(B)** SL42 and **(C)** SL48.

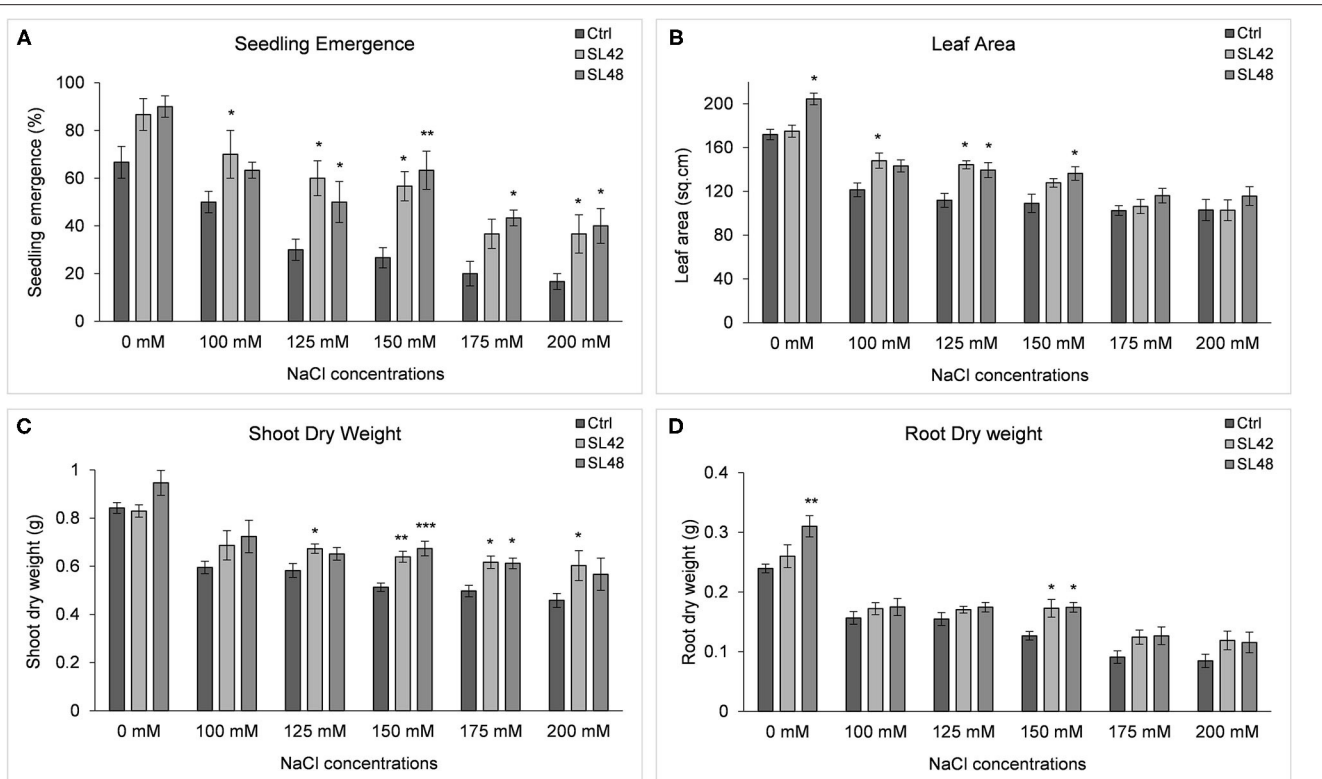
Seedling emergence rate at 8<sup>th</sup> DAP was significantly higher for the treatment SL42 at all the salt concentrations from 100 ( $P = 0.0326$ ) to 200 mM ( $P = 0.0153$ ) NaCl than the control treatment. It was also increased by treatments SL48, SL49, and SL55 at different salt levels but the statistical significance varied. Growth variables of soybean treated with the four isolates, grown under a range of salt concentrations were measured at 28<sup>th</sup> DAP (**Supplementary Figure 15**) and plant growth was greatly reduced at 175 and 200 mM NaCl. Leaf area was significantly increased by the treatments with SL48 at 0, 125, and 150 mM NaCl ( $P = 0.01$ ) and SL42 at 100 ( $P = 0.022$ ) and 125 mM NaCl compared to control treatments. This corresponded to the increase in shoot biomass, which was significantly higher than the control for treatments SL42 at 125, 150 ( $P = 0.0016$ ), 175, and 200 mM NaCl and SL48 at 150 ( $P < 0.0001$ ) and 175 mM NaCl. Shoot dry weight was improved by SL48 and SL42 at other NaCl concentrations as well, albeit not significantly. Treatment with SL49 has significantly increased shoot biomass at 150 ( $P = 0.02$ ) and 175 mM NaCl. Root dry weight was significantly higher

at 0 mM NaCl for the treatments SL48 ( $P = 0.001$ ) and SL49 and at 150 mM for the treatments SL42 ( $P = 0.0015$ ), SL48 ( $P = 0.0011$ ), and SL55 ( $P = 0.002$ ). The root dry weight was also increased by the bacterial treatments at higher salt concentrations (175 and 200 mM NaCl). The results indicated that the strains SL42 and SL48 have greatly improved soybean growth under a range of salt stress conditions (**Figure 5**). Though salt stress of 100–150 mM NaCl had significant differences between the control and bacterial treatments, 150 mM NaCl provided a much clearer distinction related to salinity stress in the shoot ( $P = 0.0004$ ) and root biomass ( $P = 0.0036$ ).

## Co-inoculation of Nodule Isolates Improves the Growth and Development of Soybean

Two of the bacterial isolates, *Rhizobium* sp. SL42 and *Hydrogenophaga* sp. SL48 were co-inoculated with *Bradyrhizobium japonicum* 532C and the soybean plants were grown under optimal or 150 mM NaCl conditions. Inoculation with *B. japonicum* alone served as the control as



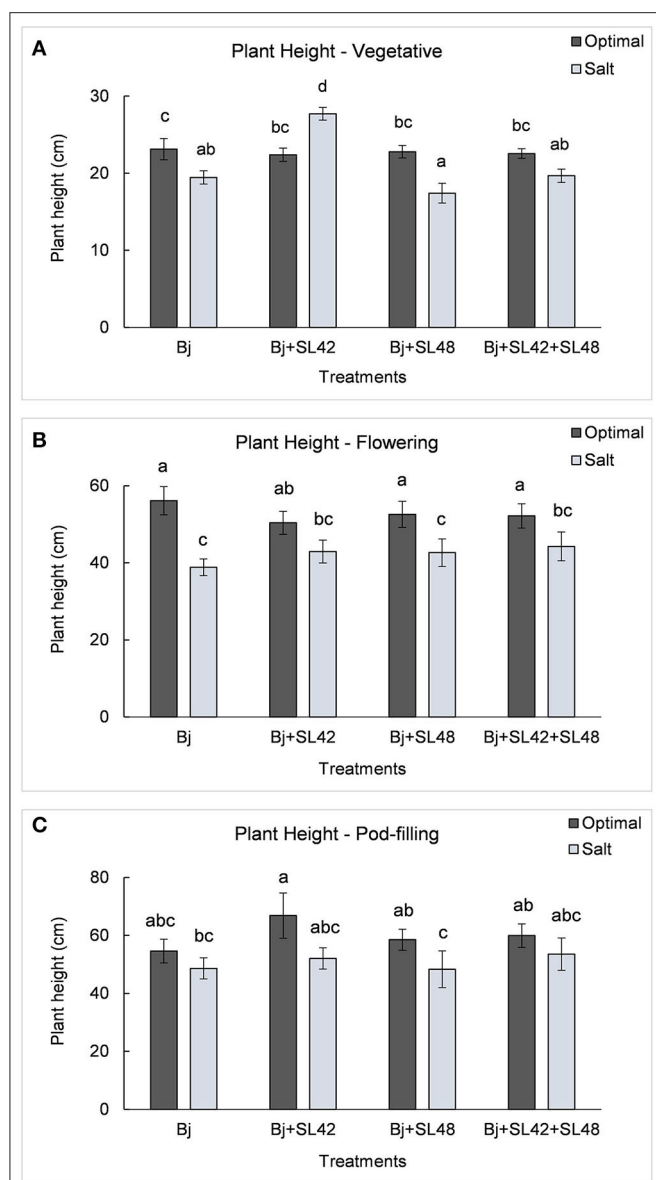


**FIGURE 5 |** Growth variables of soybean, (A) Seedling emergence rate measured on 8<sup>th</sup> DAP and growth variables of soybean (B) Leaf area, (C) Shoot dry weight, and (D) Root dry weight measured at 28<sup>th</sup> DAP under increasing salt concentrations (0, 100, 125, 150, 175, and 200 mM NaCl). The seeds were treated with 10 mM MgSO<sub>4</sub> as control or bacterized with strains SL42 and SL48. Values represent mean  $\pm$  SE ( $n = 6$ ). Significant differences (increase) between the bacterial treatments and the respective control treatments of a particular salt concentration are indicated by asterisk above the data points, \* $p \leq 0.05$ , \*\* $p \leq 0.001$ , \*\*\* $p \leq 0.0001$  ( $\alpha = 0.05$ ).

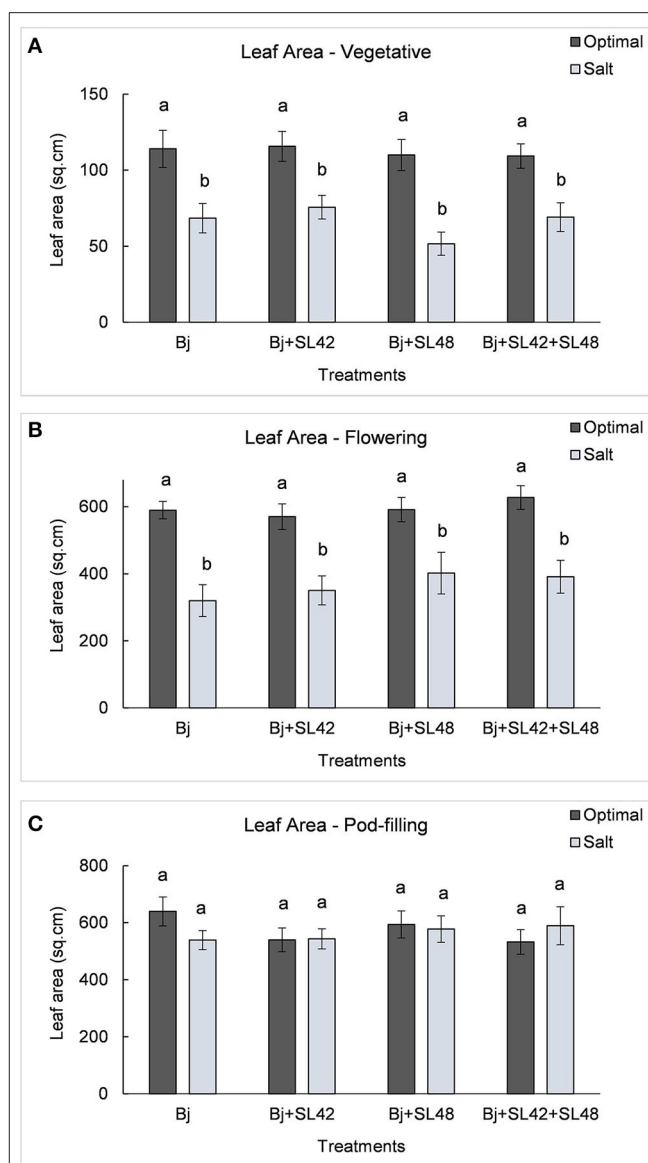
it is the standard N<sub>2</sub>-fixing symbiont of soybean. Even though growth variables under optimal conditions were insignificantly different for plants inoculated with *B. japonicum* because of the uninhibited nitrogen fixation, co-inoculation with SL42 and SL48 enhanced plant growth in most cases. However, under salt stress, there were substantial differences between the co-inoculation treatments and the *B. japonicum* control and the co-inoculation treatments resulted in higher growth than the control in all respects.

During the vegetative and flowering stages, the growth variables were all significantly different ( $P < 0.0001$ ) between the optimal and salt stress conditions. At the vegetative stage, plant height ( $P = 0.0001$ ), shoot biomass ( $P = 0.2764$ ), and root dry weight ( $P = 0.0935$ ) were increased by the *B. japonicum*+SL42 treatment compared to the control, *B. japonicum* (Bj) under salt stress. Shoot biomass was also increased by the *B. japonicum*+SL42+SL48 treatment under both optimal and salt stress conditions. During the flowering stage, plant height ( $P = 0.1934$ ), leaf area ( $P = 0.1562$ ) (Figure 7), shoot biomass ( $P = 0.0872$ ), and root dry weight ( $P = 0.1766$ ) were all higher for the *B. japonicum*+SL42+SL48 treatment, and also for the other co-inoculation treatments than the *B. japonicum* control under salt stress. The treatment

of *B. japonicum*+SL42+SL48 was also the highest under optimal conditions (except for plant height), although the difference was more notable under salt stress. At the pod-filling stage, shoot biomass was increased by the treatments of *B. japonicum*+SL42 ( $P = 0.1001$ ) and *B. japonicum*+SL42+SL48 ( $P = 0.3866$ ), as was the case with other growth variables, compared to the *B. japonicum* control under salt stress. The treatment of *B. japonicum*+SL42 also improved plant growth under optimal conditions (except for leaf area index). Plant height (Figure 6) and leaf area (Figure 7) of soybean increased exponentially up to the pod-filling stage and vegetative growth was stationary as the plants reached maturity. During the harvest stage, the shoot biomass was considerably reduced due to the senescence of leaves and not much of a difference among the treatments were observed. Overall, the co-inoculation treatments have resulted in increased shoot dry weight by 1.6 and 18.3% at vegetative, 11.9 and 27% at flowering, 7.1 and 7.5% at pod-filling, 7.5 and 4.6% at harvest under optimal and salt stress conditions, respectively (Figure 8). The root dry weight under salt stress was particularly increased by the treatment of *B. japonicum*+SL42, by 28% at vegetative, 16% at flowering, 9% at pod-filling, and 24.5% at harvest stages (Figure 9).



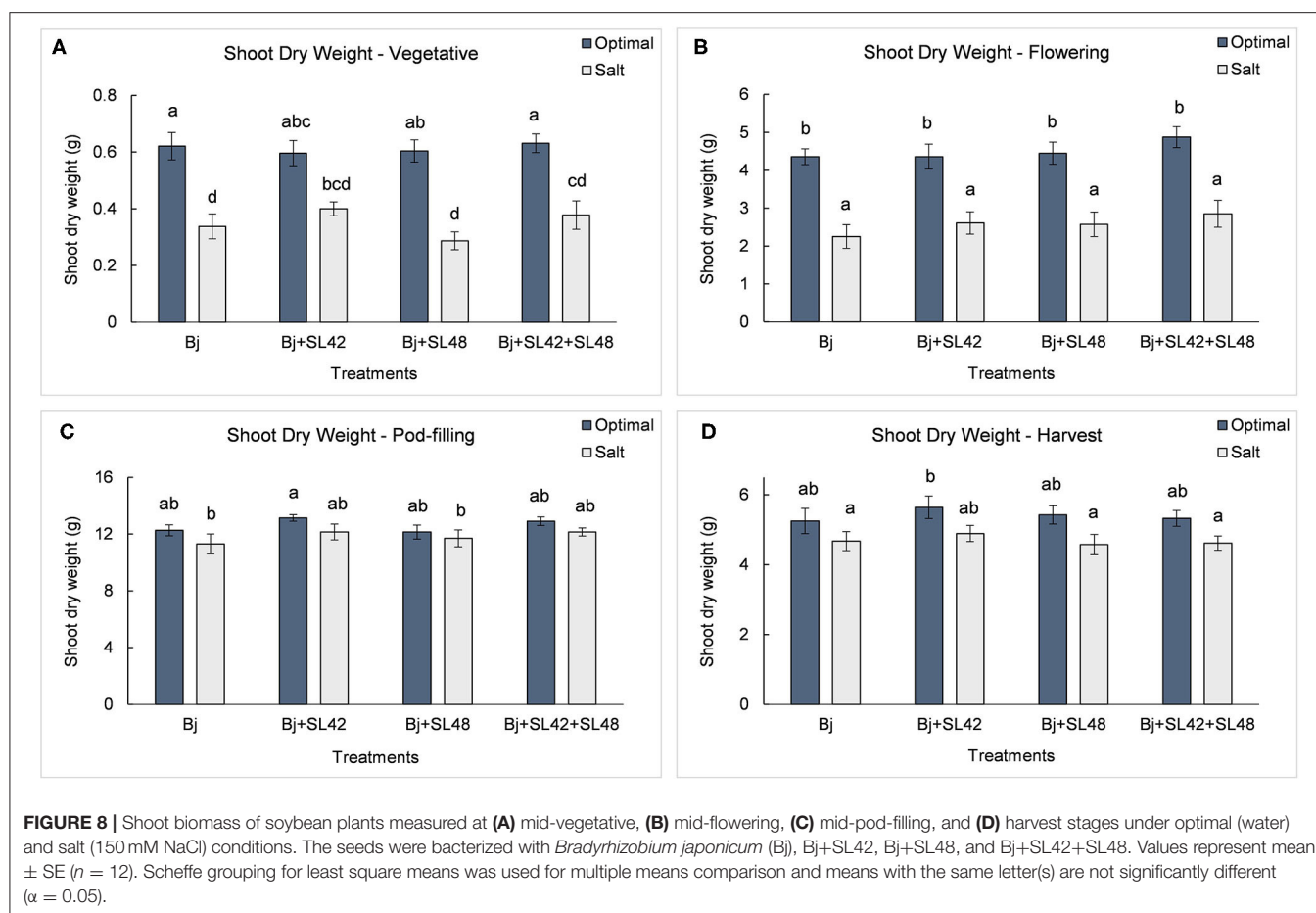
**FIGURE 6 |** Height of soybean plants measured at (A) mid-vegetative, (B) mid-flowering, and (C) mid-pod-filling stages under optimal (water) and salt (150 mM NaCl) conditions. The seeds were bacterized with *Bradyrhizobium japonicum* (Bj), Bj+SL42, Bj+SL48 and Bj+SL42+SL48. Values represent mean  $\pm$  SE ( $n=12$ ). Scheffe grouping for least square means was used for multiple means comparison and means with the same letter(s) are not significantly different ( $\alpha = 0.05$ ).



**FIGURE 7 |** Leaf area of soybean plants measured at (A) mid-vegetative, (B) mid-flowering, and (C) mid-pod-filling stages under optimal (water) and salt (150 mM NaCl) conditions. The seeds were bacterized with *Bradyrhizobium japonicum* (Bj), Bj+SL42, Bj+SL48 and Bj+SL42+SL48. Values represent mean  $\pm$  SE ( $n = 12$ ). Scheffe grouping for least square means was used for multiple means comparison and means with the same letter(s) are not significantly different ( $\alpha = 0.05$ ).

The yield variables, seed weight and seed number were increased by all three co-inoculation treatments compared to *B. japonicum* alone under both optimal and salt stress conditions (Figure 10). The treatment of *B. japonicum*+SL42 increased seed weight by 4.3% ( $P = 0.7207$ ) and seed number by 10.5% ( $P = 0.2788$ ) under salt stress. The other treatments, *B. japonicum*+SL48 and *B. japonicum*+SL42+SL48 increased seed weight by 7.4% ( $P = 0.449$ ) and 8.1% ( $P = 0.3347$ ), under optimal conditions and 3.6% ( $P = 0.7145$ ) and 3.1%

( $P = 0.8686$ ) under salt stress, respectively. Even though seed weight and seed number were less in salt stress than the optimal conditions, the difference between the corresponding treatments was small. The harvest index is the proportion of seed dry weight to the aboveground biomass and the treatments with *B. japonicum*+SL48 and *B. japonicum*+SL42+SL48 had higher harvest indices ( $P = 0.1621$ ) than that of the treatments with *B. japonicum* and *B. japonicum*+SL42, under both optimal and salt stress conditions.

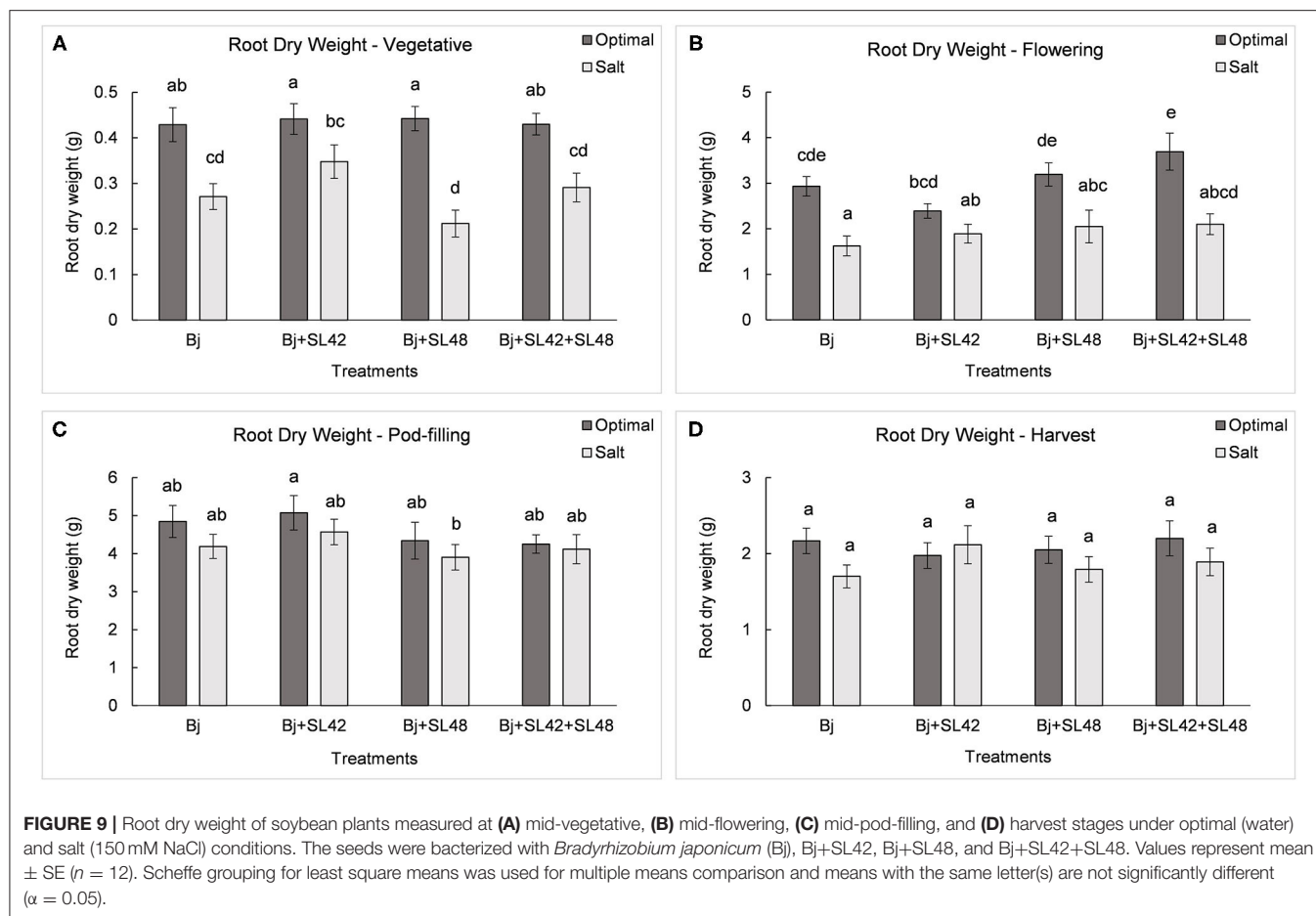


## Nutrient Composition Analysis of Plant Tissues and Seeds

The nutrient analysis provided an interesting perspective on how the nutrients were translocated between various plant tissues throughout the developmental stages. Nitrogen concentration was largely unvarying in the vegetative and flowering stages, except that shoot N concentration was greater than that of roots. No significant difference was observed in nitrogen concentration between the treatments since all of them were inoculated with *B. japonicum*. At pod-filling, N concentration under salt stress was lower than optimal in leaves, shoot, and roots but almost equal in the pods and at harvest, it was less in the shoot and pods but more or less equal in the roots (Supplementary Table 3). This is reflected in the seed quality where the protein concentration was lower under salt stress (34%) than optimal (37%) conditions. Nitrogen assimilation was calculated as a ratio of total N concentration in the tissues to the dry weight, and it was significantly reduced ( $P < 0.0001$ ) with the salt stress at all the developmental stages. At the vegetative stage, N assimilation did not vary among treatments and was corresponding to the amount of fertilizer applied under optimal conditions (60 mg per plant) since the nodules will be still developing at this stage and not fully functional yet. As the plants developed, biological nitrogen fixation was actively occurring, evident by the high N assimilation. During

the flowering stage, treatment of *B. japonicum*+SL42+SL48 had increased N assimilation under optimal and salt conditions. At the pod-filling stage, inoculation with *B. japonicum*+SL42 showed higher N assimilation under optimal conditions, yet the treatment of *B. japonicum*+SL42+SL48 resulted in the highest N assimilation under salt stress. At the harvest stage, N assimilation was relatively higher for the *B. japonicum*+SL42 treatment under both optimal and salt-stressed conditions (Table 2).

Phosphorous concentration was higher under salt stress than optimal conditions at the flowering and pod-filling stages, but more or less equal at the vegetative and harvest stages (Supplementary Table 4). The treatment of *B. japonicum*+SL42+SL48 had the lowest P concentration in the shoot and pods at the harvest stage but highest in the seeds under salt stress. Potassium concentration under salt stress was lower and sodium concentration was higher than optimal conditions at the vegetative stage (Supplementary Tables 5, 6). The plants at this stage had higher salt accumulation relative to their biomass and hence, the  $K^+/Na^+$  ratio (Table 3) was lower. The treatment of *B. japonicum*+SL42 had higher K concentration and lower Na concentration in the shoot than the *B. japonicum* control, and so, the shoot  $K^+/Na^+$  ratio was significantly higher ( $P = 0.006$ ) under salt stress. Potassium concentration under salt stress was higher in the leaves, shoot, and pods but lower in the roots at flowering, pod-filling, and



**TABLE 2 |** Total Nitrogen assimilation in shoot and root tissues of soybean through the developmental stages.

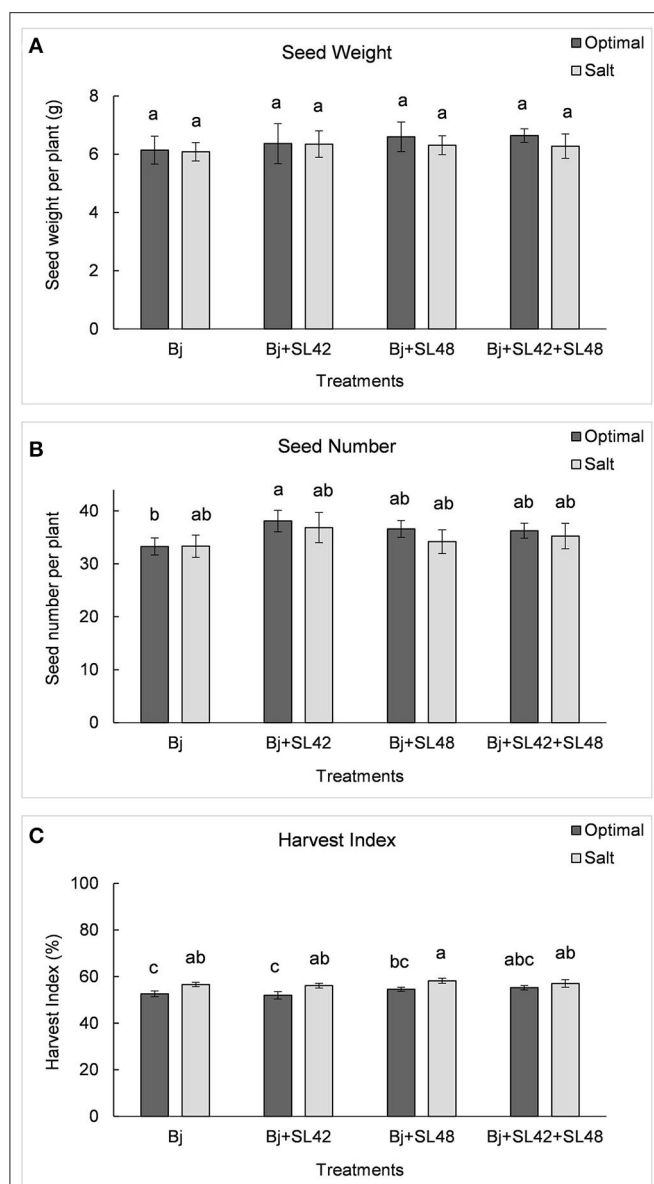
Treatments	Optimal	Salt	Optimal	Salt
Vegetative stage		Flowering stage		
	<i>P</i> = 0.542	<i>P</i> = 0.3922	<i>P</i> = 0.3499	<i>P</i> = 0.7125
Bj	55.62 ± 6.36	35.31 ± 5.26	572.77 ± 24.96	322.98 ± 21.40
Bj+SL42	60.52 ± 1.04	39.64 ± 7.44	561.20 ± 48.26	360.97 ± 18.38
Bj+SL48	60.80 ± 5.50	21.92 ± 2.44	613.69 ± 63.51	357.90 ± 18.52
Bj+SL42+SL48	58.58 ± 6.51	32.71 ± 3.31	679.05 ± 57.68	360.59 ± 45.72
Pod-filling stage		Harvest stage		
	<i>P</i> = 0.4104	<i>P</i> = 0.3142	<i>P</i> = 0.5496	<i>P</i> = 0.622
Bj	1795.54 ± 73.41	1407.42 ± 60.49	285.99 ± 37.43	213.10 ± 12.95
Bj+SL42	1849.71 ± 91.22	1497.74 ± 86.39	294.78 ± 27.36	225.96 ± 20.87
Bj+SL48	1728.23 ± 41.03	1455.76 ± 57.62	259.59 ± 32.88	196.21 ± 5.92
Bj+SL42+SL48	1778.99 ± 96.30	1551.74 ± 70.02	293.55 ± 25.86	202.31 ± 12.76

Values represent mean  $\pm$  SE ( $n = 12$ ).

harvest stages than optimal conditions. Sodium concentration was relatively higher under salt stress and much of the Na was accumulated in the roots, as compared to the shoot and leaves. This explains the low  $K^+/Na^+$  ratio in roots versus the high  $K^+/Na^+$  ratio in the shoot and leaves. The treatment of *B. japonicum*+SL48 had lower Na concentration in the leaves

and significantly higher ( $P < 0.0001$ )  $K^+/Na^+$  ratio than the *B. japonicum* control under salt stress at the flowering stage. The  $K^+/Na^+$  ratio was significantly increased ( $P < 0.01$ ) by the *B. japonicum*+SL42 treatment under salt stress in the leaves during the pod-filling stage. Treatment with *B. japonicum*+SL48 increased K concentration and decreased Na concentration in





**FIGURE 10 |** Yield variables of soybean plants measured after harvest **(A)** seed weight, **(B)** seed number, and **(C)** harvest index under optimal (water) and salt (150 mM NaCl) conditions. The seeds were bacterized with *Bradyrhizobium japonicum* (Bj), Bj+SL42, Bj+SL48, and Bj+SL42+SL48. Values represent mean  $\pm$  SE ( $n = 12$ ). Scheffe grouping for least square means was used for multiple means comparison and means with the same letter(s) are not significantly different ( $\alpha = 0.05$ ).

the shoot and pods, which resulted in higher  $K^+/Na^+$  ratios at the harvest stage. Calcium concentration in the shoot and roots were relatively higher under salt stress than optimal conditions at the flowering, pod-filling and harvest stages, indicating that the plants were also utilizing  $Ca^{2+}$  to maintain ionic balance under salinity stress (Supplementary Table 7).

Seed nutritional composition analysis showed that the moisture, protein, and fiber concentrations decreased, and fat, ash, and potassium concentrations increased under salt

stress conditions. Sodium concentration was also significantly higher under salt stress ( $P = 0.0079$ ). The treatment of *B. japonicum*+SL42+SL48 had the highest protein, fiber, phosphorus, potassium, calcium, sodium, and magnesium concentrations and the lowest moisture, fat, and ash concentrations under salt stress (Supplementary Table 8). Diversity in the nodule bacteria of soybean was observed at  $10^{-4}$  and  $10^{-5}$  dilutions and the colonies were disparate between optimal and salt-stressed plants (Supplementary Figure 16). Colonies similar to the morphology of SL42 and SL48 were prominent in the co-inoculation treatments, specifically, the *B. japonicum*+SL42 treatment had predominantly SL42 colonies under salt stress. Also, the bacterial population was higher in the *B. japonicum*+SL42+SL48 treatment than the control, *B. japonicum*.

## DISCUSSION

Rhizobia and legumes have established their mutualistic association over 100 million years of coevolution and the association between different lineages of both the rhizobia and the legume has diverged to be species-specific and spatially-specific (Parker, 1999). This mutualistic specificity also holds true for the host relationship with other members of the phytomicrobiome, including other bacteria in the nodules. The nodules of *Amphicarpaea bracteata* have endophytic bacteria other than its *Bradyrhizobium* symbiont. The vastness in the diversity of these bacteria suggests that they might be effectively functioning as plant growth promoting rhizobacteria (PGPR) in their host. The tested isolates do not coexist with *B. japonicum* in nature (or at least not known yet) and in this study, they were introduced to a related host intended to exert beneficial effects. Co-inoculation of PGPR with rhizobia was reported in various legume plants and proposed to be used as inoculants (Bai et al., 2002a).

Seed germination is the initiation of plant growth and favorable conditions are necessary for successful germination and subsequent seedling emergence. The rate of germination and the time to seedling emergence are important in terms of crop establishment at the beginning of the growing season. Seedling emergence and younger seedlings are more prone to risk from salinity since root development occurs in the topsoil, which generally has higher accumulation of soluble salts (Almansouri et al., 2001). Salt was pre-applied to vermiculite before planting the soybean seeds in the greenhouse, so as to mimic the salinity stress under field conditions where salt is already present in the topsoil and the seeds have to undergo the process of germination and development in the presence of salt. The seedling stage of the soybean plant is considered to be more sensitive than seed germination (Hosseini et al., 2002) and that is why the effect of salinity stress was acute and precise in the screening experiments where the plants were grown up to the mid-vegetative stage. Salinity stress inhibited seed germination, affected seedling growth, reduced biomass accumulation and decreased seed weight of soybean (Essa, 2002). The plants exhibited symptoms of salinity stress, the seedling

**TABLE 3 |** Distribution of  $K^+/Na^+$  in different plant tissues through the developmental stages of soybean.

Treatments	Optimal	Salt	Optimal	Salt
<b>Vegetative: Shoot</b>		<b>Flowering: Shoot</b>		
	$P = 0.5220$	$P = 0.0065$	$P = 0.4234$	$P = 0.6128$
Bj	254.51 ± 68.90	47.66 ± 9.70	665.28 ± 224.79	142.83 ± 25.72
Bj+SL42	197.71 ± 35.67	76.11 ± 19.68	783.88 ± 215.66	185.02 ± 40.09
Bj+SL48	159.00 ± 30.97	29.46 ± 3.33	872.86 ± 171.62	194.68 ± 55.18
Bj+SL42+SL48	219.71 ± 56.53	54.47 ± 13.11	1184.70 ± 425.62	231.78 ± 62.76
<b>Vegetative: Root</b>		<b>Flowering: Root</b>		
	$P = 0.4619$	$P = 0.8506$	$P = 0.2347$	$P = 0.8806$
Bj	3.30 ± 0.40	1.82 ± 0.10	4.75 ± 0.34	2.16 ± 0.23
Bj+SL42	3.19 ± 0.10	1.69 ± 0.24	4.56 ± 0.35	2.24 ± 0.13
Bj+SL48	2.86 ± 0.18	1.88 ± 0.07	5.23 ± 0.34	2.15 ± 0.33
Bj+SL42+SL48	3.11 ± 0.15	1.97 ± 0.42	5.51 ± 0.40	2.27 ± 0.35
<b>Pod-filling: Leaves</b>		<b>Flowering: Leaves</b>		
	$P = 0.0072$	$P = 0.0088$	$P = 0.6510$	$P < 0.0001$
Bj	215.98 ± 62.23	232.97 ± 26.94	425.80 ± 27.13	317.67 ± 21.29
Bj+SL42	272.06 ± 3.14	599.77 ± 176.51	365.97 ± 43.21	357.96 ± 91.51
Bj+SL48	431.80 ± 99.21	219.03 ± 42.57	502.20 ± 69.57	515.35 ± 142.80
Bj+SL42+SL48	686.93 ± 90.96	331.00 ± 102.04	373.02 ± 75.04	445.21 ± 194.94
<b>Pod-filling: Shoot</b>		<b>Harvest: Shoot</b>		
	$P = 0.5937$	$P = 0.1282$	$P = 0.0906$	$P = 0.404$
Bj	786.69 ± 226.81	92.10 ± 36.67	40.71 ± 7.08	1.83 ± 0.57
Bj+SL42	523.81 ± 139.14	38.31 ± 5.75	77.01 ± 20.80	1.26 ± 0.38
Bj+SL48	499.56 ± 113.14	68.86 ± 10.89	60.02 ± 12.82	2.31 ± 0.69
Bj+SL42+SL48	645.64 ± 97.23	61.80 ± 20.85	67.53 ± 12.35	2.35 ± 0.54
<b>Pod-filling: Pods</b>		<b>Harvest: Pods</b>		
	$P = 0.4089$	$P = 0.1799$	$P = 0.257$	$P = 0.2863$
Bj	1029.94 ± 130.26	366.12 ± 84.35	393.44 ± 113.12	63.94 ± 14.32
Bj+SL42	821.22 ± 137.93	689.29 ± 101.93	609.17 ± 191.17	38.03 ± 13.11
Bj+SL48	1032.80 ± 440.95	436.10 ± 48.10	387.16 ± 71.89	86.54 ± 20.10
Bj+SL42+SL48	602.90 ± 159.18	664.29 ± 176.20	384.78 ± 64.55	58.60 ± 12.43
<b>Pod-filling: Root</b>		<b>Harvest: Root</b>		
	$P = 0.0184$	$P = 0.2422$	$P = 0.9461$	$P = 0.088$
Bj	4.66 ± 0.41	1.24 ± 0.10	2.40 ± 0.27	0.59 ± 0.05
Bj+SL42	3.99 ± 0.30	1.43 ± 0.15	2.48 ± 0.11	1.22 ± 0.50
Bj+SL48	5.41 ± 0.45	1.44 ± 0.10	2.78 ± 0.51	0.52 ± 0.08
Bj+SL42+SL48	5.63 ± 0.46	1.19 ± 0.09	2.82 ± 0.86	0.44 ± 0.03

Values represent mean ± SE (n = 12).

emergence was slower, and the growth was less compared to the optimal conditions. The mechanisms underlying the inhibition of soybean seed germination and growth by salinity stress are only partially understood (Zhang et al., 2014). Salt stress leads to the up-regulation of ABA and ethylene biosynthesis and down-regulation of GA during seed germination and auxin and cytokinin during plant growth (Shu et al., 2017). The PGPR are reported to modulate phytohormone signaling involved in salinity stress; a rhizobacterium *Sphingomonas* sp. LK11, known to secrete phytohormones (auxins and gibberellins) and trehalose had significantly increased plant growth under drought-induced osmotic stress in soybean (Asaf et al., 2017). Another rhizobacterium, *Arthrobacter woluensis* AK1 was

shown to ameliorate salinity stress by decreasing ABA content, regulating antioxidant activities and salt tolerance genes and reduced sodium uptake in soybean (Khan et al., 2019). Several isolates, including SL42, SL48, and SL49 have significantly improved seed germination and shoot biomass under salt stress and similar results were observed in the consecutive greenhouse trial as well. The isolates also produced IAA and ACC deaminase, which at least partly explains the observed plant growth promotion and stress tolerance.

Since the bacteria were isolated from the nodules of *A. bracteata* that has *Bradyrhizobium* sp. as the symbiont, they have co-existed in the nodules. Hence, the behavior of nodule bacteria was speculated to be potentially complementary when

co-inoculated with a related symbiont in soybean. The plants were grown up to the harvest stage and samples were collected at every growth stage to discern the effect of the isolates on the salinity response of soybean. Soybean has varying water requirements throughout its growing season and rapid root and shoot growth are noted from mid-vegetative to mid-pod-filling stages when the water requirements are also the highest. Though the plant is moderately tolerant and able to withstand short periods of drought and salinity stress, they affect development and crop yield and the plant is most susceptible to the stressors during the vegetative and flowering stages (FAO, 2002). Shoot dry weight of soybean was more affected by salt stress than root dry weight, as reported previously (Essa, 2002) and above-ground plant growth was significantly reduced ( $P < 0.0001$ ) by salinity during the vegetative and flowering stages when the plant was suffering from chronic salt stress. At the later development stages (pod-filling and harvest) this difference was seldom noticed because the plants would have developed tolerance mechanisms and acclimatized to the stress with time (Munns and Tester, 2008). The degree of salt tolerance in soybean differs among developmental stages (Phang et al., 2008). The plant is sensitive to salinity at early growth stages, but this doesn't necessarily mean it will also be sensitive at later growth stages. The results would probably vary if the plants were exposed to another surge of salinity stress at the later growth stages. For soybean, both flowering and pod-filling stages are responsive to water availability and significant yield loss occurred when the plants were exposed to drought at these developmental stages (Westgate and Peterson, 1993). Soluble salts are usually localized in the sub-surface layers and the concentration of these salts reduces water availability and the roots may be exposed to salt-contaminated soil water table (Rengasamy, 2002). Nevertheless, it is interesting to note that the salt stress is contained in a closed system in the greenhouse and salt volume was applied proportionately to the field capacity of the pot volume. Under field conditions, the intensity of the stress fluctuates depending on other environmental factors such as precipitation or evapotranspiration.

The plants were supplied with a low N fertilizer and the nitrogen fixation was predominantly carried out by *Bradyrhizobium japonicum*. The decrease in nitrogen accumulation under salinity stress was due to the inhibition of root nodulation and biological nitrogen fixation. The number of root nodules and root hair curling were constrained by salt stress (Tu, 1981). The N content of the pods dramatically decreased from the pod-filling to harvest stages indicating the translocation of N to the pods and then to the seeds. The protein content of the seeds was reduced under salinity stress whereas, the oil content was increased. Despite the decline in photosynthesis, translocation of assimilates to the sink tissues were largely maintained in soybean under drought stress (Huber et al., 1984). Phytohormone signaling coordinates partitioning of the assimilates between source and sink, and thereby maintains growth, development and function (Perez-Alfocea et al., 2010). The co-inoculation treatments resulted in higher seed weight and seed number than the control under salt stress and allowed the plants to at least partially overcome the effects of stress on reproduction. The isolates might regulate signaling events in the

plants during the initial osmotic phase but later shift towards balancing ionic stress under salinity. Potassium is a key nutrient in maintaining ion homeostasis under salinity and the ratio of  $K^+$  to  $Na^+$  is determined by the rate of  $K^+$  assimilation. The high cytosolic  $K^+/Na^+$  ratio is critical for plant salinity tolerance and the function of  $K^+$  transporters is regulated by osmolytes and calcium signaling. Ionic homeostasis is maintained by excluding  $Na^+$  and  $Cl^-$  and restricting their accumulation in plant tissues and compartmentalizing the toxic ions in vacuoles (Shabala and Cuin, 2008). The ability of the plants treated with isolates SL42 and SL48 to maintain a high  $K^+/Na^+$  ratio through various growth stages is indeed an indication of induced salinity tolerance. Follow-up studies are in progress to understand the mode of action of the isolates and the adaptive mechanisms elicited in the plants. The primary reason for using vermiculite as the sole potting medium is that it is inert, ruling out the possibility of interference by organic matter (including microflora) usually present in the soil or peat-based potting medium. This has proved to be an effective testing tool for salt stress mitigation by the bacterial inoculation and plant nutrient uptake from the added fertilizer. The observations of nodule bacterial colonies indicated that SL42 and SL48 predominantly inhabited the nodules of soybean and also supported the resident nodule phytomicrobiome population. Indigenous microbial communities influence the survival of inoculated bacteria and vice-versa (Trabelsi and Mhamdi, 2013). However, the tested strains are a part of the native habitat, so that the potential concern for altering the ecosystem function of soil microbial communities is diminished. They have a competitive advantage over the resident soil microbiota since they provide a synergistic plant-microbe interaction with soybean. Considering that many other factors are at play under a field condition, extensive field trials are needed to determine the beneficial effects of these microbes on soybean growth and yield in local agriculture production systems.

## CONCLUSIONS

Soybean cultivation has reached its northern hemisphere limit and expansion/extension of cultivation both spatially and temporally will be possible when the plants can further acclimatize to the native conditions. Co-inoculation with native nodule bacterial strains can help in the adaptation and expression of particular traits such as salt/drought tolerance or cold acclimatization induced by the bacteria can benefit the associated plant. Early plant response mechanisms to these stresses overlap each other, which means inoculation with these bacteria can be an asset to sustainable soybean production under the Canadian agricultural scenario. Moreover, such growth promoting technology as this might invigorate native soil properties (both abiotic and biotic), create synergy with the native soil microflora, assist in the reduction of chemical inputs and advance crop productivity. However, multiple field trials are required to demonstrate the potential of these isolates to boost yield by growth promotion and stress alleviation. Even though adaptation to salinity stress depends on various factors including the plant's innate potential and the environmental conditions, implementing a cost-effective strategy of PGPR inoculation to

enhance stress tolerance will be fruitful and helpful to meet the rising demands for global food production.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

GI conducted the research, collected data, and prepared the manuscript. TS helped GI for statistical analysis of the data. DS helped GI in editing the manuscript and providing feedback. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.617978/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## NOMENCLATURE

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NCBI BLAST (NCBI BLAST, RRID:SCR\_004870)

Clustal Omega (Clustal Omega, RRID:SCR\_001591)

ClustalW2 (ClustalW2, RRID:SCR\_002909)

iTOL (iTOL, RRID:SCR\_018174)

WinRHIZO (WinRHIZO, RRID:SCR\_017120)

SAS 9.4 (Statistical Analysis System, RRID:SCR\_008567)



# Distinct Root Microbial Communities in Nature Farming Rice Harbor Bacterial Strains With Plant Growth-Promoting Traits

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A nature farming system is an ecological farming practice that entails cultivating crops without using chemical fertilizers and pesticides. To understand the diversity and functions of root microbiomes associated with nature farming systems, we compared the root microbial community of rice under nature farming conditions with those under conventional farming conditions. High-throughput amplicon analysis demonstrated a higher abundance and greater diversity of the root microbiome under unfertilized nature farming conditions than under conventional conditions. The application of chemical fertilizers reduced the microbial diversity and abundance of some beneficial taxa important for plant growth and health. Subsequently, we isolated and identified 46 endo- and epiphytic bacteria from rice roots grown under nature farming conditions and examined their plant growth-promoting activity. Six potential isolates were selected for plant growth assessment in insoluble P- and K-containing media. Most of the isolates promoted rice growth, and *Pseudomonas koreensis* AEPR1 was able to enhance rice growth significantly in both insoluble P- and K-containing media. Our data indicated that nature farming systems create a distinct root microbiome that is comparatively more diverse and supports plant growth under low-input cultivation practices than under conventional practices. The potential isolates could be exploited as sources with potential applications in sustainable agriculture.

**Keywords:** nature farming, root microbiome, plant growth-promoting bacteria, rice, sustainable agriculture

## INTRODUCTION

The root microbiome plays a key role in plant growth and productivity, making it an important factor in agroecosystems (Edwards et al., 2015; de Vries and Wallenstein, 2017; Wasai and Minamisawa, 2018). Notably, plant root-associated microbiomes have attracted attention in recent years owing to their roles in maintaining plant productivity by contributing to conferring plant biotic and abiotic stress and resilience through various mechanisms (Tkacz and Poole, 2015; Ding et al., 2019). These include plant growth promotion (PGP) through fixation or solubilization of growth-limiting nutrients, growth promotion by the production of plant growth hormones and antagonism of plant pathogens through the production of antibiotics and resource competition.

The utilization of plant growth-promoting microbes may serve as a sustainable alternative to the use of chemical fertilizers (Vejan et al., 2016; Backer et al., 2018). Therefore, understanding how root-associated microbial communities respond to farming management practices is of great agronomic interest.

Conventional farming heavily relies on the use of agrochemicals such as synthetic fertilizer and pesticide to boost agricultural productivity to meet the global crop production demand. The intensification of agrochemical application, however, has led to adverse impacts. These include increases in greenhouse gas emissions, nutrient leaching, soil degradation, loss of plant and soil microbes, increased susceptibility of crops to pests and diseases and loss of biodiversity (Xu et al., 2018; Guo et al., 2019). Concern over these problems has led to the search for alternative practices to ensure more sustainable food production and environmental conservation practices. Of these practices, organic farming is the most adopted in many countries worldwide as demand for sustainability increases (Eyhorn et al., 2019), and this includes “nature farming,” which is the major organic farming practice in Japan. The cultivation practices of nature farming systems are more or less similar or comparable with other organic farming practices conducted in other countries to improve soil biological and chemical properties as well as crop production (Li et al., 2012; Hartmann et al., 2015; Wang et al., 2016). “Nature farming,” proposed by Mokichi Okada (1882–1955) in Japan in 1935, is an ecological farming system based on sound agronomic principles (Xu, 2006). One of the concepts of nature farming systems is to advocate a lack of reliance on the application of agrochemicals in cultivating crops (Okada, 1991, 1993). The farming strategies in nature farming systems to improve plant and soil health include not relying on the application of agrochemicals such as fertilizer and pesticides, reducing soil tillage and plowing, using mulches with crop residue, crop rotation, intercropping and incorporating biofertilizer. These farming systems may enhance the abundance and diversity of plant and soil microbes, maintain soil physicochemical properties and improve the quality and safety of food production (Lenc et al., 2015; Gu et al., 2017; Liao et al., 2019).

Under nature farming systems, plants mostly rely on the root microbiome for plant health and productivity. However, we still have limited knowledge on the effects of nature farming systems on the root microbial community in crop plants. Little is known about the root microbial community associated with nature farming and its plant growth-promoting potential under low-input conditions, and no study has been performed on the root microbial community of naturally farmed rice plants in Japan. A previous study reported that rice fields managed with organic farming practices have a distinct bacterial community composition compared with that of conventionally farmed fields (Suzuki et al., 2019). The root microbial abundance was significantly higher in organically managed crops than in conventionally managed crops (Lenc et al., 2015), and a higher abundance of nutrition-related bacteria was observed (Wang et al., 2016). Organic farming harbors a much more complex root microbial structure than that of conventional farming with highly significant connectivity (Banerjee et al., 2019). The culturable

bacterial and fungal endophytes associated with plants grown under organic farming were reported to be able to promote tomato growth and yield (Xia et al., 2015, 2019).

In the current study, we used high-throughput sequencing of 16S rRNA gene markers to evaluate the root microbial community composition of naturally farmed rice plants. We also aimed to identify culturable bacterial isolates associated with nature farming practices that are likely to have plant growth-promoting traits that might be beneficial to plant growth, health, and yield. This study determined the root microbial community and its plant growth-promoting function in rice plants growing under nature farming conditions in Japan.

## MATERIALS AND METHODS

### Soil Preparation

A nature farming soil sample was collected on 26th November 2019 from the field of Ohito Farm (Shizuoka Prefecture, 35°02' N, 139°00' E, 357 AMSL), which has continuously practiced non-chemical fertilizer and non-pesticide application for 17 years. A conventional farming soil sample was collected on 3rd December 2019 from Fuchu Honmachi Paddy Field (Tokyo, 35°41' N, 139°29' E, 46 AMSL). Soil samples were collected at a depth of 0–15 cm. Subsequently, each of the soil samples (40 L) was divided into two parts to be used as unfertilized and fertilized soil-treated samples. A 1/5000 Wagner pots were filled with 2.5 L of soil, and fertilized soils were mixed with 2.6 g pot<sup>-1</sup> potassium oxide (60% K<sub>2</sub>O), 8.9 g pot<sup>-1</sup> superphosphate (17% P<sub>2</sub>O<sub>5</sub>), and 4.16 g pot<sup>-1</sup> ammonium sulfate (21% NH<sub>4</sub>SO<sub>4</sub>).

### Physicochemical Properties of Soil

Soil pH and electric conductivity (EC) were determined in deionized water (H<sub>2</sub>O) and 1 M KCl at a soil-to-solution ratio of 1:5. Total carbon (TC) and total nitrogen (TN) were quantified using the dry combustion method with an NC analyzer (SUMIGRAPH NC TR22, Sumika Chemical Analysis Service, Ltd., Osaka, Japan). Inorganic N in the soil was promptly extracted from 6 g of soil with 2 M KCl for 30 min. Then, the suspension was centrifuged and filtered through filter paper (Advantec No. 5C; Advantec, Tokyo, Japan). Several drops of 1 mg kg<sup>-1</sup> copper(II) bromide solution were added to the extract and kept at 4°C. Then, N-NH<sub>4</sub> in the extract was analyzed by the modified indophenol blue method (Rhine et al., 1998) using a spectrophotometer (UV-1200; Shimadzu Co., Ltd., Kyoto, Japan), and N-NO<sub>3</sub> in the extract was analyzed by flow injection analysis using a flow-through visible spectrophotometer (S/3250; Soma-Kogaku Co., Ltd., Tokyo, Japan) connected to a double-plunger pump (PE-230; Aqua-lab Co., Ltd., Tokyo, Japan) equipped with a copperized cadmium column to reduce nitrate to nitrite in the sample solutions. Available phosphate (Available-P) was evaluated by the Bray 2 method (Nanzyo, 1997).

### Rice Pot Experiment

We used two rice (*Oryza sativa* L.) cultivars, namely, the Koshihikari and Asahi cultivars. Koshihikari is one of the main cultivars of paddy rice in Japan, and we used this cultivar as a model of a modern variety. The Asahi cultivar is one of the



native varieties separated from the old cultivar Hinode since 1909, and it is optimized under low-nutrient soil conditions (Takahashi and Yoshida, 2008). The rice seeds were surface-sterilized by immersion in 70% ethanol for 30 s, followed by soaking in 5% sodium hypochlorite solution for 20 min and washing five times with sterilized distilled water. Subsequently, the sterilized seeds were soaked in PPM solution for 48 h at 4°C and washed three times with sterilized distilled water. The seeds were incubated for 48 h at 28°C under dark conditions for germination. The germinated seeds were sown directly into prepared pots, with four plants per pot and a total of four replications per experiment. Plants were grown in a controlled-environmental chamber (Koito-tron Koito-industries, Japan) at Tokyo University of Agriculture and Technology and kept at 28°C for 16 h and 25°C for 8 h with 60% humidity for 30 days, and plants were watered with sterilized distilled water.

### DNA Extraction and Amplicon Analysis

DNA extraction was carried out according to Ikeda et al. (2004) with slight modification. Root samples were washed thoroughly under running tap water to remove soil debris. Ten-gram root samples were first lyophilized and ground to a fine powder with liquid nitrogen. Then, 0.3 g of ground root sample was suspended in 350 µl of DNA extraction buffer [0.5 M Tris (pH 8.0), 0.1 M EDTA, 0.1 M NaCl, 2% SDS] and 350 µl of 0.3 M sodium phosphate buffer (pH 8.0) in 2 ml screw-capped tubes. After adding 0.5 g of  $\phi$ 0.1 mm zirconia/silica beads (BioSpec product, Inc., Bartlesville, USA), the tubes were processed for 20 s four times using a FastPrep-24™ instrument (MP Biomedicals, Santa Ana, California, USA). The tubes were centrifuged for 5 min at 13,000 rpm and room temperature. Then, 600 µl of supernatant was collected into a new tube, and 120 µl of 8 M potassium acetate was added. After 5 min of incubation, the tubes were centrifuged for 5 min at 13,000 rpm. The supernatants were transferred into new tubes and mixed with 0.1 volumes of 3 M sodium acetate (pH 5.2) and 0.5 volumes of isopropanol. After incubation for 5 min at room temperature, the tubes were centrifuged at 13,000 rpm at room temperature. The pellets were washed with 500 µl of 70% ethanol twice and suspended in 100 µl of sterilized Milli-Q water. DNA was assessed for quantity and quality by using a Nanodrop 2000 (Thermo Fisher Scientific, USA) and stored at –20°C until sequencing.

Amplicon sequencing of the V3/V4 region was carried out with the MiSeq platform at Bioengineering Lab. Co. (Sagamihara, Japan). The primers V3/V4f\_MIX and V3/V4r\_MIX were used to amplify the bacterial V3/V4 region (341f-805r). The first PCR was prepared in a final volume of 20 µl comprised of DNA template (100 ng) using an AmpliTaq Gold 360 polymerase kit (Thermo Fisher Scientific, USA), and the PCR conditions were set according to the manufacturer's instructions. The PCR products were purified using AMPure XP (Beckman Coulter) and quantified using Synergy H1 (Bio Tek) and the QuantiFlour dsDNA system. A quality check of the libraries was performed using the fragment analyzer and sdDNA 915 reagent kit (Advanced Analytical Technologies). The libraries were pooled together and loaded into an Illumina MiSeq instrument following the manufacturer's instructions (Illumina, San Diego, CA, USA).

The Quantitative Insights into Microbial Ecology (QIIME 2.0) toolkit was used to process the raw high-throughput sequencing data. Then, the high-throughput sequencing data obtained from the 16S amplicon were analyzed using MicrobiomeAnalyst (Dhariwal et al., 2017), and the OTUs were annotated as SILVA labels. The amplicon data were subjected to rarefaction curve, microbial alpha- and beta-diversity and relative abundance analyses using the *ranacapa* and *phyloseq* packages, respectively. Preferential microbial taxa were compared across treatments using linear discriminate analysis (LDA) effect size (LEfSe, LDA score > 4.0). The raw sequencing reads were submitted to DRA/DBJ under accession no. DRA010459.

### Isolation and Characterization of Bacteria From Rice Roots Grown in Nature Farming Soil

For isolation of endophytic and epiphytic bacteria, roots were collected from plants grown on unfertilized nature farming soils. The isolation of epiphytic bacteria was carried out by immersion of 0.5 g root samples into 10 ml of 0.05% Tween 20 solution, and 100 µl of the solution was plated onto NB agar medium (Eiken Chemical Co., Ltd. Tochigi, Japan) for bacteria and incubated at 28°C for 7 days. Next, the roots were surface-sterilized with 70% ethanol for 30 s and 1% sodium hypochlorite for 1 min and washed five times using sterilized distilled water for the isolation of endophytic bacteria. The root tissue was macerated in a sterilized mortar in the presence of 1 ml of 0.85% sodium chloride solution. The extract was serially diluted, and 100 µl of diluted suspension was spread onto NB and PDA media, followed by incubation at 28°C for 7 days. Colonies with different morphologies based on size, shape and color were selected and subcultured on fresh NB agar media to obtain pure cultures. Stock cultures of each isolated strain were preserved in 50% glycerol and kept at –80°C. These strains were routinely grown in NB agar medium as necessary.

The bacterial isolates were identified by targeting 16S rDNA. Colony PCR was conducted by using the primer sets V3V4f 5'-CCTACGGGNGGCWGCAG-3' and V3V4r 5'-GACTACHVGGGTATCTAATCC-3' using GoTaq (Promega). PCR was conducted at 95°C for 3 min and 30 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and 30 s extension at 72°C using the Veriti 96-well thermal cycler (Applied Biosystems; California, USA). After electrophoresis, the bands were cut and purified using a FastGene™ Gel/PCR Extraction kit following the manufacturer's protocol. The DNA concentration and purity were examined using a NanoDrop 2000 UV-Vis spectrophotometer (Thermo Fisher Scientific, USA) and sent for sequencing. The 16S rRNA gene sequences of the bacterial isolates associated with nature farming practices were compared with bacterial sequences in the GenBank database through the BLAST program, and MEGA X version 10.1 was used to estimate the phylogenetic relationship with the obtained sequences. The sequences obtained in this study have also been deposited at the DDBJ under accession number.

## Plant Growth Promotion (PGP) Assays

### Indole-3-Acetic Acid (IAA) Production

For the IAA evaluation, bacterial isolates (adjusted OD<sub>600</sub> = 0.01) cultured in NB medium supplemented with 100 mg L<sup>-1</sup> L-tryptophan were inoculated with bacterial isolates and incubated for 48 h in the dark at 28°C. Bacterial cell suspensions were centrifuged at 10,000 rpm for 15 min, and the concentration of IAA in the supernatant was measured using the Salkowski colorimetric technique (Glickmann and Dessaux, 1995) by measuring absorbance at 530 nm with a microplate reader (Spectra MaxParadigm, molecular device Japan Co., Ltd, Osaka, Japan).

### P- and K-Solubilizing Activities

Five microliters of a bacterial suspension (adjusted OD<sub>600</sub> = 0.01) was spotted onto National Botanical Research Institute's Phosphate (NBRIP) medium (Nautiyal, 1999) containing 0.5% tricalcium phosphate [Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>] and slightly modified Aleksandrov medium (Hu et al., 2006) containing 0.2% sericite mica for insoluble phosphate and potassium solubilization screening, respectively. Plates were incubated at 28°C for 7 d in the dark. The P- and K-solubilizing activities of each isolate were evaluated by measuring the size of the clear zone around the colony and comparing it with previously isolated P- and K-solubilizing isolates, *Paenibacillus rhizosphaerae* (NBRC109635) and *Micrococcus yunnanensis*, respectively.

### Siderophore Production

Five microliters of the bacterial suspension (adjusted OD<sub>600</sub> = 0.01) was spotted onto slightly modified Chrome-azuro S (CAS) medium (Hu, 2011). Plates were incubated at 28°C for 2 d in the dark. The siderophore production activity of each isolate was evaluated by measuring the size of the orange or yellow zone around the colony.

### Effects of Bacterial Isolates on Rice Growth

Seed coats were removed from rice seeds (cv. Nipponbare). The dehusked seeds were surface-sterilized with 70% ethanol for 30 sec, shaken in 5% (w/v) sodium hypochlorite for 10 min and washed five times with sterilized distilled water. The sterilized seeds were incubated for 2 d at 28°C under dark conditions for germination.

Bacterial isolates were grown on NB medium at 28°C for 48 h and washed twice with sterilized distilled water. Bacterial cells were resuspended in sterilized distilled water to a final density of 10<sup>9</sup> CFU mL<sup>-1</sup>. Bacterial suspensions were diluted 10-fold to a final density of 2 × 10<sup>7</sup> CFU mL<sup>-1</sup>. Pregerminated sterilized seeds were soaked in the prepared bacterial suspension for 2 d at 28°C in the dark. *P. rhizosphere* and *M. yunnanensis* isolates were used as positive strains, and isolate KENR28 was used as a negative strain in our study.

Inoculated seeds were transferred into plant boxes (CUL-JAR300; Iwaki, Tokyo, Japan) containing 100 mL of 25% semisolid modified phosphate-deficient Hoagland and 25% semisolid modified potassium-deficient Hoagland medium supplemented with 2 g l<sup>-1</sup> Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub> as the sole P source and 2 g l<sup>-1</sup> sericite mica as the sole K source. Non-inoculated seeds planted on modified P-deficient and K-deficient Hoagland

media acted as negative controls. Five replicates of all controls and inoculated treatments were conducted. The plants were incubated in a plant growth chamber (LPH-240SP; NK system, Osaka, Japan) under 16-h light: 8-h dark conditions at 25°C for 7 days. Growth parameters were analyzed to assess the response of bacteria to inoculation, including plant height, root length, shoot biomass and root biomass.

## Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. Different lowercase letters represent significantly different values ( $p < 0.05$ ).

## RESULTS

### Soil Physicochemical Properties

Soil samples were collected from naturally and conventionally farmed fields and subjected to soil nutrient analysis before the cultivation of crops began. The soil pH of nature farming soil was slightly lower than that of conventional farming soil (Supplementary Table 1). The total carbon and nitrogen contents were observed to be slightly higher in the nature farming soil. Moreover, the nature farming soil had lower soil nitrate nitrogen (NO<sub>3</sub>-N), ammonium nitrogen (NH<sub>4</sub>-N) and available P contents than the conventional farming soil.

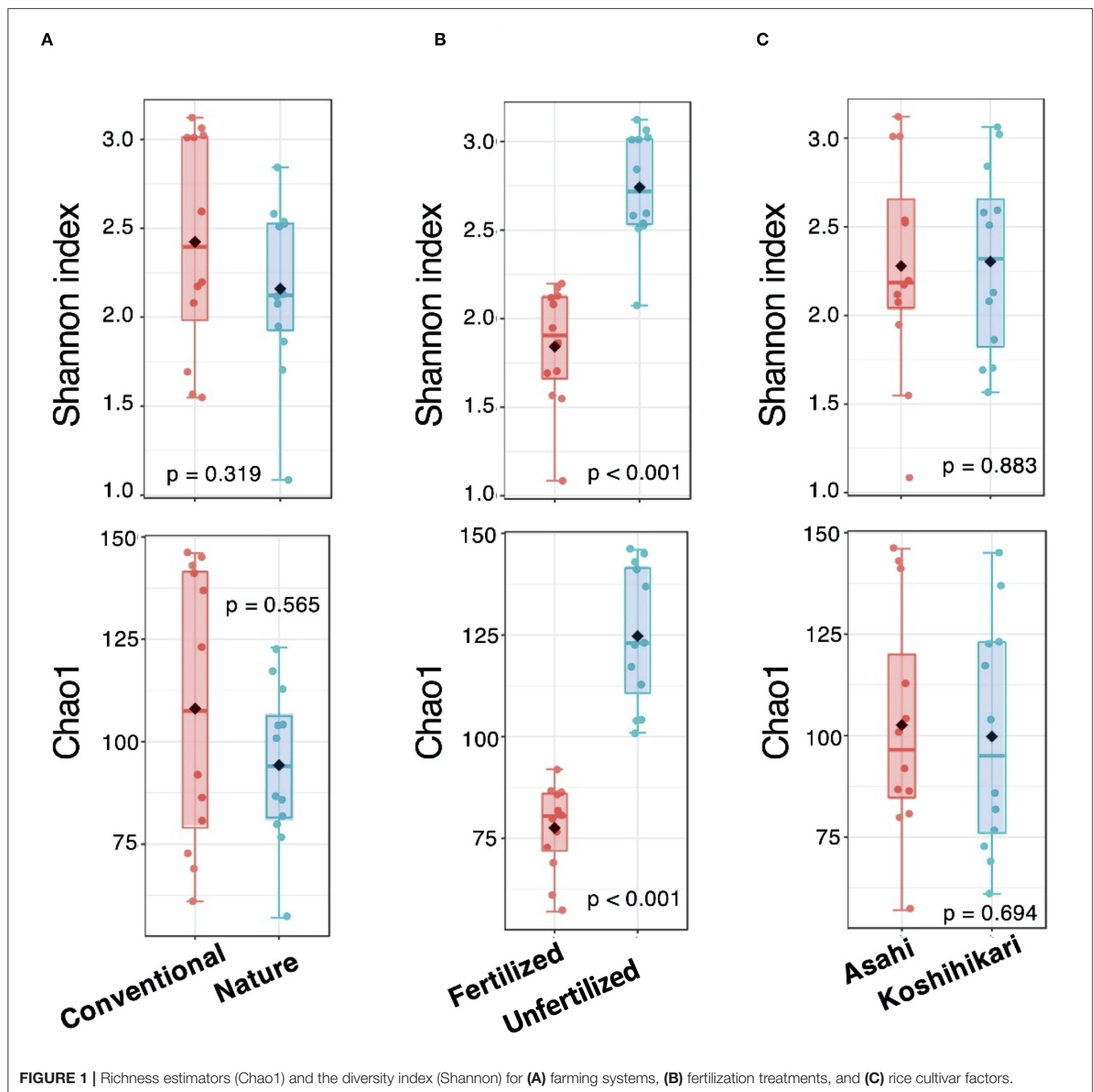
### Diversity of the Root Microbial Community Under Nature Farming and Conventional Farming Conditions With Fertilization Treatments

A total of 1,340,485 16S rRNA sequences were obtained with a median read count per sample of 56,446 (range: 39,586–69,191) obtained from the 24 root samples (two farming systems, two fertilization treatments and two rice cultivars; three replicates each). The reads were clustered into 1,225 microbial OTUs. Rarefaction curves increased steadily as the number of sequences increased (Supplementary Figure 1), indicating that the sample size was sufficient for subsequent data analysis.

### Root Microbial Diversity as a Function of Farming Practice, Fertilization Treatment and Rice Cultivar

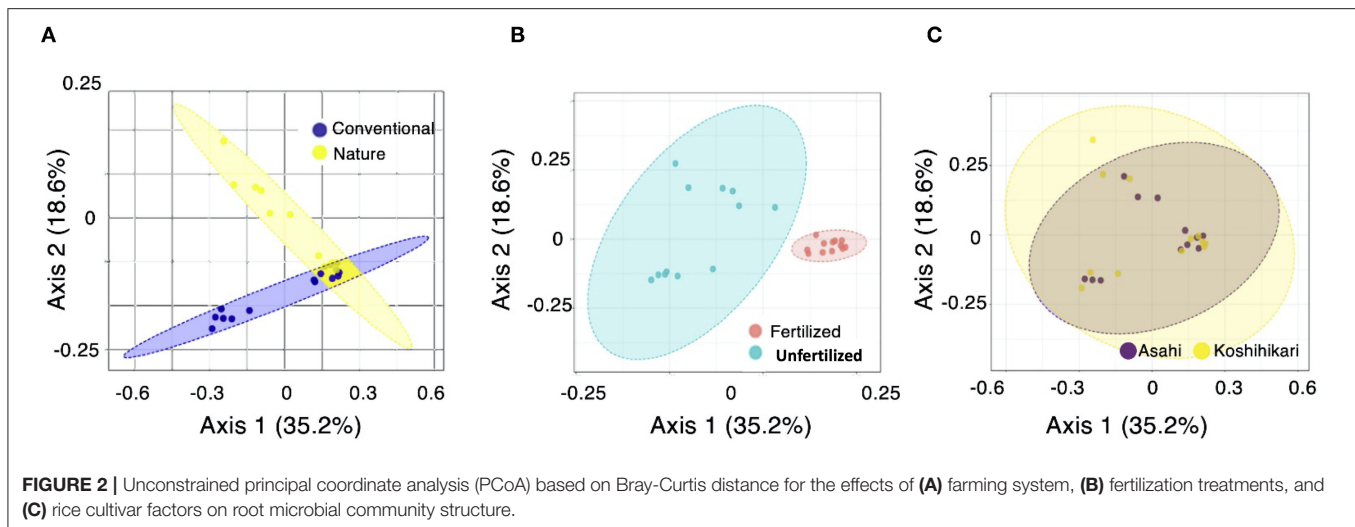
Univariate analysis showed that microbial abundance (Chao1) and diversity (Shannon index) were significantly different ( $p < 0.005$ ) within the fertilization treatments irrespective of farming system and rice cultivar (Figure 1). The fertilized groups had lower microbial abundances and diversity than the unfertilized groups (Figure 1B). However, there was no significant difference in root microbial richness or diversity between the farming systems or rice cultivars (Figures 1A,C).

Unconstrained principal coordinate analysis (PCoA) indicated that the bacterial community clearly clustered based on fertilization treatments, and PERMANOVA revealed that fertilization treatments ( $p < 0.001$ ) led to significant shifts in the bacterial communities in both farming



systems ( $p < 0.004$ ) (Supplementary Table 2). There was a significant effect of farming practice (nature farming vs. conventional farming) on the root microbial community structure of rice (Figure 2A). Unfertilized nature farming has a distinct root microbial community composition from fertilized conventional farming (Figure 2B). However, the rice cultivars Asahi and Koshihikari showed no significant difference in root microbial community structure (Figure 2C). Our results suggested that the application of chemical fertilizer influenced the root microbial community across the farming systems.

To clarify the root microbial community composition within the two farming systems, the phylum-level dataset was analyzed first. The four most dominant phyla across the treatments were V5, Proteobacteria, Firmicutes and Bacteroidetes (Figure 3A). The application of chemical fertilizer reduced the abundances of Proteobacteria, Firmicutes, Chloroflexi, Spirochaetes and certain other phyla, irrespective of farming system or rice cultivar (Figure 3B). The root microbial community composition within the farming systems (Figure 3C) and rice cultivars (Figure 3D) showed no significant difference in their relative abundance of phyla.



## Root Microbial Community Composition Between Unfertilized Nature Farming and Fertilized Conventional Farming Soils

The unfertilized nature farming soil showed a distinctly different root microbial community structure than that of fertilized conventional farming soil, which was significantly influenced by the application of chemical fertilizer. The microbial community composition analysis between the unfertilized nature farming and fertilized conventional farming soils, irrespective of rice cultivar, revealed that the abundances of Proteobacteria, Firmicutes, Chloroflexi and Spirochaetes were lower under fertilized conventional farming conditions (Figure 4A). This was supported by LEfSe analysis (LDA score > 4.0), which showed that the unfertilized nature farming soil had higher numbers of differential taxa than the fertilized conventional farming soil (Figure 4B). The relative abundances of the OTUs of uncultured bacteria (LDA score = 5.32) were observed to be the highest under unfertilized nature farming conditions. This was followed by the genera *Anaromyxobacter*, *Haliangium*, *Sideroxydans*, *Methylobacter*, *Candidatus Methylospira*, *Bradyrhizobium*, *Piscinibacter*, *Curvibacter*, and *Acidibacter*. Notably, the preferential taxa under fertilized conventional farming conditions were *Allorhizobium*, *Asticcacaulis*, *Dyella*, *Thermomonas*, *Massilia*, *Devosia*, *Azospirillum*, and *Magnetospirillum*.

## Identification of Bacterial Isolates Associated With Rice Roots Grown Under Nature Farming Conditions

A total of 46 bacteria were isolated from rice roots grown in the nature farming soil. Based on 16S rRNA identification, the isolates were categorized into 16 different genera and grouped into six groups according to the phylogenetic tree (Figure 5): G1, *Pseudomonas*; G2, *Enterobacter*, *Kosakonia*, *Flavobacterium*, *Klebsiella*, *Aeromonas* and *Leclercia*; G3, *Achromobacter*; G4, *Sinorhizobium* and *Brevundimonas*, G5, *Microbacterium*; and

G6, *Bacillus*, *Paenibacillus*, *Lysinibacillus*, *Ureibacillus* and *Brevibacillus*. Of the 46 isolates, 27 isolates were endophytic bacteria and 19 isolates were epiphytic bacteria. The dominant genera were *Pseudomonas*, *Bacillus* and *Paenibacillus* at 35, 20, and 11%, respectively.

## Evaluation of Plant Growth-Promoting Traits of Bacterial Isolates Associated With Nature Farming Rice Roots

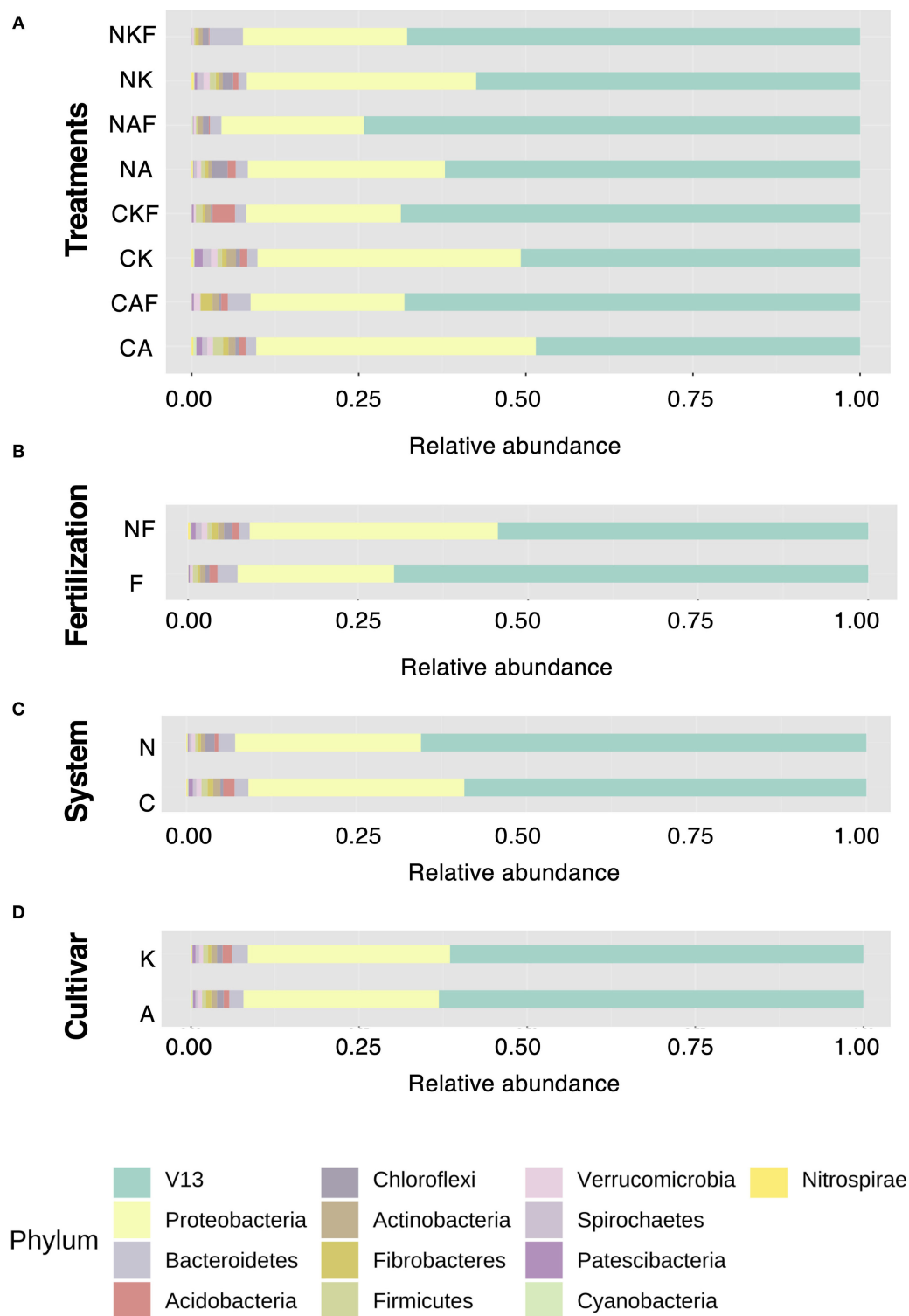
We evaluated plant growth-promoting (PGP) traits, such as IAA production, P solubilization, K solubilization and siderophore production, of the isolates (Table 1). From the IAA production assays, 13 of the isolates exhibited IAA production traits through the observation of pink or red color development. AENR23 and KEPR3, which were closely identified as *B. viscosa* and *L. adecarboxylata*, respectively, showed notable ability to produce IAA according to color change observations.

A total of 23 bacterial isolates were able to solubilize insoluble  $\text{Ca}_3(\text{PO}_4)_2$  based on the visibility of the halo zone formed around the colony. The size of the clear zone varied between 0.7 and 1.4 cm among the isolates (Table 1, Supplementary Figure 2). Most of the isolates showed better phosphate solubilization activity than that of the previously reported phosphate solubilizer bacterium *P. rhizosphere* (0.6 cm). AENR18, AENR22 (*K. cowanii*) and AEPR2 (*F. acidificum*) isolates formed the largest halo zone, with a diameter of 1.4 cm.

From the potassium solubilization assays, 25 isolates exhibited solubilization activity of insoluble sericite mica and showed better activity than that of the previously reported potassium solubilizer bacterium *M. yunnanensis* (0.7 cm) (Table 1, Supplementary Figure 3). The size of the halo zone formed varied between 0.7 and 2.3 cm among the isolates. AEPR2 and AEPR3 isolates, which were closely identified as *F. acidificum* and *E. tabaci*, formed the largest halo zone, with a diameter of 2.3 cm.

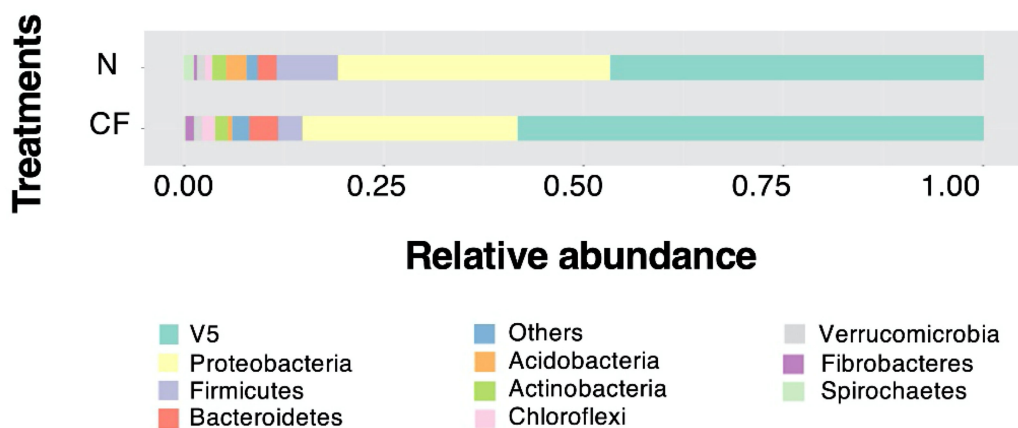
For the siderophore production assay, 22 isolates exhibited siderophore production activity by the formation of a yellow



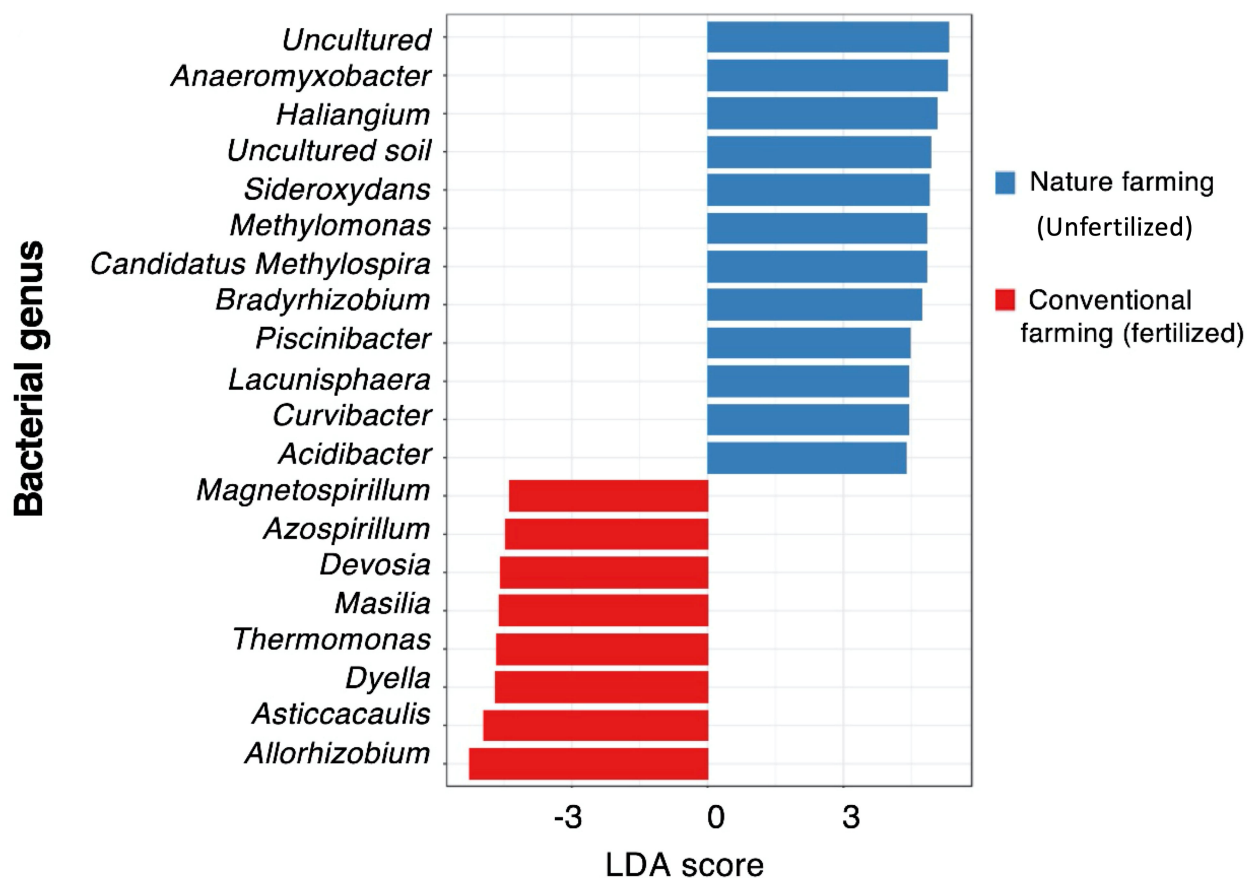


**FIGURE 3 |** Relative abundance of dominant phyla **(A)** across the samples, **(B)** fertilization, **(C)** farming systems, and **(D)** rice cultivars. **(A)** NKF, Koshihikari grown in nature farming soil treated with chemical fertilizer; NK, Koshihikari grown in nature farming soil without chemical fertilizer; NAF, Asahi grown in nature farming soil treated with chemical fertilizer; NA, Asahi grown in nature farming soil without chemical fertilizer; CKF, Koshihikari grown in conventional farming soil treated with chemical fertilizer; CK, Koshihikari grown in conventional farming soil without chemical fertilizer; CAF, Asahi grown in conventional farming soil treated with chemical fertilizer; and CA, Asahi grown in conventional farming soil without chemical fertilizer. **(B)** NF, no application of chemical fertilizers; F, application of chemical fertilizer. **(C)** N, Nature farming soil; C, conventional farming soil. **(D)** K, Koshihikari cultivar; A, Asahi cultivar. V13 refer to taxonomy labels which not assigned or other category cannot be projected to lower taxonomy.

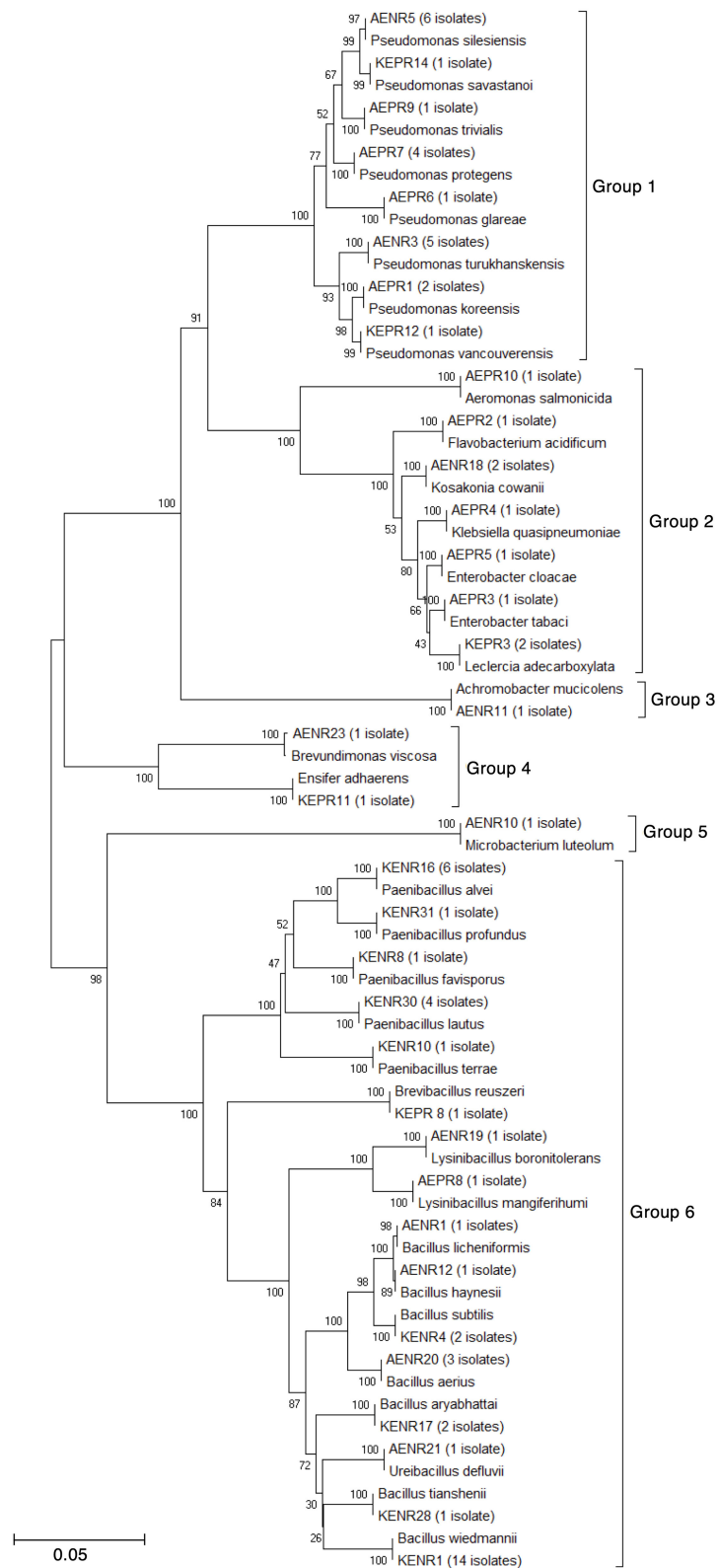
A



B



**FIGURE 4 |** Root microbial community composition between unfertilized nature farming and fertilized conventional farming. **(A)** Relative abundance of dominant phyla under unfertilized nature farming (N) and fertilized conventional farming (CF) conditions. **(B)** Linear discriminate analysis (LDA) of the effect size (LEfSe) of the preferential taxa at the genus level under unfertilized nature farming and fertilized nature farming conditions. Only taxa with LDA > 0.4 are shown. V5 refer to taxonomy labels which not assigned or other category cannot be projected to lower taxonomy.



**FIGURE 5 |** Phylogenetic tree of bacterial isolates associated with rice roots grown under unfertilized nature farming.

**TABLE 1** | Details of isolates, closest relatives, and PGP traits.

Group	Code	Rice cultivar	Type	Closest identification	IAA <sup>a</sup>	P-solubilization <sup>b</sup>	K-solubilization <sup>c</sup>	Siderophore production <sup>d</sup>
Group 1	AENR3	Asahi	Endophyte	<i>Pseudomonas turukhanskensis</i>	-	0.8 ± 0.05	0.9 ± 0.03	2.0 ± 0.03
	AENR5	Asahi	Endophyte	<i>Pseudomonas silesiensis</i>	+	0	0	0
	AENR7	Asahi	Endophyte	<i>P. silesiensis</i>	-	0	0.8 ± 0.03	1.8 ± 0.06
	AENR16	Asahi	Endophyte	<i>P. silesiensis</i>	-	0	0.7 ± 0.03	1 ± 0.06
	AEPR6	Asahi	Epiphyte	<i>Pseudomonas glareae</i>	-	1.0 ± 0.05	1.9 ± 0.66	1.5 ± 0.23
	AEPR7	Asahi	Epiphyte	<i>Pseudomonas protegens</i>	-	0.7 ± 0.03	1.9 ± 0.15	1.4 ± 0.00
	KEPR6	Koshihikari	Epiphyte	<i>P. protegens</i>	-	0.7 ± 0.03	1.4 ± 0.31	1.8 ± 0.05
	KEPR15	Koshihikari	Epiphyte	<i>P. protegens</i>	-	0.9 ± 0.06	1.5 ± 0.23	1.6 ± 0.08
	AEPR1	Asahi	Epiphyte	<i>Pseudomonas koreensis</i>	+	0.8 ± 0.03	1.1 ± 0.06	2.9 ± 0.06
	KEPR7	Koshihikari	Epiphyte	<i>P. koreensis</i>	-	0	0	0
	AEPR9	Asahi	Epiphyte	<i>Pseudomonas trivialis</i>	-	0.8 ± 0.00	1.1 ± 0.13	2.2 ± 0.00
	KEPR12	Koshihikari	Epiphyte	<i>Pseudomonas vancouverensis</i>	+	0	0	0
	KEPR14	Koshihikari	Epiphyte	<i>Pseudomonas savastanoi</i>	-	0.9 ± 0.0	1.6 ± 0.08	1.9 ± 0.03
	KEPR1	Koshihikari	Epiphyte	<i>P. turukhanskensis</i>	-	0.7 ± 0.03	0.8 ± 0.03	2.0 ± 0.00
	KEPR9	Koshihikari	Epiphyte	<i>P. turukhanskensis</i>	-	1.0 ± 0.0	1.3 ± 0.03	1.2 ± 0.57
	KEPR10	Koshihikari	Epiphyte	<i>P. turukhanskensis</i>	-	0.8 ± 0.0	0.8 ± 0.03	2.0 ± 0.0
Group 2	AENR18	Asahi	Endophyte	<i>Koskania cowanii</i>	-	1.4 ± 0.03	1.8 ± 0.3	0
	AENR22	Asahi	Endophyte	<i>K. cowanii</i>	-	1.4 ± 0.05	2.2 ± 0.13	0
	AEPR2	Asahi	Epiphyte	<i>Flavobacterium acidificum</i>	+	1.4 ± 0.03	2.3 ± 0.13	1.2 ± 0.03
	AEPR4	Asahi	Epiphyte	<i>Klebsiella quasipneumoniae</i>	-	1.1 ± 0.03	1.6 ± 0.3	2.3 ± 0.05
	AEPR3	Asahi	Epiphyte	<i>Enterobacter tabaci</i>	-	0.8 ± 0.05	2.3 ± 0.24	2.7 ± 0.03
	AEPR5	Asahi	Epiphyte	<i>Enterobacter cloacae</i>	-	1.1 ± 0.03	1.7 ± 0.43	2.4 ± 0.11
	AEPR10	Asahi	Epiphyte	<i>Aeromonas salmonicida</i>	-	1.1 ± 0.03	1 ± 0.03	1.5 ± 0.03
	KEPR3	Koshihikari	Epiphyte	<i>Leclercia. adecarboxylata</i>	+	1.1 ± 0.03	1.9 ± 0.08	0
Group 3	AENR11	Asahi	Endophyte	<i>Achromobacter mucicolens</i>	+	0	0	0
Group 4	AENR23	Asahi	Endophyte	<i>Brevundimonas viscosa</i>	+	0	0	0
	KEPR11	Koshihikari	Epiphyte	<i>Ensifer adhaerens</i>	-	0.8 ± 0.03	0.8 ± 0.0	1.2 ± 0.0
Group 5	AENR10	Asahi	Endophyte	<i>Microbacterium. luteolum</i>	+	0	0	0
Group 6	AENR1	Asahi	Endophyte	<i>Bacillus licheniformis</i>	-	0.9 ± 0.0	1.2 ± 0.3	1.4 ± 0.03
	AENR12	Asahi	Endophyte	<i>Bacillus haynesii</i>	+	0	0	0
	AENR20	Asahi	Endophyte	<i>Bacillus aerius</i>	-	0	0	0
	AENR21	Asahi	Endophyte	<i>Ureibacillus defluvii</i>	-	0.8 ± 0.05	0.8 ± 0.03	2.1 ± 0.03

(Continued)



TABLE 1 | Continued

Group	Code	Rice cultivar	Type	Closest identification	IAA <sup>a</sup>	P-solubilization <sup>b</sup>	K-solubilization <sup>c</sup>	Siderophore production <sup>d</sup>
	AENR19	Asahi	Endophyte	<i>Lysinibacillus boronitolerans</i>	-	0.9 ± 0.03	0.8 ± 0.06	2.1 ± 0.03
	AEPR8	Asahi	Epiphyte	<i>Lysinibacillus mangiferihumi</i>	+	0	0	0
	KEPR8	Koshihikari	Epiphyte	<i>Brevibacillus reuszeri</i>	-	0	0	0
	KENR1	Koshihikari	Endophyte	<i>Bacillus wiedmannii</i>	-	0	0	0
	KENR11	Koshihikari	Endophyte	<i>B. wiedmannii</i>	-	0	0	0
	KENR14	Koshihikari	Endophyte	<i>B. wiedmannii</i>	-	0	0	0
	KENR4	Koshihikari	Endophyte	<i>Bacillus subtilis</i>	-	0	0	0
	KENR17	Koshihikari	Endophyte	<i>Bacillus aryabhatai</i>	-	0	0	0
	KENR28	Koshihikari	Endophyte	<i>Bacillus tianshenii</i>	-	0	0	0
	KENR30	Koshihikari	Endophyte	<i>Paenibacillus lautus</i>	+	0	0	0
	KENR31	Koshihikari	Endophyte	<i>Paenibacillus profundus</i>	+	0	0	0
	KENR8	Koshihikari	Endophyte	<i>Paenibacillus favisporus</i>	-	0	0	0
	KENR16	Koshihikari	Endophyte	<i>Paenibacillus alvei</i>	+	0	0	0
	KENR10	Koshihikari	Endophyte	<i>Paenibacillus terrae</i>	-	0	0	0
	P+	-	-	<i>Paenibacillus rhizosphere</i>	NA	0.6 ± 0.03	NA	NA
	K+	-	-	<i>Micrococcus yunnanensis</i>	NA	NA	0.7 ± 0.03	NA

<sup>a</sup>IAA: observed based on the formation of pink color.

<sup>b</sup>P-solubilization: Unit represent size of halo zone (cm) caused by dissolution of Tricalcium phosphate.

<sup>c</sup>K-solubilization: Unit represent size of halo zone (cm) caused by dissolution of potassium mineral (sericite mica).

<sup>d</sup>Siderophore production: Unit represent size of yellow zone (cm).

zone around the colony. The size of the yellow zone formed varied between 1.0 and 2.9 cm among the isolates (Table 1, Supplementary Figure 4). AEPR1 (*P. koreensis*) and AEPR3 (*E. tabaci*) isolates recorded the largest yellow zone diameters of 2.9 and 2.7 cm, respectively.

Among the 46 isolates, 35 isolates showed IAA production, P- and K-solubilization and siderophore production abilities in qualitative assays. Twenty isolates exhibited three PGP traits, and the remaining isolates exhibited two or fewer PGP traits. Most of the isolates belonging to G1 (*Pseudomonas* sp.) exhibited more PGP traits than isolates from the other groups. However, only 2 isolates, AEPR1 and AEPR2, which were closely identified as *P. koreensis* and *Flavobacterium acidificum*, respectively, were capable of carrying out all four assays.

## Effects of Bacterial Inoculation on Rice Growth in Low-Nutrient Media

Six potential isolates (AENR22, AEPR1, AEPR2, AEPR6, AEPR7, and KEPR14) were selected for the plant inoculation test based on their PGP activity. The inoculation effects of the selected isolates

were evaluated on rice plants grown in low-nutrient media for 7 days.

Under phosphate-deficient conditions, most of the isolates significantly promoted shoot height and root length (Table 2). Among the isolates, AEPR1 (*P. koreensis*) significantly increased the shoot height and root length by 15.4 and 9.3 cm, respectively. This was followed by KEPR14 (*P. savastanoi*), which also significantly increased the root length (9.3 cm) and shoot biomass (80.2 mg). There was no significant difference in the root biomass of the inoculated and non-inoculated plants. However, most of the inoculated plants showed an increase in root biomass compared with that of the control treatment. Most of the isolates associated with nature farming were able to promote rice growth under p-deficient conditions.

For the potassium-deficient media (Table 2), among the treatments, only AEPR7 (*P. protegens*) significantly increased the shoot height. AEPR7 increased the shoot height by 15.3 cm compared to the control at 13.5 cm. AENR22 significantly decreased the root length to 5.4 cm compared to the control at 8.2 cm. The inoculated plants showed no significant difference

**TABLE 2 |** Effects of selected isolates on rice plants under P-deficient and K-deficient media.

Treatments	Shoot height (cm)	Root length (cm)	Shoot biomass (mg)	Root biomass (mg)
<b>P-deficient media</b>				
AENR22	14.9 ± 1.8 ab	8.8 ± 0.9 ab	72.2 ± 16.9 ab	63.6 ± 8.6 a
AEPR1	15.4 ± 2.0 a	9.3 ± 2.4 a	56.8 ± 2.3 ab	59.6 ± 8.4 a
AEPR2	12.6 ± 1.1 bc	8.2 ± 1.5 ab	64.4 ± 21.5 ab	66.2 ± 11.6 a
AEPR6	12.7 ± 1.6 bc	8.1 ± 0.6 ab	70.2 ± 23.8 ab	60.0 ± 10.6 a
AEPR7	10.4 ± 0.8 cd	7.8 ± 1.1 abc	69.8 ± 4.9 ab	61.2 ± 6.8 a
KEPR14	13.3 ± 3.3 ab	9.3 ± 0.9 a	80.2 ± 22.4 a	72.0 ± 14.3 a
<i>P. rhizosphere</i>	10.3 ± 1.4 cd	7.1 ± 1.3 bc	73.2 ± 19.9 ab	53.4 ± 16.6 a
KENR28	6.9 ± 1.5 e	7.2 ± 0.5 bc	51.2 ± 17.0 b	21.8 ± 13.3 b
Control	9.6 ± 0.4 d	6.2 ± 1.1 c	54.8 ± 8.2 b	58.8 ± 21.2 a
<b>K-deficient media</b>				
AENR22	12.7 ± 1.3 d	5.4 ± 0.4 b	50.6 ± 11.8 b	21.2 ± 7.4 a
AEPR1	15.1 ± 0.7 ab	10.6 ± 1.1 a	66.6 ± 10.5 a	20.0 ± 5.9 a
AEPR2	14.6 ± 0.6 abc	8.5 ± 3.8 a	61.6 ± 4.3 ab	20.8 ± 2.4 a
AEPR6	14.7 ± 1.2 abc	9.9 ± 2.1 a	50.6 ± 12.8 b	23.6 ± 8.4 a
AEPR7	15.3 ± 1.0 a	8.5 ± 2.8 a	55.6 ± 6.6 ab	21.2 ± 4.2 a
KEPR14	13.2 ± 1.7 cd	9.0 ± 2.3 a	55.2 ± 8.7 ab	17.8 ± 9.0 a
<i>M. yunnanensis</i>	14.6 ± 1.0 abc	9.7 ± 0.7 a	61.8 ± 6.4 ab	19.2 ± 2.8 a
KENR28	11.9 ± 0.5 d	5.0 ± 0.9 b	50.0 ± 6.2 b	16.2 ± 3.3 a
Control	13.5 ± 1.3 bcd	8.2 ± 1.6 a	54.4 ± 9.0 ab	18.8 ± 5.2 a

in shoot and root biomass compared with the control treatment. Among all the tested isolates, AEPR1, which was closely identified as *P. koreensis*, was able to promote rice seedling growth on both phosphorus- and potassium-limited media. Plants inoculated with isolate KENR28 showed the least activity in promoting rice growth compared to other tested isolates associated with nature farming because of its lower phosphorus and potassium solubilization activity.

## DISCUSSION

Root microbiomes host a large variety of bacteria, many of which are associated with the host plant and enhance plant nutrition, stress tolerance or health. Previous studies have focused on soil microbial communities under organically or naturally managed farms (Li F. et al., 2017; Liao et al., 2018, 2019), but a limited number of studies have reported on plant-associated microbes. Elucidating whether farming practices, such as nature farming vs. conventional practices that employ chemical fertilizers, can influence root microbial communities is therefore of considerable interest. The present study targeted the root microbial community associated with rice plants cultivated under nature farming practices.

Overall, our results showed that the diversity and structures of the root microbial community were significantly influenced by fertilization practices, in which unfertilized nature farming had distinctly more diverse and abundant root microbial community structures than fertilized conventional farming. We further explored the differential microbial taxa that may possess important ecological functions in corresponding farming

systems. Moreover, we identified bacterial isolates associated with nature farming systems that have PGP traits and are able to promote rice growth under low nutrient conditions. Our results will contribute to the augmentation of the available knowledge on root-associated microbes of rice plants, especially those of nature farming.

## Root Microbial Community Composition Under Different Farming Practices

Root microbial abundance and diversity are fundamental to plant health and productivity. Our results revealed lower microbial richness and diversity under chemical fertilizer application. Huang et al. (2019) reported that long-term application of chemical fertilizers reduced the richness and diversity of soil microbes. On the other hand, organically managed fields had more positive effects on alpha-diversity parameters than conventionally managed fields (Hartmann et al., 2015; Gu et al., 2017; Liao et al., 2019). Xia et al. (2015) reported that the diversity of the endophytic community associated with plants was higher under organic practices than under conventional practices. The microbial diversity difference between these two systems might have differences in the rhizosphere microbiome that were influenced by the presence of chemical fertilizers, which could presumably result in differential bacterial colonization into the plant roots. We suggest that the application of chemical inputs may be the main factor that alters root microbial alpha-diversity.

Our findings demonstrated that farming type led to a significant variation in the root microbiome structure, although the microbial alpha-diversity exhibited relatively low variability,

and this effect could be influenced by the application of inorganic fertilizers. This was in line with previous findings in which long-term fertilization greatly influenced the root microbial community structure, although their microbial alpha-diversity was less impacted by different cropping systems (Hartman et al., 2018; Bai et al., 2020). The variation in microbial community structures may not necessarily influence microbial diversity or richness, as the changes in some taxonomic groups may be compensated by changes in other groups (Hartmann and Widmer, 2006). Edwards et al. (2015) also reported that organic cultivation had a significant separation from eco-farming or conventional cultivation practices and that this effect was exhibited across the rhizosphere compartment consisting of the rhizosphere, rhizoplane and endosphere. Taken together with previous findings, our results showed that avoidance of chemical fertilization was favorable in shaping the root microbial community.

### Root Microbial Community Composition Between Unfertilized Nature Farming and Fertilized Conventional Farming

Our results demonstrated that the application of chemical fertilizer significantly influenced the bacterial community structure. Notably, the unfertilized nature farming soil was enriched for Proteobacteria, Firmicutes, Chloroflexi and Spirochaetes, whereas the fertilized conventional farming soil was enriched in Actinobacteria. Bai et al. (2020) also reported that the phylum Proteobacteria was less diverse in the rhizosphere and root endosphere of walnut with the long-term use of chemical fertilizer, while Actinobacteria was enriched in these regions with fertilization. However, a study by Dai et al. (2018) reported that the abundance of Proteobacteria was significantly increased in response to the addition of chemical fertilizer, which differs from our results.

Furthermore, at the genus level, we observed that the bacterial community structure in the rice roots differed between unfertilized nature farming and fertilized conventional farming soils. LEfSe analysis revealed several taxa to be keystone taxa in unfertilized nature farming and fertilized conventional farming systems, which were linked to special functions. Some of the genera that were more abundant when associated under unfertilized nature farming management play an important role in plant health and development. For example, *Haliangium* produces haliangicin as an antifungal compound that can inhibit the growth of a wide spectrum of fungi (Fudou et al., 2001), and *Bradyrhizobium* is known as PGPR and is able to fix atmospheric nitrogen (Greetatorn et al., 2019). A greater abundance of iron-reducing bacteria, including the *Sideroxydans*, *Acidibacter* and *Anaeromyxobacter* genera, was found under unfertilized nature farming conditions than under conventional farming conditions (Hori et al., 2010; Falagán and Johnson, 2014; Jin et al., 2017). Unfertilized nature farming also enriched the genera belonging to Betaproteobacteria, including *Curvibacter* and *Piscinibacter*. Most Betaproteobacteria have been reported as PGPR (Vacheron et al., 2013; Bruto et al., 2014). It has been reported that the relative abundances of *Curvibacter* and

*Anaeromyxobacter* were greater in unfertilized soils than in fertilized soils (Kumar et al., 2018; Hui et al., 2019). Chen et al. (2020) also reported that the relative abundance of the *Piscinibacter* decreased over a long-term monocropping practice of peanut crops. Our results demonstrated the enrichment of the beneficial bacterial community with unfertilized nature farming. Another interesting result that emerged from this study was that the uncultured bacterial genera (LEfSe score > 4.0) were enriched under unfertilized nature farming, revealing the complexity of the root microbiome under unfertilized nature farming.

Some of the enriched genera under fertilized conventional farming, including *Dyella*, *Thermomonas*, *Massilia*, and *Devosia*, have been previously reported to be found under long-term fertilization conditions (Zhao et al., 2013; Zheng et al., 2017; O'Brien et al., 2018). The genus *Dyella* was reported to play an important role in mineral weathering and the bioremediation of hydrocarbon-contaminated soil (Kong et al., 2013; Zhao et al., 2013). The results reveal that unfertilized nature farming might enrich beneficial microbes responsible for plant growth-promoting activity. Low input management was more favorable than fertilization in shaping the beneficial bacterial community structure to improve plant health and growth, highlighting the vital importance of suitable management strategies.

### Culturable Bacteria Associated With Nature Farming Systems and Their PGP Characteristics

In the present study, we isolated bacterial strains from rice roots grown under a nature farming system and examined their potential as plant growth-promoting rhizobacteria. The present study revealed that the genera *Pseudomonas*, *Bacillus*, and *Paenibacillus* were the most predominant groups isolated from nature farming systems. These genera were previously reported to be the most dominant and most commonly found in various plant studies (Zahid et al., 2015). Previous studies also reported *Pseudomonas* and *Bacillus* as the dominant genera isolated from organic farming (Xia et al., 2015; Armalyte et al., 2019). Rodrigues et al. (2018) reported *Burkholderia*, *Bacillus* and *Rhizobium* as the most dominant genera of diazotrophic bacterial isolated from organically grown sugarcane crops. Xia et al. (2015) also managed to identify *Stenotrophomonas* sp., *Micrococcus* sp., *Denococcus* sp. and *Burkholderia* sp. to be associated with organic farming systems that are not isolated in our study.

Our results also showed that most of the isolates belonging to the *Pseudomonas* genus were able to yield positive results in all the PGP tests conducted. This was in line with a previous study that reported that the production of all PGP traits was higher among *Pseudomonas* genera than among other bacterial groups isolated from organic farming (Sagar et al., 2017). They reported that *Pseudomonas* isolates possessed IAA production; phosphorus solubilization; siderophore, ammonia and hydrogen cyanide production; and ACC deaminase activity. Many studies have reported the potential of *Pseudomonas* as a PGP in other crops (Islam et al., 2014; Sharma et al., 2014; Vacheron et al., 2016; Qessaoui et al., 2019). In our study, *Pseudomonas* isolates showed higher siderophore production activity than those of

other genera. *Pseudomonas* has been previously reported to exhibit siderophore production and antifungal activity against fungal phytopathogens (Sasirekha and Srividya, 2016; Abo-Zaid et al., 2020). Costa et al. (2006) reported that *Pseudomonas* strains isolated from organically managed maize possessed siderophore production activity and antifungal activity against *Ralstonia solanacearum*.

Most of the isolates belonged to the Bacilli group in our study, especially *Bacillus*, *Paenibacillus* and *Lysinibacillus*, which exhibited IAA production activity. The production of IAA by bacteria may aid in plant growth, such as cell elongation and rooting. Our results are in accordance with a previous study that reported that Bacilli possessed the ability to produce IAA (Wagi and Ahmed, 2019). In phosphorus and potassium solubilization assays, the isolate identified as *F. acidificum* exhibited the highest P- and K-solubilizing activity among other isolates. Phosphorus- and potassium-solubilizing bacteria play an important role in converting insoluble phosphate or potassium into their soluble form for plant uptake. Similar findings on the phosphorus solubilizing ability of the *Flavobacterium* group have been reported (Walitang et al., 2017); however, studies on the roles of *Flavobacterium* as a potassium solubilizer are limited. Isolates belonging to the Enterobacteriaceae family, such as *Enterobacter*, *Leclercia*, *Kosakonia*, and *Klebsiella*, were also reported to exhibit phosphorus and potassium solubilization and siderophore production (Chakdar et al., 2018; Pramanik et al., 2018; Bendaha and Belaouni, 2019; Khani et al., 2019). *Klebsiella* sp. isolated from the organic farming system were reported to be efficient at solubilizing phosphate, producing phytohormones and siderophores and inhibiting the mycelial growth of various phytopathogenic fungi (Melo et al., 2016).

## Plant Growth Promotion Effects of Bacterial Isolates Associated With Nature Farming Systems

Based on the PGP assays, we selected six isolates for the plant growth test and evaluated their growth potential on rice plants under low-nutrient media. These isolates positively promoted different growth parameters of rice seedlings. In the plant growth test, AEPR1, which is closely identified as *P. koreensis*, showed a greater increase in rice seedling growth on both deficient media than the other isolates. *P. koreensis* was previously reported to enhance the growth of various crops, including rice, sugarcane and vegetable crops (Li H. B. et al., 2017; Hafez et al., 2019; Kang et al., 2019). Moreover, the production of biosurfactants by *P. koreensis* suppressed *Pythium ultimum* in tomato and *Phytophthora infestans* in potato (Hultberg et al., 2010a,b). Kaur and Reddy (2013) reported the potential of other *Pseudomonas* sp. isolated from an organic field, including *Pseudomonas plecoglossicida*, which possesses phosphate solubilization abilities and significantly increases plant growth, organic carbon and available P in soils as well as P uptake in plants. The present study suggested the strong potential of these selected bacterial isolates to benefit agriculture, not only through the enhancement of crop growth and yield but also through a reduction in chemical inputs. Further works

are required to determine the antagonistic activity and the effectiveness of these bacteria to promote plant growth under field conditions.

The present study provides information on the impacts of unfertilized nature farming on the diversity, composition and PGP role of the root microbial community, which has the potential to improve crop production in the face of increasing chemical fertilizer use. However, it should be noted that our study has limitations, as we only used one source of nature farming soil and focused on comparing the effects of chemical fertilizer application in nature and conventional farming systems. In this regard, our study might not represent the root microbiomes of other nature farming fields and only provides insight into the root microbiomes influenced by chemical fertilizer but not the effects of other farming practices. Previous studies have shown that geographical location and different nature farming management strategies may have distinct effects on plants and soil microbes (Gardner et al., 2011; Lenc et al., 2015; Liao et al., 2019; Zhang et al., 2019; Mohammad Golam Dastogeer et al., 2020). Our study may provide information on root microbial communities, which could serve as a reference for assessing root microbiomes under different farming practices and geographical locations of nature farming systems. We also did not investigate the fungal communities associated with the roots, but future studies might consider examining the effects of nature farming practices on the fungal communities along with their function as PGPs to better understand the root microbiomes and their characteristics and roles under nature farming. The current study also did not investigate the relationship between soil physiochemical properties and root-associated microbes; thus, future studies might need to examine the roles of root microbes in enhancing soil fertility, which is essential for plant health.

In this study, we highlighted the impacts of agricultural systems and chemical fertilizer application on the root microbial communities. On the basis of root microbial richness and diversity, unfertilized nature farming appeared to have a significant advantage over the fertilized conventional farming and also present a distinct root microbial community structure. We demonstrated that different farming practices significantly changed the relative abundance of Proteobacteria, Firmicutes, Chloroflexi, Spirochaetes, and Actinobacteria. Fertilization appeared to have strong impacts on shifting the richness, diversity and microbial community structure of rice root microbiome, which could be important in plant growth and health. In addition, differential taxa were enriched under unfertilized nature farming, suggesting unfertilized management provides a conducive environment for the enrichment of various bacterial taxa. The enriched taxa in unfertilized nature farming, such as *Haliangium*, *Bradyrhizobium* and iron-reducing bacteria, have previously been reported to improve plant health and development. In contrast, fertilized conventional farming enriched the abundance of genera related to mineral weathering and bioremediation of hydrocarbon contaminated soil. Moreover, we identified 46 end- and epiphytic bacteria associated with rice roots grown under unfertilized nature



farming, with 35 of it possessing PGP traits for such as IAA production, phosphorus- and potassium solubilization, and siderophore production. Among all the tested isolates, *P. koreensis* AEPR1 appeared to have better activity in enhancing the rice seedling growth on both phosphorus- and potassium-limited media.

The current study will help in understanding the diversity, community structure and functions of root microbial communities in nature farming crops. This study further suggests that PGP bacterial isolates associated with nature farming systems can be promising components of integrated plant health and disease management. The development of such beneficial microbial inocula can serve as agro-inputs in nature or organic farming systems of various crops. Taken together, this study indicates that unfertilized nature farming practices beneficially alter the root microbiome and could be regarded as appropriate management for sustainable agricultural production.

## DATA AVAILABILITY STATEMENT

The raw sequencing reads were submitted to DRA/DDBJ under accession no. DRA010459.

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## AUTHOR CONTRIBUTIONS

GS, MY, and SO designed research. GS, MY, and YN performed research. CL, KD, HT, HN, and SD contributed new reagents and analytic tools. GS, MY, CL, and KD analyzed data. GS, MY, KD, and SO wrote the paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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# Genetic and Morphological Diversity of Indigenous *Bradyrhizobium* Nodulating Soybean in Organic and Conventional Family Farming Systems

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Organic farming systems are gaining popularity as agronomically and environmentally sound soil management strategies with potential to enhance soil microbial diversity and fertility, environmental quality and sustainable crop production. This work aimed at understanding the effect of organic and conventional farming on the diversity of soybean nodulating bradyrhizobia species. Field trapping of indigenous soybean *Bradyrhizobium* was done by planting promiscuous soybeans varieties SB16 and SC squire as well as non-promiscuous Gazelle in three organic and three conventional farms in Tharaka-Nithi County of Kenya. After 45 days of growth, 108 nodule isolates were obtained from the soybean nodules and placed into 13 groups based on their morphological characteristics. Genetic diversity was done by polymerase chain reaction (PCR) targeting 16S rDNA gene using universal primers P5-R and P3-F and sequencing was carried out using the same primer. High morphological and genetic diversity of the nodule isolates was observed in organic farms as opposed to conventional farms. There was little or no genetic differentiation between the nodule isolates from the different farms with the highest molecular variation (91.12%) being partitioned within populations as opposed to among populations (8.88%). All the isolates were identified as bradyrhizobia with close evolutionary ties with *Bradyrhizobium japonicum* and *Bradyrhizobium yuanminense*. Organic farming systems favor the proliferation of bradyrhizobia species and therefore a suitable environmentally friendly alternative for enhancing soybean production.

**Keywords:** soybeans, bradyrhizobia, genetic diversity, organic and conventional farming, Kenya

## INTRODUCTION

Human population has risen steadily over time, soils lost fertility and arable land are scarce leading to increased pressure on agricultural systems to provide food for the ever-increasing population. This has led to drastic land-use changes that involve intensive agricultural practices that has affected biodiversity driven ecosystem processes on the local and global scale (Foley et al., 2005).



Most African countries have agriculture as a main component of their economies and organic agriculture plays an important role for not only assuring sustainable food security but also essential for environmental management (Grandi, 2008; Mutoni et al., 2017). Organic farming enhances the population and diversity of beneficial indigenous bradyrhizobia in the soil which in turn boosts soybean production through biological nitrogen fixation (Wongphatcharachai et al., 2015). Therefore, it is crucial to evaluate the effects of organic and conventional farming systems on soil microbial diversity since farming systems may either increase or suppress the diversity and activity of soil microorganisms.

Conventional farming system of soybean involves the use of inorganic fertilizers, herbicides and pesticides and other farm inputs to optimize production. Most of these inputs contribute significantly to environmental degradation (Abou-Shanab et al., 2017) and are not sustainable in smallholder farming systems. Soil nutrient management strategies can enhance soybean production without detrimental effects to the environment (Hellal and Abdelhamid, 2013). Soil microorganisms that inhabit the plant rhizosphere are important as pest biocontrol agents, enhancement of nutrient utilization and promotion of plant growth (Igiehon et al., 2019). Conventional farm management practices have caused soil erosion, increased salinization, pollution due to agrochemicals, degrading effect on the environment, danger to food security; quality and safety, reduced biodiversity and lack of sustainability in agriculture. The adverse effects of prolonged use of inorganic agrochemicals in conventional farming system have led to increased demands to make agricultural systems more sustainable by reforming agricultural practices. These concerns and negative impacts posed by conventional farming gave birth to organic farming.

Organic farming is a system which avoids the use of synthetic inputs but embrace biological system of nutrient mobilization and plant protection. Organic farming system is characterized by strict limitation of chemical fertilizers, herbicides and pesticides, where soil management is through addition of organic materials and other strategies such as crop rotation (IFOAM, 2006). This mode of farming relies more on agro-ecological services than on the use of expensive external inputs. Therefore, compared to conventional system, organic farming is sustainable, cost effective, environmentally friendly, and enhances soil fertility and biodiversity.

Beneficial soil microorganisms are critical in organic farming systems due to their potential to promote soil fertility and maximize on nutrient cycles. Symbiotic bradyrhizobia-legume interaction that results to biological nitrogen fixation (BNF) can be a suitable alternative for improvement of soybean production (Thuita et al., 2012). Inoculation of soybean with effective indigenous or exotic nitrogen fixing bacteria has been touted as the most environmentally friendly way of improving its production (Ulzen et al., 2016; Ndusha et al., 2019). However, the inoculant isolates should be selected based on their effectiveness in fixing nitrogen and their competitiveness. Introduction of exotic inoculant strains may experience indigenous isolates competition in the soil that limits their nitrogen fixing ability leading to little or no realization of their potential (Heerwaarden

et al., 2018). Other than nitrogen fixation, some bradyrhizobia species produce indole-3-acetic acid (IAA) which enhances the survival of soybean crops (Igiehon et al., 2019). Soybean varieties have been reported to express host specificity and therefore, fail to form symbiotic interactions with indigenous bradyrhizobia when introduced in farms for the first time (Thuita et al., 2012). Tropical Glycine cross (TGx) genotypes of soybean that were developed by International Institute of Tropical Agriculture (IITA) are promiscuous and capable of effective symbiotic interaction with indigenous bradyrhizobia communities in African soils (Chibeba et al., 2017). Soils with no previous history of soybean cultivation may not have suitable population of bradyrhizobia for appropriate nodulation of the crop, that poses another challenge to the reliance on indigenous bradyrhizobia isolates for soybean production enhancement (Abbasi et al., 2008).

Farming practices including tillage, use of organic and inorganic fertilizers and cropping patterns adjust the soil physicochemical parameters, which in turn influence the diversity and number of soil microorganisms (Schmidt et al., 2019). Various studies have demonstrated that the diversity of bradyrhizobia in agroecosystems is as a result of interaction between the bradyrhizobia and legume species, other bradyrhizobia as well as biotic and abiotic factors in that ecosystem (Yan et al., 2014). The diversity of soybean nodulating bradyrhizobia in agroecosystems is not only related to the crop genotype and geographical location of the site but also the soil conditions including pH, available phosphorus and organic matter (Yan et al., 2014).

Farmers in Sub-Sahara Africa mainly cultivate traditional crops as the main bulk of their agricultural production. However, non-traditional crops like soybean can be a worthy investment for enhanced agricultural productivity (Chianu et al., 2009). Soybean is one of the most traded legumes in the world accounting for over 84.5% of the traded grain legumes (Varshney et al., 2019). It is a highly traded crop due to its importance, as a major source of vegetable oil and a rich protein source for human food and animal feeds (Abou-Shanab et al., 2017). Kenya requires 120,000 metric tons of soybean grain per year. However, it produces only 2007 metric tons covering the deficit through importation (Murage et al., 2019). Between the years 2013 to 2016, Sub Saharan African countries imported 6.8 million tons of soybean grain of which Kenya imported 18% of this volume. This signifies continually rising demand for soybean in Africa (Muriithi et al., 2016).

As a result of climate change, global crop production is estimated to decline owing to stressful biotic and abiotic factors (Raza et al., 2019). Soybean is a hardy crop that can easily be cultivated in soils varying greatly in texture, fertility and in fluctuating climatic conditions (Cely et al., 2016). Soybean yield losses due to soil deficiencies have been reported. However, they differ depending on the nutrients; with soil N deficiency contributing 10 percent of the yield losses (Hellal and Abdelhamid, 2013).

Soybean is an exotic crop that originated in China and introduced in Kenya in the early 1960s (Abate et al., 2008). In Kenya, the legume is cultivated as a food diet and for

income by poor rural smallholder farmers (Ndungu et al., 2018). However, the growth of soybean in Eastern Kenya by smallholder farmers has not been sustainable in the past one decade (Njeru et al., 2013). Several factors such as nutrient deficiency in soil (Caliskan et al., 2008), inappropriate use of fertilizers (Brahim et al., 2017), and poor soil management practices (Samson et al., 2019) pose a threat to farmers in their cultivation efforts. Although field studies show that organic farming and inoculation tend to increase soybean production; the range of morphological and genetic diversity of indigenous bradyrhizobia that nodulate soybean under the two farming systems in Eastern Kenya has not been tested fully. Moreover, little attention has been focused on the possibility of raising the inoculum potential of indigenous bradyrhizobia by appropriate agricultural management practices; a strategy that would be fundamental in crop production. Knowledge of how indigenous bradyrhizobia strains are affected by agronomic practices is a crucial step toward optimizing utilization of bradyrhizobia in sustainable crop production. Additionally, a better understanding of the diversity of bradyrhizobia strains, their interactions in different agronomic practices and role is important in the development of sustainable agriculture, increased food production, enhanced soil fertility and biodiversity.

In this research, we tested the hypotheses that bradyrhizobia strains that nodulate soybeans under organic and conventional farming are morphologically and genetically diverse. The objectives of this study were to (i) assess the morphological characteristics of soybean nodulating indigenous bradyrhizobia isolated from nodules under organic and conventional farming in Eastern Kenya and (ii) evaluate the genetic diversity of indigenous bradyrhizobia nodulating soybean.

## MATERIALS AND METHODS

### Experimental Site and Soil Characteristics

Field experiments were conducted in six family farming systems in Tharaka-Nithi County, Kenya at 0°19'49.7"S and 37°38'52.9"E, three strictly organic and conventional, respectively. All the organic farms had 3 years after conversion. The organic farmers are trained and certified. The area is in the Upper midland 2 and 3 (UM2 and UM3) agroecological zones and has an altitude of ~1,373 m above sea level. It experiences bimodal rains, which range from 1,200 to 1,400 mm and a mean temperature of 20°C annually. The long rains are from March to June, and the short rains are from October to December. The soils are Humic Nitisols, commonly called the red Kikuyu loams; deep, well weathered, typically acidic, and free draining with a friable clay texture and moderate to high fertility (Jaetzold et al., 2006).

### Soil Sampling and Analysis

Soils samples were collected in September 2015 from the six selected organic and conventionally managed farms. The upper 20 cm of soil was sampled from six different points along, diagonally and across every selected farm prior to the onset of short rain. Samples from each farm were mixed thoroughly to make a homogenous composite sample. The soils were air-dried,

ground and passed through a 2-mm sieve prior to analysis. Soil pH was determined using a pH meter in a prepared soil-water suspension ratio of 1:2.5. The soil organic carbon was determined by Walkley-Black combustion method (Ashworth et al., 2014) while percentage nitrogen was determined following the Kjeldahl method (Hanon K9840 Kjeldahl apparatus) (Vauclare et al., 2013). The available phosphorus (P) and potassium (K) were determined according to Mehlich-3 (M-3) procedures (Furseth et al., 2012).

### Trapping of Bradyrhizobia in the Farms

The farms were plowed and prepared for planting in October 2015 prior to the onset of short rains. Field trapping of indigenous bradyrhizobia was carried out in all the six farms using soybeans obtained from Kenya Seed Company Limited, Nairobi. Three soybean varieties; two promiscuous (SC squire and SB 16) and one non-promiscuous (Gazelle) were planted in the identified organic farms designated as HO, JO and GO as well as in conventionally managed farms designated as HC, JC and GC during October to December short rain season. The experiment was set up in a split plot design with farm management as the main factor and soybean variety as the sub-factor and in triplicate, giving a total of 18 treatments. None of the farms had previous history of bradyrhizobia inoculation or soybean cultivation. Organic farms had no recent history of herbicide, pesticide and inorganic fertilizer application while conventional farms had a long history of herbicide, pesticide and inorganic fertilizer application. Forty-five (45) days after crop emergence, three healthy soybean plants were randomly selected and harvested from each of the treatment for nodulation and shoot dry weight analysis. A total of 54 soybean plants were sampled and cut at the cotyledonary node separating shoots from the roots. The roots were carefully washed with distilled water and nodules from each sampled plant were detached, collected, nodule number per plant determined and packed in sterile sample vials containing cotton wool and silica gel to prevent desiccation. The nodules and shoots were transported to the Microbiology laboratory at Kenyatta University where they were air-dried and dry weight assessed. Nodules were stored at 4°C for bradyrhizobia isolation. At physiological maturity (after 125 days), three plants per plot were randomly selected, manually harvested, pods detached and threshed. The recovered seeds were dried to a constant dry weight and weight recorded in g plant<sup>-1</sup>.

### Bradyrhizobia Isolation, Culture, and Preservation

In the laboratory, undamaged nodules showing red or pink coloration were selected from the preserved nodules. The air-dried intact nodules were placed in sterile distilled water and allowed to imbibe water for 1 h. The nodules were then rinsed with sterile distilled water and immersed in ethanol (95% v/v) for 30 s to reduce the surface tension and remove air bubbles from the tissues. The nodules were then sterilized using 3.8% sodium hypochlorite solution for 4 min and finally rinsed with six changes of sterile double-distilled water (Thuita et al., 2012). After rinsing, 10 nodules from each of the treatment samples were picked and crushed with a sterile glass rod in

a drop of sterile distilled water using a blunt-tipped pair of flame-sterilized forceps. A loopful of the nodule suspension was aseptically streaked onto a Petri dish plate containing Yeast Extract Mannitol Agar (YEMA) media supplemented with 25 µg/ml Congo red and incubated at 28°C in the dark. Daily observations on colony emergence were made after incubation for 5 days and well-isolated colonies were streaked on YEMA plates containing Congo red. The single colonies were identified and purified by sub-culturing on YEMA media supplemented with Congo red (Mungai and Karubiu, 2011).

## Morphological Characterization of the Indigenous Nodule Isolates

After purification, the cultural and biochemical characteristics of the nodule isolates were established through presumptive tests. The indigenous bradyrhizobia isolates were authenticated, confirmed as nodule-forming bacterial strains and grouped based on morphological characteristics (Odee et al., 1997). The nodule isolates were also evaluated for their ability to alkalinize or acidify the media by growing them on YEMA media containing 25 mg/l of bromothymol blue. The Gram reaction of each isolate was determined using standard Gram staining procedures. Bradyrhizobia do not absorb or they absorb little Congo red in the dark. This characteristic was used to identify possible bradyrhizobia colonies after culturing the isolates in YEMA media supplemented with 0.25 g/ml Congo red stain and incubating in the dark (Bala et al., 2002; Chianu et al., 2009). After confirming the purity of each single type of colony, the isolates were maintained as cultures and stored on YEMA slants in McCartney screwed cap bottles at 4°C.

## DNA Extraction and Purification

DNA extraction was done from randomly selected representative nodule isolates in each group using a modified CTAB genomic DNA extraction procedure. Three-day old pure bacteria isolate plate culture colonies were put into 1.5 ml microcentrifuge tubes containing 600 µl of normal saline. The mixture was then vortexed at maximum speed for 1 min followed by centrifugation at 13,000 rpm for 5 min. The liquid phase was then decanted off and the process repeated several times to get rid of all the extracellular polysaccharides. The cell pellets were resuspended through vortexing in 600 µl of CTAB genomic lysis buffer.

The incubation of the samples was carried out in a water bath at 65°C for 1 h. The samples were then centrifuged at 13,000 rpm for 10 min and the liquid phase pipetted into sterilized 1.5 ml micro centrifuge tubes. Six hundred microliters of chilled absolute alcohol were then added and the samples incubated at −20°C in a freezer. This was followed by centrifugation of the samples at 13,000 rpm for 5 min and the liquid phase discarded leaving behind DNA pellets. To clean the DNA pellets, 600 µl of chilled 70% alcohol was added followed by centrifugation of the samples at 13,000 rpm for 3 min. The alcohol was then decanted off gently. After air-drying, the DNA pellets were dissolved in 60 µl of elution buffer (TE). Storage of the DNA was done at −20°C.

DNA yield was quantified employing a NanoDrop® ND-1000 spectrophotometer (Inqaba Biotech, South Africa) using the convention that one absorbance unit at 260 nm equals 50 µg/ml

DNA. The purity of the DNA was judged on the basis of A260:A280 ratio and its integrity and overall quality judged by inspection of the genomic DNA band on the agarose gel. On running the agarose gel stained with SYBR green, DNA was resolved in 1% agarose gel and 80 V for 30 min followed by observation of the DNA in a gel documentation system.

## PCR Amplification of 16S rDNA

Polymerase chain reaction (PCR) was done on the genomic DNA after dilution and standardizing concentration to 50 ng/µl. The PCR machine used was 9800 Fast Thermal Cycler from Applied Biosystems. PCR targeting the 16S rDNA was done using universal primers P3 (forward, >5'-ATTAGATACCCTGGTAGTCC-3') and P5 (reverse, >3'-GGTTACCTTGTTACGACTTC-5'). The PCR reaction mix contained 12.5 µl one Taq 2X mastermix with standard buffer from Biolabs, 1.25 µl of 10 µM primer P3F, 1.25 µl of 10 µM primer P5R, 9.0 µl sterile nuclease free PCR water, and 1.0 µl of sample DNA. After optimization, the PCR conditions were as follows: an initial DNA denaturation at 94°C for 3 min then denaturation at 94°C for 45 s, annealing at 53°C for 45 s, and extension at 72°C for 2 min (35 cycles). The final extension was carried out at 72°C for 5 min. Amplified DNA was held at 4°C.

## Gel Electrophoresis and 16S rDNA Sequencing

The PCR products stained with SYBR-Green were separated by gel electrophoresis in 1.5% agarose in 0.5X TAE buffer at 100 V for 1 h. A 100 bp DNA ladder (thermoscientific) was used to estimate the molecular sizes of the bands. Gel visualization was done using a UV trans-illuminator and photographed using a digital camera. The PCR products were purified using QIAquick® PCR purification Kit following the manufacturer's instructions and run again on an agarose gel to confirm the presence of the product. After purification of the PCR products, Sanger sequencing targeting the 16S rDNA was done using primers P3 (forward) and P5 (reverse) in MacroGen Inc (Netherlands).

## Data Analysis

Qualitative data on the isolate characteristics were coded into numeric characters and then used to cluster the nodule isolates. The cluster analysis based on morphology was done using the neighbor-joining method and Euclidean similarity index using PAST software version 3 (Hammer et al., 2001). Principal component analysis displaying the relationship between the isolates was also done using PAST version 3 and DARwin version 6.0.17 software. Nodule isolate diversity indices were determined using PAST software. Dendrogram on morphological diversity was drawn using PAST software version 3. Redundancy analysis (RDA) was done using Canoco software version 5 to determine the relationship between soil characteristics and nodule isolate diversity.

Sequence editing was done using Bioedit software. Consensus sequences were created using DNA Baser software followed by comparison with the sequences in the NCBI database using Basic Local Alignment Search Tool (BLAST). The consensus sequences



and aligned matching sequences from the NCBI database were then aligned using Muscle in Mega X software, MEGA X (Kumar et al., 2018). Aligned sequences were then used to construct a neighbor-joining phylogenetic tree employing the Maximum Composite Likelihood method (Tamura et al., 2004). Genetic diversity and conversion of sequence data to haplotypes was done using DNA SP6 software. The haplotype data was then used to determine the isolates' genetic differentiation and molecular variance using Arlequin 3.5.2.2 software.

## RESULTS

### Soil Characteristics and Plant Growth Parameters

Generally, the physicochemical properties of the soil varied depending on farm management practice. Interestingly, the organic farm soils had higher nutrient and chemical content compared to conventional farm soils. The soil tested characteristically acidic with conventional farm soils recording lower pH (4.57) than the organic farm soils (5.70). On average, the organic soil contained 2.82% organic carbon (OC), 0.27% available nitrogen (N), 27.73 ppm available phosphorus (P), 1.00 cmol kg<sup>-1</sup> potassium (K) and 4.86% soil organic matter (SOM) which was higher than in conventional soil that had 2.54% OC, 0.22% N, 25.50 ppm P, 0.96 cmol kg<sup>-1</sup> K and 4.38% SOM (Table 1).

Farm management practices had influence of soil acidity, nutrient levels and various soybean growth parameters. Organic farm management practice significantly enhanced soybean nodulation, growth and yield production. Soybean grown in less acidic organic farm soils recorded significantly higher nodule number ( $p < 0.0001$ ), nodule dry weight ( $p < 0.0001$ ), shoot dry weight ( $p < 0.0001$ ) and seed dry weight ( $p = 0.001$ ) compared to these in slightly more acidic conventional farms. No significance difference was noted among the individual farms in terms of nodulation. However, the three organic farms (GO, HO, JO,) recorded higher nodule number and nodule dry weight compared to the three conventional farms (GC, HC, JC). A significant variation was noted in shoot dry weight ( $p = 0.015$ ) and seed dry weight ( $p = 0.037$ ) among the individual organic and conventional farms (Table 2).

### Morphological Characterization of the Indigenous Nodule Isolates

In this study, 108 pure isolates were obtained from the root nodules of soybean grown in the field trapping experiment and placed into 13 groups based on their morphological characteristics. A majority of the isolates (constituting 62%) had colonies visible after 5 days and characterized by alkalinizing YEMA media supplemented with Bromothymol blue (BTB) which turned color from deep green to blue. Minority of the isolates (accounting for 38%) turned BTB medium from deep green to yellow due to production of acid substances in the

medium which is a typical characteristic of rhizobia that show visible growth after only 3 days (Table 3).

In addition, all the isolates absorbed little or no Congo red in the dark. During Gram staining reaction, the isolates obtained had color change that signified they were Gram negative. The isolates were rod shaped with varied colony diameter, i.e. 15.4% formed small colonies ( $\leq 0.5$  mm), while 30.8% had diameters  $\geq 2.5$  mm. The isolates with larger colonies with a diameter ranging between 2 and 4 mm and constituted 53.8% of the isolates showed production of copious extracellular polysaccharides on the growth medium (Table 3). The colony size/diameter was correlated with growth rate. The rhizobial that had visible colonies after 3 days produced large colony sizes, while those that had colonies visible after 5 days tended to produce small or very small sized colonies.

The colonies were shiny, with circular shape while the elevation was convex, domed, or raised. All colonies had entire margins and colony appearance was either opaque or translucent and the color was white, creamy, milky, and watery. The colony texture was either gummy, soft gummy and firm gummy with rod shaped cells (Table 3).

### Morphological Grouping of the Nodule Isolates

The morphological features of the isolates had notable variations and the 108 isolates obtained were placed into 13 groups (GRP) designated as GRPa to GRPm based on their morphological characteristics. The most abundant isolates were in group GRPh accounting for 42.05% of the total isolates while group GRPb followed with 33.28%. Groups GRPl, GRPg, and GRPm had the rarest isolates accounting for 1.07, 0.74, and 0.38%, respectively (Table 4). Organic farms had higher representation in the 13 groups compared to conventional farms. Among the organic farms, farm GO had the highest representation at 85% while farm GC scored the highest (69%) among the conventional farms as derived from Table 4.

A neighbor-joining tree using Euclidian similarity index and principal component analysis using the same similarity index partitioned the isolates into five morphological groups. In the neighbor-joining tree there were five clusters (A, B, C, D, and E). Isolate groups GRPe, GRPk and GRPa partitioned in cluster A. Groups GRPb and GRPh partitioned in cluster B. Group GRPc was independent in cluster C. Groups GRPg, GRPm, GRPf, and GRPj partitioned in cluster D while groups GRPi, GRPl, and GRPd were in cluster E (Figure 1A). The clustering of the groups in the neighbor-joining tree was similar to the clustering of the isolate groups using principal component analysis (Figure 1B). The isolates were partitioned in the PCA and appeared in the four quadrats with the same grouping pattern as the neighbor-joining tree (Figure 1B).

### Diversity of the Nodule Isolates Based on Morphological Characteristics

The number of isolate taxa varied according to the farm management practices. The three organic farms GO, HO, and JO had the highest number of isolate taxa between 10 and 11



**TABLE 1** | Soil characteristics in organic and conventional farms during the experimental period.

Properties	Organic farms				Conventional farms			
	GO	HO	JO	Average	GC	HC	JC	Average
pH	5.89	5.47	5.73	5.70	4.75	4.42	4.54	4.57
OC (%)	2.88	2.73	2.85	2.82	2.63	2.44	2.56	2.54
SOM (%)	4.97	4.71	4.91	4.86	4.53	4.21	4.41	4.38
N (%)	0.28	0.25	0.27	0.27	0.23	0.21	0.22	0.22
K <sup>+</sup> cmol/kg	1.01	0.97	1.01	1.00	0.98	0.94	0.95	0.96
P (ppm)	28.9	26.9	27.4	27.73	25.9	24.9	25.7	25.50

OC, organic Carbon; SOM, soil organic matter; N, Nitrogen; K, Potassium; P, Phosphorus; GO, HO and JO are organic farms, while GC, HC and JC are conventional farms.

**TABLE 2** | Effect of farm management on soybean growth parameters.

	Nodule number (plant <sup>-1</sup> )	Nodule dry weight (g plant <sup>-1</sup> )	Shoot dry weight (g plant <sup>-1</sup> )	Seed dry weight (g plant <sup>-1</sup> )
<b>Farm management</b>				
Organic	7.778 ± 1.081a	0.125 ± 0.011a	3.618 ± 0.175a	5.408 ± 0.242a
Conventional	4.333 ± 0.640b	0.077 ± 0.010b	2.882 ± 0.109b	4.492 ± 0.178b
<b>Farms</b>				
GO	9.111 ± 2.124a	0.137 ± 0.036a	4.159 ± 0.378a	5.890 ± 0.278a
JO	8.222 ± 2.152a	0.129 ± 0.034a	3.477 ± 0.218ab	5.379 ± 0.422ab
HO	6.000 ± 1.404a	0.108 ± 0.025a	3.219 ± 0.222ab	4.956 ± 0.228ab
GC	5.222 ± 1.362a	0.092 ± 0.022a	3.076 ± 0.151b	4.799 ± 0.313ab
JC	4.333 ± 1.000a	0.076 ± 0.011a	2.923 ± 0.205b	4.491 ± 0.291ab
HC	3.444 ± 0.973a	0.065 ± 0.001a	2.636 ± 0.201b	4.186 ± 0.322b
<b>P-Value</b>				
Farm management	<0.0001	<0.0001	<0.0001	0.001
Farm	0.612	0.898	0.015	0.037
Farm Management*farm	0.001	0.124	0.086	<0.001

Means followed by a different letter(s) within the same column differ significantly at  $p < 0.05$  using Tukey's HSD test.

while the conventional farm HC had the lowest number of taxa (6). According to Shannon (H) diversity index all the organic farms had the highest diversity of bradyrhizobia isolates while conventional farm bradyrhizobia isolates had the lowest diversity. Based on Shannon diversity index (H), farm GO had the highest diversity index (1.78) while farm HC had the lowest diversity index of 1.121 (Table 5). The use of Simpson 1-D diversity index revealed that organic farms GO and JO had the highest diversity of bradyrhizobia isolates while conventional farms bradyrhizobia isolates population had the lowest. The organic farm JO had the highest diversity of rhizobia isolates at 0.75 while conventional farms HC had the lowest bradyrhizobia diversity at 0.60 based on Simpson diversity index. The rhizobia diversity evenness also varied according to the farm management practices. The lowest rhizobia diversity evenness was observed in conventional farm JC having a low of 0.4658 while organic farm JO had the highest isolate distribution evenness at 0.5749 (Table 5). The conventional farms (GC, HC, and JC) had the highest species dominance D while the organic farms (GO, HO, and JO) had the lowest species dominance. The highest dominance was observed in conventional farm HC with 0.3998 while organic farm JO had the lowest Dominance-D at 0.249 (Table 5).

A neighbor-joining cluster diagram based on morphological diversity of the nodule isolates partitioned the farms into two

main clusters (A and B). Organic farms GO, HO, and JO clustered together in cluster A while conventional farms HC, JC, and GC clustered together in cluster B (Figure 2).

## Relationship Between Soil Characteristics and Nodule Isolate Diversity

Based on redundancy analysis (RDA) with soil characteristics as the explanatory variable, the soil characteristics accounted for 98.7% of the variation. The variation as explained in the first four axes was 96.96% (Axis 1), 98.13% (Axis 2), 98.72% (Axis 3), and 98.75% (Axis 4). The Eigen values for the first four axes were 0.9696 (Axis 1), 0.0117 (Axis 2), 0.0059 (Axis 3), and 0.0002 (Axis 4) with the first axis having the highest Eigen values. The soil chemical parameters, P, K, organic carbon, soil organic matter, and pH correlated positively with the number of taxa per farm, the Shannon diversity index and Evenness of isolate distribution in the farms. Species dominance had negative correlation with all the measured soil chemical parameters (Figure 3).

## Genomic DNA Extraction and Polymerase Chain Reaction

Using the procedures described in DNA extraction, good concentration, and quality of DNA was realized for all the 38 representative samples. The PCR process targeting 16S rDNA of

**TABLE 3 |** Colony characteristics of indigenous bradyrhizobia isolates obtained from soybean nodules from organic and conventional farms.

Isolate colony characteristic	13 bradyrhizobia nodule isolate groups (GRP)												
	a	b	c	D	e	f	g	h	i	j	K	l	m
Cell shape	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod	rod
Colony shape	cr	cr	cr	cr	cr	cr	cr	cr	cr	cr	cr	cr	cr
Margin	s	s	s	s	s	s	s	s	s	s	S	s	s
Gram stain	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve	-ve
Elevation	rsd	cvx	cvx	rsd	cvx	rsd	dm	cvx	cvx	rsd	cvx	cvx	cvx
BTB reaction	y	b	b	y	b	b	b	y	y	b	y	b	b
Congo Red A	cna	cna	cna	cna	cna	cna	cna	cna	cna	cna	cna	cna	cna
Colony size	4.0	3.5	2.75	3.0	2.0	1.0	0.5	3.5	3.0	1.5	2.5	3.0	0.5
Color	crw	crm	ww	mw	crw	mw	mw	crm	mw	mw	crw	mw	mw
Transparency	t	t	t	t	t	t	o	t	t	t	t	t	o
Texture	sag	sag	sag	sag	sag	sag	fag	sag	sag	sag	fag	sag	fag
Colony nature	shy	shy	shy	shy	shy	shy	shy	shy	shy	shy	shy	shy	shy

cr, circular; s, smooth; -ve, gram negative; rsd, raised; cvx, convex; dm, domed; y, yellow; b, blue; cna, congo red non-absorber; crw, creamy white; mw, milky white; ww, watery white; o, opaque; t, translucent; sag, soft and gummy; fag, firm and gummy; shy, shiny; colony size, colony diameter in mm.

**TABLE 4 |** Percentage abundance of bradyrhizobia isolates obtained from soybean nodules grown in organic and conventional farms.

Isolate morphological group	Percentage of isolates per farm						% isolate per group
	Organic farms			Conventional farms			
	GO	HO	JO	GC	HC	JC	
GRPa	2.62	2.79	3.52	2.66	0.00	2.48	2.34
GRPb	28.63	29.48	28.53	33.98	39.87	39.98	33.28
GRPc	3.75	3.76	3.05	3.56	2.48	2.55	3.28
GRPd	3.59	3.23	4.23	3.29	3.34	2.95	3.43
GRPe	3.64	3.82	3.47	3.31	0.00	3.64	2.97
GRPf	3.32	3.63	4.90	3.12	0.00	0.00	2.49
GRPg	0.00	4.44	0.00	0.00	0.00	0.00	0.74
GRPPh	39.59	39.47	39.26	42.60	48.74	43.66	42.05
GRPi	5.11	5.52	6.44	3.86	0.00	0.00	3.49
GRPj	0.00	3.86	0.00	0.00	2.47	2.44	1.59
GRPk	4.21	0.00	3.51	3.62	3.10	2.30	2.89
GRPl	3.28	0.00	3.09	0.00	0.00	0.00	1.07
GRPm	2.26	0.00	0.00	0.00	0.00	0.00	0.38
TOTALS	100.00	100.00	100.00	100.00	100.00	100.00	100.00

the bradyrhizobia isolates were amplified by using primer P3 and P5; and yielded a single DNA band of ~1,500 bp.

Genetic Diversity

Haplotype diversity was high in all populations. Population in farms GC, GO, HO and JO had high haplotype diversity (Hd) of 1 except population in farm JC (0.985). Nucleotide diversity, pi was lowest (0.711) in farm HO population and highest (0.748) in farm JO population (Table 6).

The nucleotide diversity with Jukes and Cantor correction, Pi (JC) was also highest among farm HO isolates and lowest in farm JC. The analysis showed that the isolates were genetically diverse with bradyrhizobia population from JC farm having the lowest

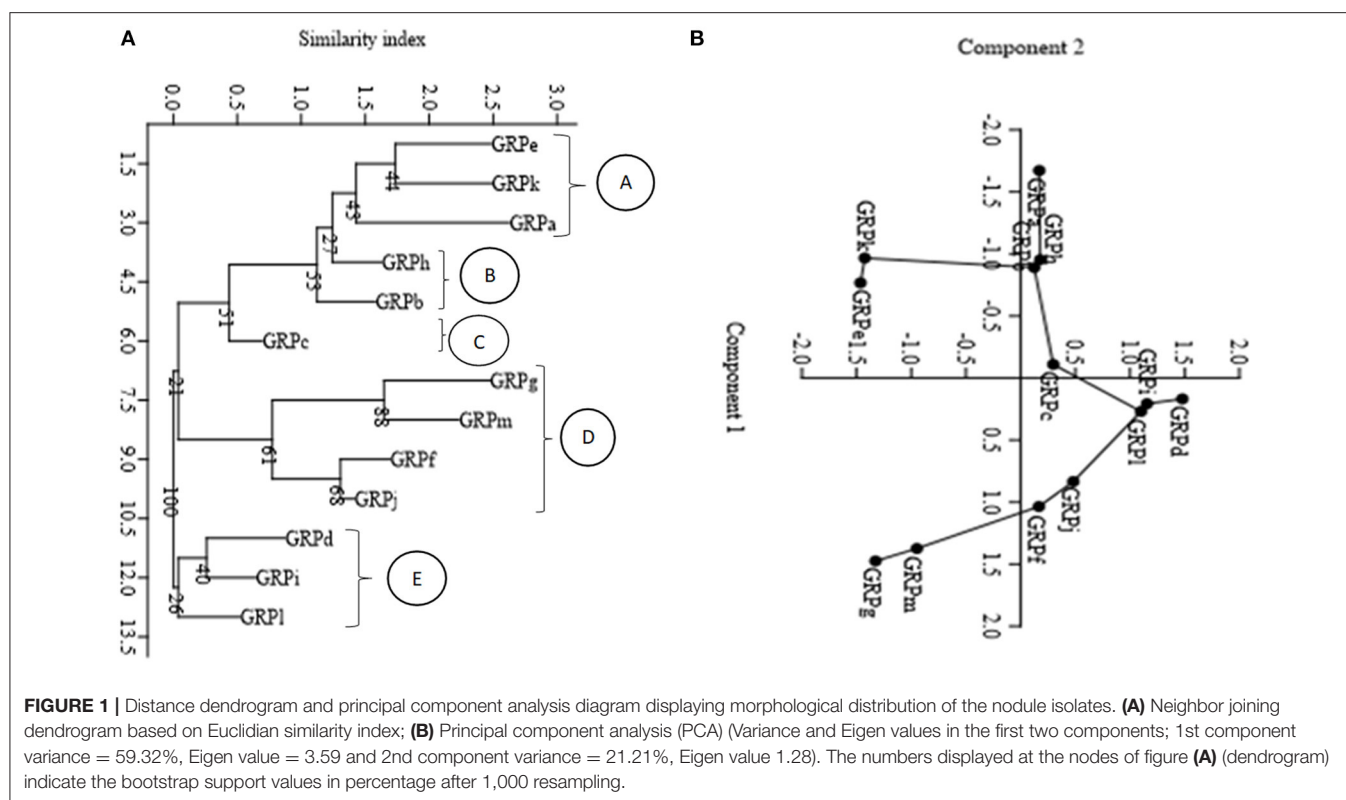
genetic diversity estimate compared to the other farms which recorded the highest diversity (Table 6).

Analysis of Molecular Variance (AMOVA)

Based on analysis of molecular variance, there was significant variation ( $p \leq 0.05$ ) between isolates from the various farms. The highest variation was within populations (91.12%) while the lowest variation was among populations at 8.88% (Table 7).

Genetic Differentiation

Pairwise differentiation analysis demonstrated that there was no significant differentiation ( $p > 0.05$ ) between most of the populations. Pairwise  $F_{ST}$  values for populations in farm GO and



**TABLE 5 |** Diversity indices of bradyrhizobia isolates obtained from soybeans nodules grown in organic and conventional farms.

Diversity Indices	GO	HO	JO	GC	HC	JC
Taxa (S)	11	10	10	9	6	8
Individuals	95	94	96	95	97	95
Dominance (D)	0.2505	0.2552	0.2494	0.3049	0.3998	0.355
Simpson (1-D)	0.7495	0.7448	0.7506	0.6951	0.6002	0.645
Shannon (H)	1.78	1.73	1.749	1.525	1.121	1.316
Evenness ( $e^{H/S}$ )	0.5389	0.5641	0.5749	0.5104	0.5114	0.4658

GC, JO and GC, JO and GO, and JO and JC were all negative (zero). However, a significant differentiation ( $p = 0.018$ ) was observed between populations in farms JC and HO with  $F_{ST}$  value of 0.041 (Table 8).

## Phylogenetic Analysis

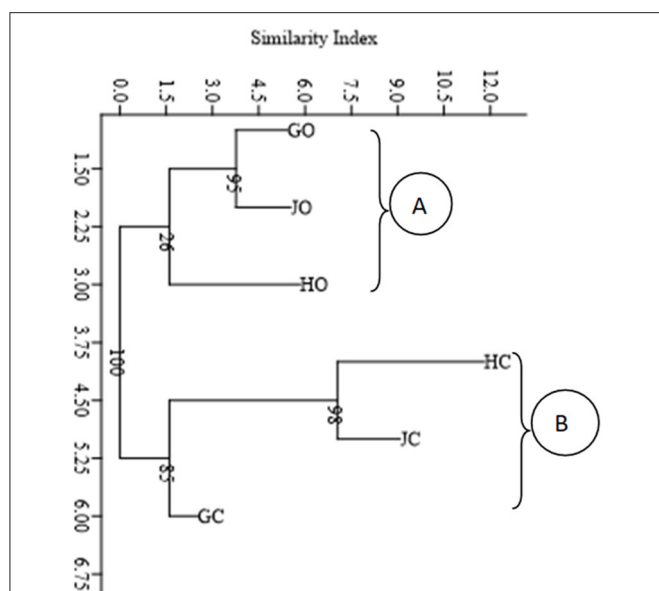
Phylogenetic analysis based on the sequence data clustered the isolates into three clusters (A, B, and C) (Figure 4). Most of the isolates clustered with *Bradyrhizobium* sp. Strain UFLA05-136 in cluster C. Cluster C isolates related closely with cluster A isolates that also had above 98% match with *Bradyrhizobium* sp. Strain UFLA05-136 after BLAST analysis. Cluster B had the least number of isolates and two sub-clusters. In the major sub-cluster in cluster B, supported by 100% bootstrap value, the isolates related closely with isolates *Bradyrhizobium* sp. CCBAU 51511 (KF114656.1:780-1457) and uncultured *Bradyrhizobiaceae* bacterium clone SIF008 (JF733143.1:431-1043) from the NCBI database. In the minor sub-cluster in

cluster B, supported by 95% bootstrap value the isolates clustered with *Bradyrhizobium* sp. strain R-46313 (FR753119.1:737-1104), *Bradyrhizobium yuanmingense* (LC460889.1:726-1013), and *Bradyrhizobium japonicum* PRY65 (AF239848.2:713-1394).

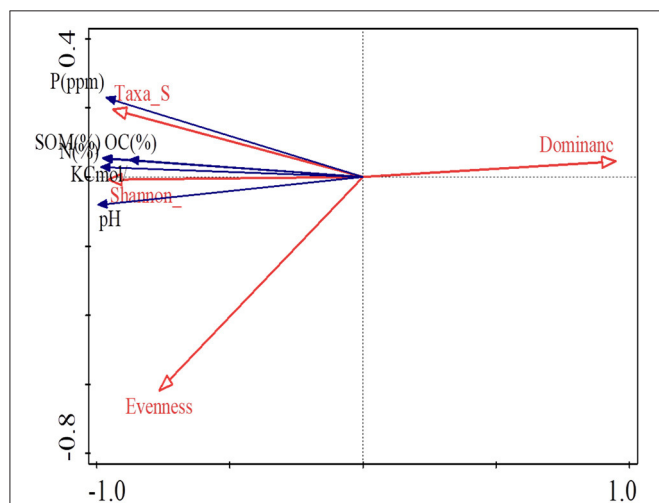
## DISCUSSION

### Soil Characteristics

The conservation of fertile agricultural soils is important for sustainable food production systems and soil nutrient contents can be maintained at an optimum level over time if good farm management practices are adhered to by farmers. The soil properties varied from farm to farm and largely depended on farm management system in practice. The conventional farm soils typically recorded higher acidity and lower fertility levels compared to organic farms; which could be due to extensive application of inorganic fertilizers and other agrochemicals, heavy leaching and runoff. Conventional farming renders the soil



**FIGURE 2 |** Cluster diagram showing morphological distribution of the nodule isolates based on Neighbor joining method and Euclidian similarity index. The numbers shown at the nodes of the dendrogram indicate the percentage bootstrap support value after 1,000 resampling.



**FIGURE 3 |** Redundancy analysis showing the relationship between the soil characteristics and nodule isolate diversity.

more acidic and less fertile due to leaching or salinization (Aktar et al., 2009). The low level of soil fertility in conventional farms as compared to organic farms; could be attributed to acidity, runoff and leaching; following the use of inorganic chemical fertilizers (Oberson et al., 2013; Hunt et al., 2019). The relatively higher acidity of the conventional soil, which is typical of highly weathered East African soils (Okalebo et al., 2002), debilitates soil nitrogen cycling and phosphorous solubility and availability. The results revealed that conventional farming systems rendered the soil less fertile due to increased soil acidity, loss of beneficial soil

microorganisms and increased nutrient leaching. The continuous application of chemical fertilizers as practiced in conventional farming promotes soil acidification, leaching and mineralization of important ions like phosphorus, nitrogen and potassium, which play a vital role in plant growth (Wyngaard et al., 2016).

The less acidic soil in organic farms may be due to application of organic fertilizers and amendments that neutralize the low soil acidity and maintain beneficial soil microorganisms that maintain soil nutrient recycling. The application of animals' manure and compost promote the bioactivity and diversity of soil microbes (Hassink, 1995) that promote nutrient cycling rate and hence enhancing soil properties and plant productivity (Gajda et al., 2000). Organic farming system that involves the application of organic fertilizer, obliterate the use of inorganic chemical fertilizers and other agrochemicals that leads to loss of biodiversity, decline in soil fertility, pollute the environment, costly and unaffordable to most resource constrained smallholder farmers. Therefore, organic farming promotes diversity of bradyrhizobia species compared to conventional system; diverse species that could be responsible for enhanced nitrogen fixation and nutrient solubility that improve levels of soil nutrient content.

## Soybean Growth Parameters

Soil properties varied from farm to farm and this explains the observed differences in various soybean growth parameters such as nodulation, plant biomass and seed yields. Generally, organic farming platform allows good nutrient management practices that are affordable, favor the soil properties, legume growth and sustainable yield production. The organic farms had better soybean performance compared with the conventional farms, which appears to result from the favorable soil conditions for biological nitrogen fixation (Pandey et al., 2017; Schneider et al., 2017). The high acidity of conventionally managed farm soil could have contributed to lower nodulation in the respective farms compared to organic farms with higher soil pH and high nodule number. Conventional farm soils are highly acidic and this hinders the establishment of effective nodulation (Kawaka et al., 2014; Koskey et al., 2017). Soil pH below 5.5 does not favor soybean production due to suppression of nodule formation and symbiotic nitrogen fixation and limits the availability of essential elements like nitrogen and phosphorous (Mairura et al., 2008). The higher seed dry weight revealed among soybean grown in organic farms in the current study resonates with Enrico et al. (2018) who documented increased soybean biomass and seed yield after intensive organic farm management practices.

Non-application of chemical fertilizers in organic farming is a smart strategy of producing organic products that are perceived to be healthier, safer and contain lower levels of agrochemical residues. The growing and unmet consumer demand for healthier organic food is high in markets worldwide and has fueled unprecedented interest in organic farming. Organic farm management practices positively influence soil fertility and biodiversity (Tuomisto et al., 2012), reduces erosion (Seitz et al., 2019) and negative environmental impacts and healthwise, organic foods contain lower levels of agrochemical residues (Smith-Spangler et al., 2012). Therefore, organic



**TABLE 6 |** Genetic diversity of bradyrhizobia isolates obtained from trapping of soybean in organic and conventional farms.

Farm population	Number of sequences	Number of segregating sites, S	Number of haplotypes, h	Haplotype diversity, Hd	Average number of differences, K	Nucleotide diversity, Pi	Nucleotide diversity with JC, Pi JC
GC	11	677	11	1	504.672	0.745	3.062
GO	6	677	6	1	505.93	0.747	3.498
HO	6	676	6	1	482.0	0.711	3.366
JO	3	640	3	1	506.33	0.748	3.521
JC	12	677	11	0.985	490.076	0.724	3.013

**TABLE 7 |** Analysis of molecular variance (AMOVA) of nodule isolates obtained from organic and conventional farms.

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	P-values*
Among populations	5	1094.486	11.67099 Va	8.88	0.03226
Within populations	54	6467.664	119.77156 Vb	91.12	<0.0001
Total	59	7562.150	131.44255		

\*Significance tests based on 1,023 permutations. Significant values at  $\alpha \leq 0.05$ .

farming is gaining popularity and significance due to its beneficial role in producing organic food with lower amounts of chemical residues, enhanced soil fertility, maintenance of environmental quality and increased biodiversity. Profitable organic farm management system is increasing in popularity due to high consumer demand for healthier food, soil fertility improvement, the need for environmental conservation and restoration of soil biodiversity (Bett and Ayieko, 2017). Additionally, organic farming involves the application of locally available, affordable and environmentally friendly organic farm yard manure; that reduce the cost of production and maintain environmental quality; hence promote sustainability in smallholder farming systems.

## Morphological Diversity of the Indigenous Bradyrhizobia Isolates

The morphological diversity of the indigenous bradyrhizobia isolates nodulating soybeans in organic and conventional farms in Eastern Kenya was confirmed by calculating the diversity indices. The morphological diversity estimates calculated showed differences in the nodule isolate diversity in the farms investigated. The nodule isolate diversity indices were higher among organic farming systems as compared to conventional farming systems. According to Liao et al. (2018), different farm management practices affect the structure of the soil which in turn influences the microbial community structure in that agro-ecosystem. Moreover, organic farming systems have been reported to enhance soil organic carbon providing much needed energy for microorganisms and hence enhancing their diversity and abundance unlike conventional farming systems (Liao et al., 2018).

According to Shannon diversity (H), Simpson 1-D and other diversity estimates, organic farms had the highest diversity of bradyrhizobia isolates. Among the different farms, organic farms

GO and JO had the highest rhizobia diversity while conventional farm HC and JC had the lowest diversity. The organic farms with the greatest bradyrhizobia diversity indices also had the highest levels of isolate distribution evenness. Similar findings were observed by César et al. (2015) when working on rhizobia isolates from stem and root nodules of *Discolobium* and *Aeschynomene*. The bradyrhizobia species dominance values were lower in farms with high rhizobia diversity in the present study. This is in agreement with research by Valley et al. (2009) who reported lower dominance values in areas with higher cowpea rhizobia diversity in Fransisco River Valley.

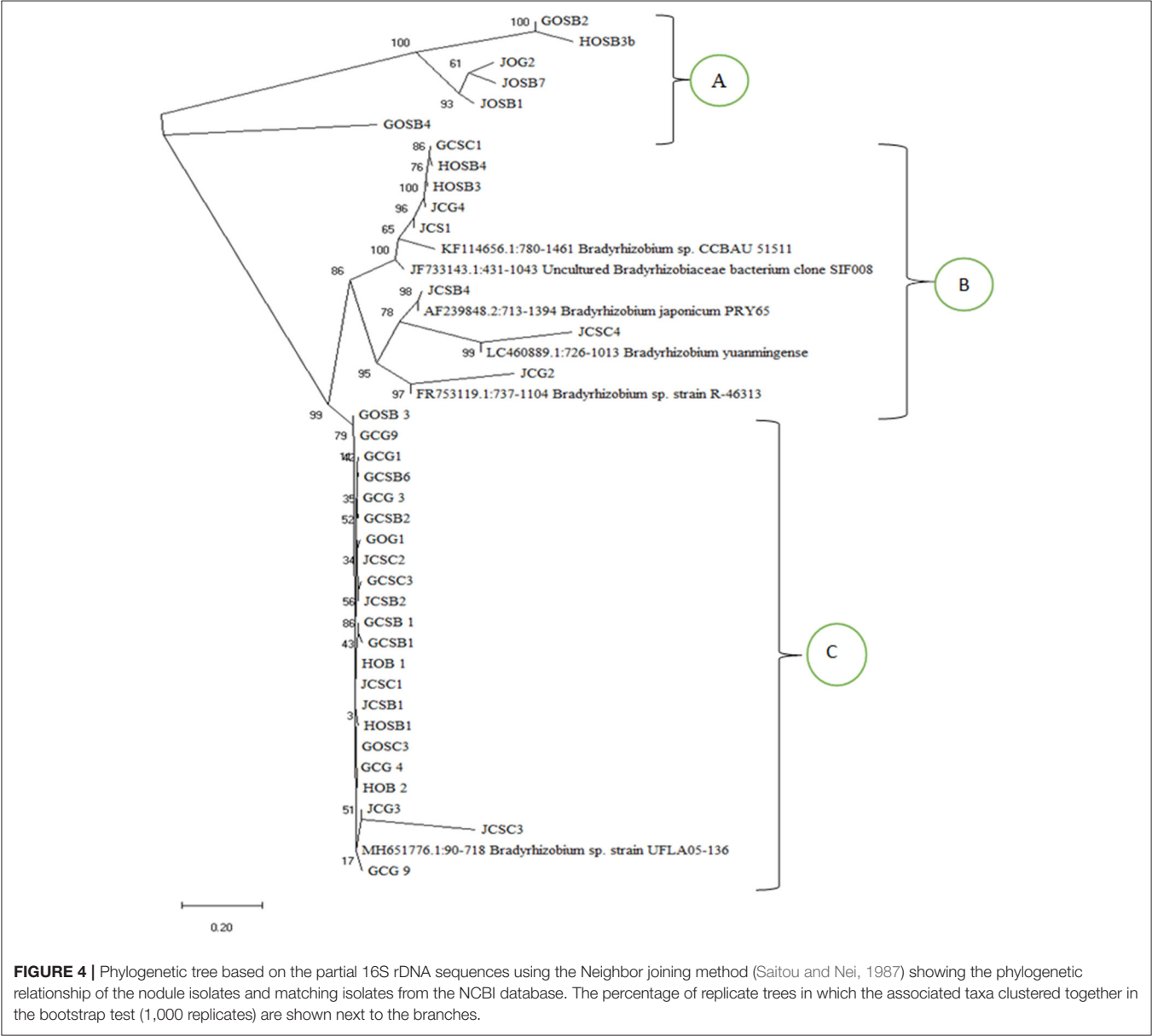
Cluster dendrogram and principal component analysis of the isolates based on their diversity indices clustered organic farms GO, HO and JO isolates together and the conventional farms GC, HC and JC with the lowest diversity of bradyrhizobia isolates in an independent group. This variation could be due to different farm management practices and the characteristics of the farms soil such as pH and nutrient content. The bradyrhizobia diversity variation based on farm management practices has also been documented in previous study findings. Working in both organic and conventional farming systems, Liao et al. (2018) noted similar clustering of microbial diversity based on the farming system. According to Yan et al. (2014), rhizobia diversity results due to different land use histories, different fertilizer supplements, planting of legume or non-legume plants and rotation or monoculture of the host plant. Mason et al. (2018), while working on effect of soil characteristics and flooding on bradyrhizobia diversity in the Philippines also emphasized on the ability of soil management to influence bradyrhizobia diversity and species dominance. Chemical soil fertilization also decreases rhizobia diversity which explains the lower diversity of rhizobia in conventional farming systems (Yan et al., 2014).

Redundancy analysis showed a positive correlation between bradyrhizobia Shannon diversity index and soil properties

**TABLE 8 |** Population pairwise ( $F_{ST}$ ) difference based on the distance method.

	GC	GO	HO	JO	JC
GC	0.00000 (*)				
GO	−0.018 (0.793±0.044) <sup>x</sup>	0.00 (*)			
HO	0.025 (0.063±0.019)	0.021 (0.28±0.043)	0.00 (*)		
JO	−0.030 (0.802±0.029)	−0.005 (0.721±0.033)	0.037 (0.252±0.049)	0.00 (*)	
JC	0.001 (0.487±0.041)	0.002 (0.414±0.049)	0.041 (0.018±0.012)	−0.018 (0.649±0.050)	0.00 (*)

<sup>x</sup>(in parentheses)  $F_{ST}$   $P$ -values based on 110 permutations. Significant values at  $\alpha \leq 0.05$ . \*denotes comparison between same individuals, so cannot give a  $p$  value.



like soil organic matter, Nitrogen, Potassium, Phosphorus, and pH. This is in agreement with the findings by other researchers who have reported positive correlation between rhizobia diversity and soil characteristics (Yan et al., 2014; Li et al., 2016). Yan et al. (2014) also reported positive correlation between soybean bradyrhizobia diversity and soil organic carbon, available potassium and phosphates. The positive correlation between soil organic matter and bradyrhizobia diversity could be due to enhanced soil organic matter, which is a key source of energy for microorganisms and usually a major indicator of the quality of soil (Li et al., 2016). Soil pH has also been reported to be an important component in the management of soybean bradyrhizobia diversity and distribution in the soil (Mason et al., 2018). Alterations in soil pH are believed to influence the bioavailability of soil carbon and nitrogen which are important nutrients for proliferation of microorganisms (Sharma et al., 2013; Han et al., 2019). The positive correlation between the bradyrhizobia diversity and soil nitrogen is contrary to findings by Lin et al. (2019), however it suggests low nitrogen content in the soils hence the need for nitrogen fixing microorganisms. The biological nitrogen fixation is a high energy demanding process that utilizes large amounts of adenosine triphosphate (ATP). This possibly explains the positive correlation between phosphorus and the trapped bradyrhizobia diversity (Han et al., 2019).

### PCR Amplification of 16S rDNA

The representative of the 108 bradyrhizobia isolates obtained from farm trapping experiments were evaluated for their genetic diversity using the 16S rDNA. The amplification of the target 16S rDNA gene resulted to a single band of 1.5 kb in size. Previous results reported that the band size obtained correspond to the expected size due to the use of primers specific to rhizobia (Zhang et al., 2014; Ondieki et al., 2017). The molecular analysis findings demonstrated a high variation in the 38 indigenous representatives isolates which is an indication that the soils from the trapping farms harbor populations of highly diverse bradyrhizobia species. Similar results were reported by Atieno et al. (2012) and Shiro et al. (2013) from other parts of the world.

### Genetic Diversity of the Soybean Nodule Isolates

Haplotype diversity and nucleotide diversity are common measures of genetic diversity of living organisms in a population. There was a high haplotype and nucleotide diversity of bradyrhizobia isolates in all the populations which is in accordance with that of previous research studies. High genetic diversity of rhizobia isolates from Lower Eastern Kenya was also reported by Ondieki et al. (2017) among nodule isolates from cowpea plant hosts. Previous study by Koskey et al. (2018) reported a high genetic diversity of rhizobia isolates from nodules of mid altitude climbing beans planted in Embu and Tharaka-Nithi regions of Eastern Kenya. High genetic diversity of soybean nodulating bradyrhizobia in Kenya was also reported by Wasike et al. (2009) in different soils. The isolates from different farms showed high nucleotide and haplotype diversity. This agrees with findings by Naamala et al. (2016) on high diversity of soybean

nodulating isolates in South Africa based on 16S rDNA analysis. The marginally lower haplotype diversity in JC populations as compared to the other populations could be due the cultivation history in the farm or even the soil characteristics as influenced by different farm management practices at the study farm. This is in line with findings by Appunu et al. (2008) and Stefan et al. (2015) who reported variations in soybean bradyrhizobia haplotype diversity with even higher diversity in areas that had no history of soybean cultivation in various agro ecological zones. The geographical locality also had an effect on all the biodiversity measures including the nucleotide and haplotype diversity that is concordant with findings by Wasike et al. (2009) who reported a similar trend in diversity of soybean nodulating bradyrhizobia from two localities in Kenya.

### Analysis of Molecular Variance (AMOVA)

The analyses of molecular variance (AMOVA) based on amplified 16S rDNA showed that most of the genetic variation of native bradyrhizobia isolates was within the bradyrhizobia populations and not among the populations. Similar findings were reported by Elboutahiri et al. (2015) who observed a larger proportion of significant genetic variation distributed within regions (89%) than among regions (11%) in *Sinorhizobium meliloti* and *Sinorhizobium medicae* obtained from drought and salt affected regions of Morocco. Risal et al. (2010) and Vaclare et al. (2013) recorded high variation of rhizobia isolates within populations and a small variation across different regions.

The AMOVA analysis results demonstrated that there is low level of differentiation and gene flow between the various farms trapping isolates obtained from different regions. Similar observations, however on climbing beans nodulating rhizobia from Embu and Tharaka-Nithi Counties of Kenya were also reported by Koskey et al. (2018). Wu et al. (2011) also reported similar findings on low variation of rhizobia isolates between populations; which increases the probability of the crop host to get a wide variety of the same indigenous nitrogen fixers in any of the farms. Stefan et al. (2015) recorded high variation of clover nodulating rhizobia isolates within populations and a small variation across different ecosystems in Northern Romania. The low differentiation between the populations signifies high gene flow between the populations (Omondi et al., 2010). The low genetic variation across regions also supports the position that geographical distance between populations of particular organisms does not always translate to increased variation and there is high possibility of limited barriers to gene flow between the populations for many years (Degani et al., 2013).

### Genetic Differentiation

A comparison of the analysis of molecular variance and the pairwise difference analysis of the current study showed no significant differentiation between most of the populations. This meant that most of the bradyrhizobia isolates were not secluded based on farm or even distance from where they were isolated (Jiang et al., 2019). In this study, the highest significant differentiation ( $p = 0.018$ ) was observed between populations JC and HO with  $F_{ST}$  value of 0.041. The study results are in line with finding by Koskey et al. (2018) who reported some differentiation

of climbing bean nodulating rhizobia isolates from different sites in Embu and Tharaka-Nithi Counties. Wu et al. (2011) also reported differentiation of soybean nodulating and non-nodulating bacteria from different ecological regions in China. Pairwise  $F_{ST}$  values for populations GO and GC, JO and GC, JO and GO, and JO and JC were all negative (zero); meaning that there was no genetic differentiation between the populations. The results are in agreement with previous study by Appunu et al. (2008) who reported no genetic differentiation between soybean nodulating bradyrhizobia from different ecoclimatic regions in India. According to Krause and Whitaker (2015), most microorganisms obey the clonal ecotype model of natural variation in their genomes due the high possibility of little or no genetic recombination within populations as a result of clonal reproduction in microorganisms. This explains the low gene flow that was observed between the populations.

Phylogenetic analysis based on the sequence data separated the isolates into three clusters. All the isolates from both organic and inorganic farming systems in the three clusters were identified as bradyrhizobia spp. This supports findings by Yan et al. (2014) who reported bradyrhizobia species to be the common micro symbionts of soybean. A majority of the isolates clustered with *Bradyrhizobium japonicum* and *Bradyrhizobium yuanminense*. These results are supported by finding by Wasike et al. (2009) that isolated the two isolates from nodules of promiscuous soybean grown in Mitunguu in Lower Eastern Kenya and therefore the isolates are resident in Kenyan soils. *Bradyrhizobium japonicum* has also been identified as symbiont of soybean in other countries in Africa including South Africa, Ethiopia and Zibambwe (Jaiswal et al., 2019). A big number of the isolates had a similar evolutionary trend with *Bradyrhizobium* sp. Strain UFLA05-136 that is a bradyrhizobia that has not yet been classified and not yet reported in African soils as a common soybean micro symbiont, but was first isolated from *Arachis pintoi* in Brazil.

## CONCLUSION

According to this study, the farm management system influences bradyrhizobia species diversity and abundance; with organic

farming systems enhancing the diversity of bradyrhizobia as compared to conventional farming systems. Eastern Kenya soils have indigenous bradyrhizobia species that can form symbiotic interaction with soybean in both organic and conventional farming systems. There was also no genetic differentiation between the nodule isolates between the organic and conventional farms which signifies the presence of similar bradyrhizobia species across the region. Organic farming system favors the proliferation of bradyrhizobia species and is therefore, a suitable environmentally friendly alternative for enhancing soybean production.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

NG, EN, JM, and RC conceived and designed the research and data collection tools, and participated in drafting the manuscript. NG collected the data and participated in data analyses. EN, JM, and RC supervised the project and data evaluation. All authors read the manuscript and approved the submission.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.606618/full#supplementary-material>

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# Yield and Production Components of Corn Under Straw of Marandu Palisade Grass Inoculated With *Azospirillum brasilense* in the Low-Land Cerrado

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The use of plant growth-promoting bacteria combined with Integrated Agricultural Production Systems (IAPS) has resulted in productivity increases in the Brazilian Cerrado region. Thus, the objective was to evaluate the effect of inoculating or not inoculating *Azospirillum brasilense* on corn and palisade grass seeds (*Urochloa brizantha* cv. Marandu) on growth, yield components, and dry matter yield of the aerial part and grains in an Oxisol cropped in ICLS and under a no-tillage system for 12 years in an irrigated area. The experimental design was a randomized block design with four replications. The treatments were composed of corn (monoculture) with inoculated seed, corn (monoculture) without inoculated seed, corn + palisade grass (intercropping) without inoculation, corn + palisade grass (intercropping) with inoculation in both seeds, corn + palisade grass (intercropping) with inoculation of corn seeds, and corn + palisade grass (intercropping) with inoculation of grass seeds. At the end of each production cycle, the yield and components of corn, corn straw biomass, and dry matter of palisade grass were evaluated. Considering the optimal conditions of soil provided by liming and fertilization at sowing and nitrogen applied in topdressing, the application of *A. brasilense* in corn seeds did not show its potential. Intercropped systems inoculated or not with *A. brasilense* on corn seeds increase grain yield, dry matter production of grass, and nutrient accumulation in the straw, providing greater sustainability to the Cerrado no-tillage system.

**Keywords:** intercropping, no-tillage system, plant growth-promoting bacteria, *Urochloa brizantha*, *Zea mays*

## INTRODUCTION

The intensification of land use for agricultural practices in the Cerrado region is increasing. However, inadequate soil management in this region has led to land degradation, with a consequent reduction in crop yields (Costa et al., 2015). In addition to degradation, the use of species not fitted to local conditions, low plant establishment before grazing, and soil fertility losses due to



excessive nutrient extraction by the animal, among other factors, contribute to the degradation of the pastures (Hungria et al., 2016).

Incrementing competitiveness in the agricultural sector through the use of intensive crops as a means of supplying the food demand of developing countries has resulted in the need to replace the extensive traditional production model with systems that enable the maximization of land use as a no-tillage system (NTS) and integrated crop-livestock system (ICLS) (Pariz et al., 2020), as issues related to mitigation of CO<sub>2</sub> emissions, water consumption, and overuse of fertilizers have become a worldwide concern (Hu et al., 2016).

However, the accumulation and maintenance of the straw in tropical regions such as the Cerrado are complicated practices due to the high temperatures and dry winter (Cavalli et al., 2018), which favor the rapid decomposition of these residues. However, mulching practices are especially important in these regions. The use of species such as palisade grass and guinea grass intercropping with grains cultures did not cause reductions in kernels' attributes and yield. Instead of this, these grass species have deep root systems that can maintain soil moisture, cycle nutrients, produce biomass, and forage for animal grazing in the off-season (Costa et al., 2020; Mateus et al., 2020). In addition, this system can reduce water losses due to evapotranspiration, as the soil is more protected against the incidence of sun and wind.

With the growing concern for maintaining natural resources and reducing possible losses of fertilizers, especially nitrogen, which are the most demanded by plants, there is a need to search for alternatives that aim to increase their efficiency or meet their need via biological N<sub>2</sub> fixation (Yan et al., 2014), aiming at agricultural and environmental sustainability.

In this sense, studies using plant growth-promoting bacteria (PGPB), such as those of the genus *Azospirillum*, aimed at reducing or even suppressing mineral fertilizers without impairing production, are necessary. Inoculation with certain strains of *Azospirillum* sp. fitted to edaphoclimatic conditions under field conditions provides greater root development, resulting in greater soil exploitation for water and nutrient absorption. Furthermore, inoculation with these PGPB provides a direct contribution to increase crop yields and enhances the effectiveness of resource production with extensive environmental benefits (Santos et al., 2019).

The genus *Azospirillum* is the most utilized for corn crop worldwide and comprises 21 species (DSMZ—Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, 2019). The strains of *A. brasilense* Ab-V5 and AB-V6 were released for use in commercial inoculants in maize and wheat in Brazil in 2009 since those strains resulted in increases in grain yield and greater absorption of nutrients (Hungria et al., 2010; Santos et al., 2019). In addition to its ability, PGPB can promote other strategies such as osmotic adjustment, maintenance of roots viability, membrane stability, and accumulation of protein and other metabolites such as proline, which helps in the maintenance of plant metabolism under drought stress (Ngumbi and Kloepper, 2016; Fukami et al., 2018).

However, it is noteworthy that this management does not always replace the use of nitrogen fertilizers and, in some cases,

does not allow the reduction of doses to obtain the highest grain yield (Silva et al., 2015). In this context, it was hypothesized that the corn-palisade grass intercropping and the use of PGPB applied in seeds increase grain yield in corn and grass dry matter productivity under irrigated conditions in the low-land Cerrado without reducing the yield of the main crop.

The objective of this study was to evaluate the effect of inoculating or not with *Azospirillum brasilense* on corn and grass seeds under monoculture or intercropping with palisade grass (*Urochloa brizantha* cv Marandu) on growth, production components, and yield of dry matter of the aerial part and grains.

## MATERIALS AND METHODS

### Site Description

The study was composed of two experiments, repeated by two growing seasons (2014/2015 and 2015/2016), carried out in the same area irrigated by a sprinkler (center-pivot) in Central-West Brazil (20° 20'S and "51° 24' 26" W, 370 m above sea level). The climate in this region is Aw, characterized as humid tropical with a rainy season in summer and dry winter, according to Koppen (Unicamp—Centro de Pesquisa Meteorológicas e Climáticas Aplicadas à Agricultura, 2016). The long-term (1956–2013) average annual maximum and minimum temperatures are 31.3 and 18.4°C, respectively. The precipitation rate, maximum and minimum temperatures, and photoperiod of the area of this study were measured (Table 1).

The soil in the experimental area was classified as Oxisol (FAO—Food and Agriculture Organization of the United Nations, 2006), clayey, and had a history of 12 years under the NTS, establishment phase, with annual and semi-perennial crops, where soybean was the previous crop.

Before initiating the study in each year, chemical and physical attributes were determined. For physical attributes, the methods described by EMBRAPA—Empresa Brasileira de Pesquisa Agropecuária (1997) were followed, and for chemical attributes, the methodology of van Raij et al. (2001) was followed. The values in the 0.00- to 0.10-m and 0.10- to 0.20-m layers are shown in Table 2.

The area was irrigated by a sprinkler (center-pivot), considering the optimal water range for the crops under study. The following equation was used to establish available water capacity (AWC):

$$AWC(mm) = [(FC - PWP)/100] \times SD \times ERZD,$$

where FC is the field capacity (%); PWP, permanent wilting point (%); SD, the soil density (kg dm<sup>-3</sup>); and ERZD, the effective root zone depth (m).

Those data were obtained from the soil water retention curve, where FC = 20.25%; PWP = 14.58%; SD = 1.48 and 0.93 kg dm<sup>-3</sup> (2014/2015 and 2015/2016 crops, respectively); and ERZD = 0.20 m. Therefore, the evaluated soil AWC was 16.78 and 11.54 mm for the 2014/2015 and 2015/2016 crops, respectively.

The water supply was transmitted with a flow of 3.3 mm h<sup>-1</sup>. Irrigation was applied every time the maximum crop evapotranspiration (ET<sub>m</sub>) reached 7.43 and 5.11 mm for the

**TABLE 1** | Rainfall, maximum and minimum temperatures, and photoperiod during the study period.

Climate characteristics	Month											
	2014–2015											
	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	Jun.	Jul.	Aug.	Sept.	Oct.
Montly rain mm	129.1	70.4	79.4	170.7	183.4	42.8	98.3	33.3	42.1	21.8	398.7	122.5
Mean max. temp., °C	31.4	32.8	35.5	31.3	31.6	32.4	28.1	27.9	33	33.2	34.3	33.2
Mean min. temp., °C	20.2	21.4	21.8	20.0	20.4	21.1	17.5	16.3	16.2	17.9	19.7	21.5
Photoperiod, h d <sup>-1</sup>	12.9	13.2	13.1	12.7	12.1	11.5	11.0	10.8	10.9	11.4	11.9	12.5
2015–2016												
Montly rain mm	304.7	194.2	211.3	116.0	244.1	94.8	134.9	62.5	3.8	139.7	57.1	100.8
Mean max. temp., °C	33.2	33.1	32.8	34.1	33.1	26.8	28.8	27.1	30.2	31.2	30.3	29.7
Mean min. temp., °C	22.9	22.6	23.1	23.3	21.7	16.0	17.1	14.6	14.5	16.3	16.6	15.9
Photoperiod, h d <sup>-1</sup>	12.9	13.2	13.1	12.7	12.1	11.5	11.0	10.8	10.9	11.4	11.9	12.5
Long-term (50-years) average												
Montly rain mm	146	211	226	178	135	81	59	30	23	23	73	125
Mean max. temp., °C	33.0	33.0	32.0	32.1	32.0	31.1	29.3	28.4	28.8	31.6	31.0	33.0
Mean min. temp., °C	22.0	22.0	20.4	20.5	22.0	17.4	14.9	13.7	13.1	14.8	19.0	21.0
Photoperiod, h d <sup>-1</sup>	12.9	13.2	13.1	12.7	12.1	11.5	11.0	10.8	10.9	11.4	11.9	12.5

**TABLE 2** | Soil physical and chemical attributes at 0.00–0.10 and 0.10–0.20 m depths in 2014/2015 and 2015/2016 crops.

Attributes	Crop			
	2014/2015		2015/2016	
	Depth (m)			
	0.00–0.10	0.10–0.20	0.00–0.10	0.10–0.20
Soil density (kg dm <sup>-3</sup> )	1.47	1.49	0.92	0.94
Macroporosity (m <sup>3</sup> m <sup>-3</sup> )	0.08	0.07	0.10	0.12
Microporosity (m <sup>3</sup> m <sup>-3</sup> )	0.34	0.34	0.35	0.34
Total porosity (m <sup>3</sup> m <sup>-3</sup> )	0.43	0.41	0.45	0.46
pH (CaCl <sub>2</sub> )	4.90	4.81	4.05	4.53
Organic matter (g dm <sup>-3</sup> )	20.05	20.56	26.33	21.08
P <sub>resine</sub> (mg dm <sup>-3</sup> )	25.63	18.76	33.73	24.03
H+Al (mmolc dm <sup>-3</sup> )	35.00	43.96	32.63	44.53
K <sup>+</sup> (mmolc dm <sup>-3</sup> )	4.41	3.21	4.28	3.04
Ca <sup>+2</sup> (mmolc dm <sup>-3</sup> )	30.46	13.88	39.50	19.30
Mg <sup>+2</sup> (mmolc dm <sup>-3</sup> )	24.45	11.75	35.83	18.30
V (%)	62.13	39.81	70.83	48.03

respective crop years (<44.3% AWC). The ET<sub>m</sub> was estimated through the following equation:

$$ET_m(\text{mm day}^{-1}) = K_c \times E_{To},$$

where  $K_c$  is the crop coefficient and  $E_{To}$  is the reference evapotranspiration.

$E_{To}$  was calculated through the following equation:

$$E_{To}(\text{mm day}^{-1}) = K_p \times ECA,$$

where  $K_p$  is the Class A tank coefficient and ECA is the Class A tank evaporation (mm day<sup>-1</sup>). Water evaporation measurement (mm) was obtained daily from a Class A tank. The  $K_p$  was calculated as proposed by Doorenbos and Pruitt (1977) based on the surrounding area, wind speed, and relative air humidity.

## Experimental Design and Treatments

The experimental design was a randomized block with four replications, and six treatments consisted of different combinations of intercropping and monoculture corn and palisade grass, as follows: corn (monoculture) with inoculated seed, corn (monoculture) without inoculated seed, corn + palisade grass (intercropping) without inoculation, corn + palisade grass (intercropping) with inoculation in both seeds, corn + palisade grass (intercropping) with inoculation of corn seeds, and corn + palisade grass (intercropping) with inoculation of grass seeds. Each plot consisted of 3.4 m in width and 20 m in length, totaling 68 m<sup>2</sup>. The usable area in each plot consisted of four central rows, which did not include 1 m at the ends of each plot and two external rows near the edge. In the second year, the plots were allocated at the same location as the first year.

Prior to the implementation of the experiment (October 2014), diazotrophic microorganisms were counted to determine the bacterial population in the number of cells per milliliter, performed by estimating the “Most Likely Number” (MPN) using the MacCrady table in NFB (*A. brasilense*) semi-solid medium according to the methodology described by Döbereiner et al. (1995), obtaining a value of  $9.0 \times 10^8$  g<sup>-1</sup> cells.

## Corn and Palisade Grass

On October 10 of 2014 and 2015, desiccation of the plants in the experimental area was carried out, aiming the eradication of weed and mulching for continuity of the

NTS, using the herbicide glyphosate [isopropylamine salt of N-(phosphonomethyl) glycine] ( $1.44 \text{ g acid-equivalent ha}^{-1}$ ) at a spray volume of  $200 \text{ L ha}^{-1}$ . On November 1 of 2015 and 2016, plants were cut with mechanical management using a plant residue crusher.

Simple hybrid DKB 390 YG corn [*Zea mays* (L.)] was used on the first growing season (2014/15). On the second growing season (2015/16), there was no availability for the same hybrid. Triple hybrid DKB 350 YG was the option for this year. The grass used for this study, in both years, was palisade grass [*Urochloa brizantha* (A. Rich.) Stapf Marandu].

To ameliorate soil acidity and according to the soil analysis, 30 days prior to sowing, dolomite limestone (40% CaO and 12% MgO) with an 85% effective calcium carbonate equivalence (85% PRNT) Dolomitic limestone was applied over the soil surface without incorporation, aiming to increase base saturation to 70% (0.00–0.20 m) as described by Cantarella and Raij (1997).

At sowing of the respective growing season, 2014/15 and 2015/16, the seeds of corn and palisade grass were inoculated or not with the diazotrophic bacterium *A. brasilense* (strains Ab-V5 and Ab-V6). The bacteria come from EMBRAPA Soybean program, selected on Brazil initially for corn and wheat crops (Hungria et al., 2010) at a dose recommended from this mentioned author of  $100 \text{ ml/25 kg}$  seeds. Inoculation occurred before sowing and in the shade.

The first growing season was sown on November 11, and the second one was sown on November 17. For both years, the corn crop was mechanically sown using a no-till drill at a depth of  $\sim 0.05 \text{ m}$  in  $0.45 \text{ m}$  spacing with  $3.0 \text{ seeds m}^{-1}$ , aiming to reach a final stand close to  $60,000 \text{ plants ha}^{-1}$ .

In both cropping years, palisade grass was sown on the same day as corn, with another type of seeder-fertilizer, with an alternating double-disc mechanism compatible with NTS, in which the seeds were placed in the fertilizer compartment in the seeder and deposited at a depth of  $0.06 \text{ m}$ , at a spacing of  $0.17 \text{ m}$ , using  $\sim 7 \text{ kg ha}^{-1}$  of viable pure seeds ( $\text{CV} = 70\%$ ) for palisade grass. Thus, the grass seeds were below the corn seeds, following the recommendations of Kluthcouski et al. (2000), with the objective of delaying the emergence of grass in relation to the grain-producing crop and reducing the likely competition among the species in the initial period of the crop development.

Due to the corn crop sowing, in both crops, respectively, the sowing mineral fertilization was performed in the furrow of the grain crop, according to the results obtained in the sampling for initial soil characterization, where  $300 \text{ kg ha}^{-1}$  of formulated 08-28-16 ( $24 \text{ kg ha}^{-1} \text{ N}$ ,  $84 \text{ kg ha}^{-1} \text{ P}_2\text{O}_5$ , and  $48 \text{ kg ha}^{-1} \text{ K}_2\text{O}$ , respectively) was applied.

At the V6 phenological stage (six fully developed corn leaves), following the recommendations of Cantarella (2007), the topdressing fertilization was carried out by manually applying  $120 \text{ kg ha}^{-1} \text{ N}$ -ammonium sulfate to the corn lines.

## Sampling and Analyses

### Proline

During the corn flowering period, in both crop years, after the emission of female inflorescence, that is, when more than 50% of the plants were clogged, and with the

presence of stigmas style (hair), the middle-thirds of 15 leaves were collected per plot for free proline quantification (01/30/2015 and 01/17/2016);  $0.5 \text{ g}$  of fresh leaf limb tissue was used, which was immediately frozen in liquid nitrogen to cease leaf metabolism. After collection, the material was properly stored in a freezer at temperatures below  $-20^\circ\text{C}$  and analyzed using the methodology described by Bates et al. (1973).

## Corn Agronomic Characteristics and Yield

Corn crop cycles for grain production were 140 and 120 days, respectively, after emergence (DAE), in the first and second year, respectively.

One day before the harvest of the 2014/2015 and 2015/2016 sowing seasons, the following morphological components were determined: basal stem diameter (BSD), plant height (PH), and height of ear insertion (HEI). Then, the number of plants and the number of ears contained in the useful area of each experimental unit (two 20-m-long central lines, excluding 1 m from each side row in each plot) were counted to determine the final plant stand (FPS).

Before harvesting (03/07/2015 and 04/01/2016, respectively), to determine the corn yield and yield components, all ears contained in the plot useful area were manually harvested. The following yield components were evaluated: principal main length (EL), principal ear diameter (ED), number of grains/ear (NGE), and 100-seed weight (W100) ( $130 \text{ g kg}^{-1}$  wet basis) were determined for 10 plants per plot that were randomly chosen from the usable area. Subsequently, the ears were mechanically threshed, and the grains were weighted, so the plot yield was calculated, extrapolated to  $\text{kg ha}^{-1}$ , and corrected for  $130 \text{ g kg}^{-1}$ .

Following corn harvesting in the respective harvests, the straw yield resulting from corn and grass dry matter cultivation was evaluated per plot. Therefore, the residues of corn plants were collected in a 2-m line/plot. The grass was collected with a metal square ( $1.0 \times 1.0 \text{ m}$ ) at a cutting height of  $0.30 \text{ m}$ , and in each sample, the cut material was dried under forced air circulation at  $65^\circ\text{C}$  for 72 h to quantify the total dry matter production of grass and corn straw, both extrapolated to  $\text{Mg ha}^{-1}$ . Subsequently, the material was ground (Willey-type) and sent to the laboratory where contents of N, P, K, Ca, Mg, and S were determined (Malavolta et al., 1997). Nutrient accumulations per hectare were calculated based on dry matter production and nutritional contents.

## Statistical Analyses

Data were normally distributed ( $W > 0.90$ ) according to the Shapiro-Wilk test, with the results indicating that all data were distributed normally ( $W > 0.9$ ). Means of the treatment were compared using the LSD test. The effects were considered statistically significant at  $p \leq 0.05$ . Statistical analyses were performed using the “R” software (2020).

## RESULTS

### Corn Agronomic Characteristics Production Attributes and Proline

The agronomics characteristics PH, HEI, and SBD were significant for both growing seasons ( $p < 0.05$ ) (Table 3). In the first year, treatments inoculated with *A. brasilense* in both seeds (corn and palisade grass) were also significant. They obtained higher growth, whereas SBD had higher results for intercropped inoculated only in corn.

At the second growing season, PH and HEI showed better values for all treatments except for the intercropped corn inoculated on corn seeds. Otherwise, for SBD, the monoculture corn inoculated showed a higher diameter. The production attributes EL, ED, NGE, and W100 were not significant for cropping systems or inoculation ( $p > 0.05$ ), showing that these features are no responses to those systems.

The PP attribute was significant for both growing seasons. In the first season ( $p = 0.02$ ), PP was higher for corn inoculates in monoculture or intercropped inoculate in both seeds. In the second season ( $p = 0.002$ ), the only treatment that presents lower PP was the intercropped inoculated in both seeds. On the other hand, PP reached the established amount of 60,000 plants  $\text{ha}^{-1}$ .

Grain yield was not significant for both seasons ( $p > 0.05$ ). However, it presents higher GY in the second year. This is related to the fact that in this year, the PP was higher than the first year, resulting in higher productivity.

Proline was significant ( $p < 0.001$ ) at the first growing season with high levels for monoculture corn without inoculation, whereas in the second growing season, proline was also significant ( $p = 0.015$ ) with lower levels only for monoculture corn without inoculation.

### Corn Straw Yield

Corn straw yield was significant for both growing seasons ( $p < 0.05$ ) (Table 4), although showing different values between years, the system that provided the highest straw production was corn intercropped with palisade grass and inoculated in corn seeds (Table 4).

There was a significant effect of intercropping corn and palisade grass for both growing seasons ( $p = 0.004$  and  $p = 0.0005$ ), respectively. Intercropping with *A. brasilense* in corn seeds inoculated promotes higher corn straw yield.

### Accumulation of Macronutrients on Corn Straw

The macronutrient accumulation in corn straw was significant for the crop systems evaluated (N, P, K, Ca, Mg, and S) for both years (Table 4). It was observed that in the first season, due to the lower MSY and yield production, the nutrient export by plants was not much, showing on average 50% more nutrients accumulated for this year. The macronutrients were not influenced by local inoculation (corn or grass seed).

In the first season, the corn monoculture presented a higher N accumulation. P and K present higher values for intercropped systems with palisade grass inoculated. Ca content was higher for corn inoculated, independent of monoculture or intercropped

system. Mg and S found better values for intercropped inoculated in corn seeds.

In the second season, corn intercropped was higher for P, K, Mg, and S, demonstrating higher values for both intercropping and monoculture, independent of the local inoculation.

## Palisade Grass

### Dry Matter

Palisade grass dry matter yield showed significant results at the first growing season ( $p < 0.05$ ) (Table 5). However, the effect of the inoculation with PGPB was independent of crop systems. For the next season, because of the better growth of corn, palisade grass shows lower values and not significant results.

## DISCUSSION

### Corn Agronomic Characteristics Production Attributes and Proline

On the first growing season, PH and HEI (Table 3) were higher when intercropping was inoculated in both seeds. Some reports found that inoculating with this genus of PGPB guaranteed greater plant height and root mass, especially in dry conditions influencing the absorption of nutrients and water from the soil (Fukami et al., 2018; Leite et al., 2018) positively. These results are lower than the results found in the second season. The reduction in PH and consequent HEI is due to the already mentioned fact that the high temperatures lasted throughout the vegetative phase (V8), a period of intense growth of the plant in height (Silva et al., 2015), which will reflect in the lower yield of corn straw.

Plant height is a morphological variable that is directly related to the population density of the crop. Smaller plants tolerate higher densities, while higher plants tolerate lower densities (Matos et al., 2017). It is also known that well-nourished nitrogen plants have a greater ability to assimilate  $\text{CO}_2$  and synthesize carbohydrates during photosynthesis, resulting in higher growth (Vogel et al., 2013).

As observed at the end of the second season, chemical characteristics of soil improved intercropped systems such as grasses associated with *A. brasilense* that present a root structure capable of absorbing a larger amount of nutrients and water (Bashan and de-Bashan, 2010) and also improve physical characteristics by way of the capacity of the root system promoting aeration as seen in Table 2.

The more favorable climate conditions in the second year resulted in better conditions of plant development, which justifies the larger plant growth. It is noteworthy that the greater plant height provides a greater productive potential of fresh and dry matter and, consequently, higher straw production for the next crop. However, above-average plant heights may result in greater ease of lodging by wind, rain, and machine traffic, causing productivity losses, a fact not found in this study.

Still, regarding the morphological characteristics, the crop and inoculation systems did not influence the BSD in both years (Table 3). Even at higher densities such as those of the second season, the crop did not show any problems such as lodging and breaking of the plants, a fact common in larger populations, as plants etiolate in search of light.



**TABLE 3 |** Means of plant height (PH), height of ear insertion (HEI), basal stem diameter (BSD), ear diameter (DE), ear length (EL), number of grains per ear (NGE), 100-seed weight (W100), plant population (PP) grain yield (GY) and proline content in intercropped or single corn, *A. brasilense* (irrigated area) inoculated or not in the seeds in 2014/15 and 2015/16 crops.

Cropping system	PH*	HEI*	BSD*	EL*	ED	NGE*	W100	PP*	GY	Proline*
	(m)		(cm)	(cm)		(ear)	(g kg <sup>-1</sup> )	(ha <sup>-1</sup> )	(Mg ha <sup>-1</sup> )	μmol g <sup>-1</sup> FM
<b>2014/15 Sowing season</b>										
Corn	2.09b	1.12b	2.4ab	19.27	5.40	628a	28.75	46.295b	5.6	1.18a
Corn+ PG	2.11b	1.11b	2.22bc	18.55	5.34	607ab	27.11	49.999b	5.3	0.93b
Corn + PG (I)	2.10b	1.09b	2.30bc	18.70	5.27	625ab	30.34	49.999b	5.2	0.87b
Corn (I) + PG	2.21b	1.13b	2.53a	18.67	5.26	605ab	27.69	46.295b	5.3	0.89b
Corn (I)	2.12b	1.13b	2.38ab	17.82	5.33	592ab	27.20	62.962a	4.9	0.76c
Corn (I) + PG (I)	2.66a	1.71a	2.06c	18.32	5.22	585b	28.40	64.814a	5.0	0.89b
Pr > Fc	>0.0001	0.005	>0.0001	0.560	0.444	0.242	0.372	0.020	0.426	>0.0001
CV (%)	4.65	5.96	6.01	5.70	2.47	4.59	7.90	12.21	8.92	7.01
<b>2015/16 Sowing season</b>										
Corn	2.82a	1.87a	2.16b	18.92b	4.70	547	28.41	74.073a	7.3	0.84b
Corn + PG	2.84a	1.84a	2.38ab	18.85b	4.97	570	26.44	77.777a	6.7	1.22a
Corn + PG (I)	2.90a	1.89a	2.48ab	18.95	4.87	540	26.95	82.406a	6.3	1.16a
Corn (I) + PG	2.44b	1.20b	2.26ab	18.75b	4.78	525	24.05	74.999a	7.4	1.33a
Corn (I)	2.84a	1.95a	2.52a	19.77ab	4.85	564	30.64	81.480a	6.2	1.47a
Corn (I) + PG (I)	2.88a	1.93a	2.44ab	20.85a	4.87	557	25.78	60.184b	6.0	1.23a
Pr > Fc	0.001	<0.0001	0.022	0.029	0.143	0.238	0.094	0.002	0.060	0.015
CV (%)	4.53	5.07	9.31	4.58	2.69	4.90	11.01	8.39	10.42	17.11

\*Means followed by different letters in the columns are different by "LSD" test at 5% probability.

In stress conditions like the first year, intercropped systems inoculated in corn seeds can promote a higher BSD that works as a reservation of nutrients. In systems with good edaphoclimatic conditions, corn monoculture shows higher PP, in dense crops, the development of plants saved nutrient reservation for its development.

These results corroborate those found by Silva et al. (2015), in that when evaluating intercropping and monoculture corn cultivation systems, at different spacing, and in the Cerrado area, they did not find differences for BSD between the systems. In addition to providing increased breakage and lodging resistance, BSD stores essential soluble solids for photoassimilate translocation and grain formation.

Opposite results for EL and ED were reported by Mumbach et al. (2017) using doses of mineral fertilizer (sowing and mulching) and inoculation with *A. brasilense*, and for the EL in a study by Moreira et al. (2019) when evaluating the economic performance of corn as a function of inoculant application forms and nitrogen fertilizer doses. This lack of responses to cropping systems may be associated with plant genotype and edaphoclimatic conditions.

The absence of significant results for NGE and W100 (Table 3) can be explained by the uniformity of data from EL and ED. Those results for W100 corroborated with the study of Silva et al. (2015), who also did not observe any significance for this production component when evaluating inoculation with *A.*

*brasilense* and nitrogen doses in the Cerrado. However, in the second year, results found for exclusive and inoculated corn were better. This is an important component for grain yield and may be altered by any type of stress occurring in the grain filling phase, in which the external factors are the most limiting to plant development (Freitas et al., 2013). In addition, Pariz et al. (2009) reported that the intercropping system of corn with other forages might compromise the translocation of photoassimilates to grains, producing lighter grains.

In that year, PP was better when inoculates in monoculture or intercropped with inoculations in both seeds (2015/16). The average plant ha<sup>-1</sup> was lower than the PP established for this study, which was 60,000 plants ha<sup>-1</sup>. This result is due to the influence of the high temperature on that year when the experiment was conducted (Table 1). The higher temperature, the faster the metabolic process, which leads to early leaf senescence. The ideal temperature for crop development from emergence to flowering is between 24 and 30°C (Cruz et al., 2010).

It can be observed from the evaluation of simultaneous sowing of corn and palisade grass, regardless of the cropping system, that there was no negative influence (Table 3) of competition between species. Corroborating the results of Araújo et al. (2018) and Garcia et al. (2013), both evaluating corn yield in association with palisade grasses, also found that intercropping systems did not influence the final plant stand. Corn PP has direct effects on

**TABLE 4 |** Remaining corn straw production (RMSP) and nutrient accumulation in corn straw in 2014/15 and 2015/16 sowing seasons.

Crop system <sup>a</sup>	2014/15 sowing season						
	RMSP*	N*	P*	K*	Ca*	Mg*	S*
	kg ha <sup>-1</sup>						
Corn	4105c	102b	12.6b	78bc	20.8b	18.0b	13.3b
Corn + PG	4738ab	100b	11.5b	54d	17.5bc	19.5b	14.5ab
Corn + PG (I)	4708ab	121ab	23.5a	101a	13.2c	18.0b	13.3b
Corn (I) + PG	6043a	109b	13.2b	87ab	31.9a	31.1a	17.5a
Corn (I)	4645ab	144a	14.0b	65cd	30.2a	20.5b	13.0b
Corn (I) + PG (I)	4787b	54c	4.9c	75bc	14.2c	12.0c	7.8c
PR > Fc	0.0004	<0.0001	<0.0001	0.002	<0.0001	<0.0001	0.0007
CV (%)	8.83	15.75	19.98	17.16	13.73	13.64	16.91
	2015/16 sowing season						
	RMSP*	N*	P*	K*	Ca*	Mg*	S*
	kg ha <sup>-1</sup>						
Corn	9478b	54bc	6.6c	20.5a	8.2c	7.6b	4.0b
Corn + PG	8626b	58b	8.6b	23.7a	8.7bc	11.5a	4.3b
Corn + PG (I)	9061b	44d	11.0a	23.5a	5.5d	8.0b	4.0b
Corn (I) + PG	11.982a	73a	8.5b	15.1b	14.7a	11.3a	5.8a
Corn (I)	9754b	46cd	5.9d	15.6b	9.7b	11.9a	5.5a
Corn (I) + PG (I)	6692c	56b	7.7bc	23.9a	9.3bc	9.3b	4.5b
Overall mean	0.0005	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
CV (%)	12.57	10.28	11.45	14.62	10.64	11.21	7.49

\*Means followed by different letters in the columns are different by "LSD" test at 5% probability.  
aPG, Palisade grass; I, Inoculated.

crop yield and also forage production (Crusciol et al., 2010). This component is related to SBD, and it is expected that the greater supply of water and nutrients will provide a larger plant size and, consequently, a higher PP.

In addition, the larger number of plants ha<sup>-1</sup> (75,000 plants ha<sup>-1</sup>) promotes greater plant growth in height (Calonego et al., 2011; Freitas et al., 2013), with a consequent increase in HEI, corroborating the results of the second year, when PP was over 60,000 plants ha<sup>-1</sup>. Another factor contributing to the larger PP is that a larger plant population can provide greater height growth, reducing evapotranspiration losses (Queiroz et al., 2016).

On the first growing season, GY has lower values because of the lower PP, a consequence of high temperatures that year, which promotes low germination. Otherwise, production showed satisfactory results, with an average of over 4.9 Mg ha<sup>-1</sup> and 7.0 Mg ha<sup>-1</sup> in the second season, and more than 116 bags ha<sup>-1</sup>. In corn, the strains of *A. brasilense* Ab-V5 and Ab-V6 resulted in an increase in grain yield that reached 27% compared with the non-inoculated control (Hungria et al., 2010). This was not observed in this study because of the different edaphoclimatic conditions.

Although Silva et al. (2015) reported that simultaneous sowing between corn and palisade grass could influence the final grain yield through competition between crops, especially after corn flowering, it was found in this study that the inoculation of corn seeds becomes viable in NTSs with correct input from plant material derived from previous crops because even with the coexistence with forage, higher results for the intercropped systems without any inoculation were achieved.

**TABLE 5 |** Dry matter yield of Palisade grass in an irrigated area after intercropped with corn in 2014/15 and 2015/16 crops.

Crop system	Palisade grass after intercropping (kg ha <sup>-1</sup> )	
	2014/2015*	2015/2016
Corn + Palisade grass	5440a	3523
Corn + Palisade grass (I)	3487b	3758
Corn (I) + Palisade grass	5962a	4285
Corn (I) + Palisade grass (I)	6381a	3761
PR > Fc	0.0052	0.530
CV (%)	16.40	18.96

\*Means followed by different letters in the columns are different by "LSD" test at 5% probability.

The most important contribution of the use of *A. brasilense* is the possibility of increasing dry matter accumulation rate, resulting in an incremented biomass and plant height, accelerating germination and benefiting the root system, and as a consequence, there may be an increase in grain yield due to greater nutrient translocation to grain and reduced competition with grass (Hungria et al., 2010).

Proline is known as an important plant osmoregulator, and its transport is assumed to influence tolerance against stress conditions. In both years, the results of free proline quantification in corn leaves had different levels (Table 2).

Proline metabolism is regulated by the balance between its anabolism and catabolism. Its deposition in parts of the plant may not occur due to an increase in biosynthesis but rather

due to its transport and utilization rate (Per et al., 2017). Under normal conditions, proline biosynthesis occurs in the cytosol, while under stress conditions, it may occur in chloroplasts (Rejeb et al., 2014). In agreement with the results observed between the evaluated years, in the first year, the greater evapotranspiration caused the stomata to close so that there was no excessive water loss.

No losses in the utilization rate and transport of the proline contents occurred in the second year as the climatic conditions were more favorable to the development of the crop, thus without stress conditions. Also, the corn cropped in a single system without inoculation showed lower levels of proline; therefore, less deposition and higher utilization rates were found.

Studies show that phytohormones such as auxins, gibberellins, cytokines, ethylene, ABA, and salicylic acid are related to plant growth, development, metabolism, and response to different stress conditions, but these results are still scarce (Iqbal et al., 2009). However, the benefits of phytohormones produced by the action of inoculation with *A. brasilense* throughout this study can be observed.

## Remaining Corn Straw and Nutrient Accumulation

The remaining corn straw in NTS on the first growing season was lower compared to the second season. Otherwise, the system that promotes the high straw production was corn intercropped with palisade grass and inoculated in corn seeds (Table 4). The lower remaining corn straw in NTS in the first growing season compared with the second one was expected. The higher temperatures in January 2015 (Table 1) in the flowering phase promotes the aborting of flowers, making the germination infeasible. It is known that moisture shortage affects stomatal functions, reducing the leaf  $\text{CO}_2/\text{O}_2$  ratio and inhibiting photosynthesis with concomitant reduction of biomass production (Lopes et al., 2011).

Otherwise, the crop system with corn intercropped with palisade grass inoculated in corn seeds promoted the higher remaining straw, once again indicating the benefits of plant growth-promoting bacteria (PGPB) in that system. This symbiosis between PGPB and plants provided yield productivity gains of grain and forage crops, resulting in a higher accumulation of dry matter after harvest, providing enough straw to maintain NTS.

Another factor that should be taken into account in the implementation of the crop in ICLS is the spatial arrangement and sowing type, which must be defined according to the objective of the system (Borghi et al., 2012) or some water restriction. High densities are recommended only when there is no water restriction (Freitas et al., 2013). The spacing used in this study was 0.45 m, with simultaneous sowing of corn and grass, providing high densities and sufficient dry matter production to maintain the NTS recommended by Kluthcouski et al. (2000), which is  $>6.0 \text{ Mg ha}^{-1}$ .

It was observed that, in the first year, due to lower MRS and GY, the nutrient exportation by plants was lower, showing, on average, 50% more nutrients accumulated for this year. The

largest accumulation of N and K stands out, confirming that this nutrient is the most absorbed and accumulated in plant tissues, corroborating the results found by Pariz et al. (2011), Costa et al. (2014), Mendonça et al. (2015), under the same edaphoclimatic conditions.

The soil in the experimental area has been cultivated in SPD for over 12 years when the immobilization of N approaches mineralization (Anghinoni, 2007). Therefore, the accumulation of O.M. and mulch started on the surface, which, combined with corn topdressing fertilization, provided a higher accumulation of this nutrient, especially when inoculated with *A. brasilense*.

The higher K extraction in intercropping systems is due to the greater competition with palisade grass, which extracts more of this nutrient from the soil. The intercropped system corn + palisade grass is unique in providing K because of the greater amount of exchangeable K in soil (Mateus et al., 2020). However, because it has no structural function in plants, K has a short half-life. Therefore, it is important for the supply of this nutrient in subsurface soil layers for succession crops (Cavalli et al., 2018).

Cropping systems and inoculation with *A. brasilense* did not influence Ca, Mg, and S. Therefore, the adoption of practices, such as intercropping and use of PGPBs, provides a higher quality of NTS, providing a greater return of nutrients to the soil through greater input of residual biomass. In addition, when the grain-producing crop is intercropped with summer forage grasses, such as *Urochloa*, it is possible to reduce the cost of pasture formation, as a food supply to ruminants in the off-season, where the availability of forage is limited (Costa et al., 2014).

## Palisade Grass

On the first growing season, the effect of inoculation with the recommended doses and strains for corn crop was independent of the inoculated species (corn or grass) (Table 5). Because palisade grass has a vigorous and deep root system, the growth-promoting effects of *A. brasilense* inoculation on root growth and biomass growth may not have manifested, especially because the crop is irrigated, determining that the plants do not need to deepen the root system in the search for water and nutrients. Similar results were observed by Barducci et al. (2009), in that upon evaluating the inoculation of species of the genera *Urochloa* and *Megathyrsus*, they reported that the greater root development of these species makes them more tolerant to water and nutrient deficits and may interfere with the hormonal effects of *Azospirillum*.

Otherwise, it is important to consider that strains of *Azospirillum* may differ in their properties to confer tolerance in drought and gains in yield (García et al., 2017). The variation between years is attributed to the overgrowth of corn plants in that year, providing greater shading of the grass, leading to its low metabolic and growth activity. These results are reinforced by Table 1, where corn straw dry matter production was higher in the second year.

When corn straw is added to grass straw production, means of 10.1 and 13.1  $\text{kg ha}^{-1}$  are observed for the first and second year, respectively. In Cerrado regions, where high temperatures and dry winter predominate, these values can supply the balance needed to maintain soil cover and nutrient cycling in this climate,

results close to those found by Sá et al. (2015), which ranged from 11.7 to 13.3 kg ha<sup>-1</sup> under the same soil and climate conditions.

In that year, when corn was intercropped with palisade grass, regardless of whether or not it is inoculated with *A. brasilense*, Modesto et al. (2019) observed a greater profitability index as well as operating profit; the positive results found in intercropped systems in the first year and in the second year were 25.6 and 59.77%, respectively, with the average difference of 34.21% due to the variables in the climatic conditions between the years that resulted in higher corn production in the second harvest. These results show that intercropping corn with palisade grass could amortize the production costs of the system and provide grass input to subsequent silage.

## CONCLUSION

Considering the optimal conditions of soil provided by liming and fertilization at sowing and nitrogen applied in topdressing, the application of *A. brasilense* in corn seeds did not show its potential. Otherwise, in the current study, intercropped systems increase grain yield, dry matter production, and nutrient accumulation in the straw, producing more biomass and providing greater sustainability to NTS in the low-land Cerrado. These results of intercropped systems have great importance, specifically in these tropical regions.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary files, further inquiries can be directed to the corresponding author.

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## AUTHOR CONTRIBUTIONS

VM conceived and designed the analysis, collected the data, contributed the data, performed the analysis and wrote the paper. MA conceived and designed the analysis, contributed the data, and wrote the paper. AN and DS collected the data, contributed the data, and performed the analysis. LF, LD, IP, and IF collected the data and performed the analysis. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2020.617065/full#supplementary-material>



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# Rhizosphere Engineering With Plant Growth-Promoting Microorganisms for Agriculture and Ecological Sustainability

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The rhizosphere is undoubtedly the most complex microhabitat, comprised of an integrated network of plant roots, soil, and a diverse consortium of bacteria, fungi, eukaryotes, and archaea. The rhizosphere conditions have a direct impact on crop growth and yield. Nutrient-rich rhizosphere environments stimulate plant growth and yield and vice versa. Extensive cultivation exhaust most of the soils which need to be nurtured before or during the next crop. Chemical fertilizers are the major source of crop nutrients but their uncontrolled and widespread usage has posed a serious threat to the sustainability of agriculture and stability of an ecosystem. These chemicals are accumulated in the soil, drained in water, and emitted to the air where they persist for decades causing a serious threat to the overall ecosystem. Plant growth-promoting rhizobacteria (PGPR) present in the rhizosphere convert many plant-unavailable essential nutrients e.g., nitrogen, phosphorous, zinc, etc. into available forms. PGPR produces certain plant growth hormones (such as auxin, cytokinin, and gibberellin), cell lytic enzymes (chitinase, protease, hydrolases, etc.), secondary metabolites, and antibiotics, and stress alleviating compounds (e.g., 1-Aminocyclopropane-1- carboxylate deaminase), chelating agents (siderophores), and some signaling compounds (e.g., N-Acyl homoserine lactones) to interact with the beneficial or pathogenic counterparts in the rhizosphere. These multifarious activities of PGPR improve the soil structure, health, fertility, and functioning which directly or indirectly support plant growth under normal and stressed environments. Rhizosphere engineering with these PGPR has a wide-ranging application not only for crop fertilization but developing eco-friendly sustainable agriculture. Due to severe climate change effects on plants and rhizosphere biology, there is growing interest in stress-resilient PGPM and their subsequent application to induce stress (drought, salinity, and heat) tolerance mechanism in plants. This review describes the three components of rhizosphere engineering with an explicit focus on the broader perspective of PGPM that could facilitate rhizosphere engineering in selected hosts to serve as an efficient component for sustainable agriculture.

**Keywords:** plant growth promoting bacteria, rhizosphere engineering, stress tolerance, microbiome, plant growth promotion

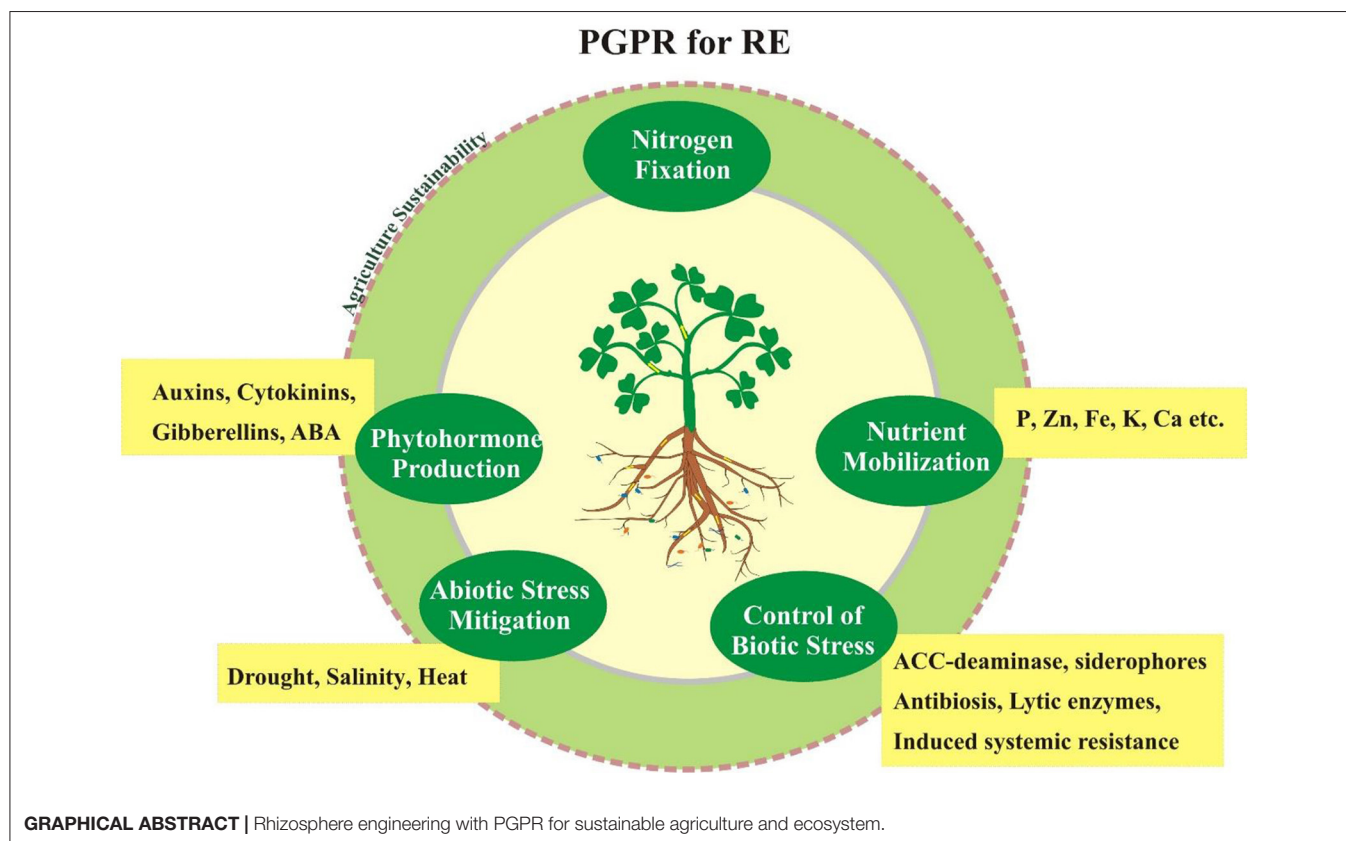
## INTRODUCTION

The development of healthy human societies depends upon the availability of food which is one of the basic needs for human beings. The rapid increase in the human population, climate changes encompassing biotic and abiotic stresses, and land scarcity have imposed an undesirable impact on global food production. Food insecurity is a chronic issue that is likely to worsen as the human population is expected to be more than 9 billion by 2050 (Kumar and Dubey, 2020). The population increase-mediated pressure in agriculture has led to intensive use of chemical fertilizers, and pesticides to get the maximum yield out of the existing agricultural lands. Of the total, around 20–30% of the applied fertilizer is taken up by the plant. Most of the crop varieties have low nutrient uptake efficiencies. Due to low nutrients use efficiency in agriculture and soil dynamics, more than 50% of applied chemical fertilizers are lost to the environment (Fageria, 2014). Furthermore, many of the plant varieties developed in the background of the “green revolution” have become non-responsive to the fertilizers and their yields are stagnant. Both nitrogen and phosphorus fertilizers added to the soil are readily volatilized, rapidly washed-off, gradually converted to un-available forms due to natural processes making the ecosphere and biosphere at higher risk for future generations. As a consequence of this, the overall agriculture production decreased along with several environmental problems such as loss of biodiversity, emission

of greenhouse gases, water pollution, and soil contamination. It has subsequently deteriorated soil biology and its health. Furthermore, the higher cost of agriculture input and low-cost benefits are also affecting the farmer’s interest. Due to these reasons, the agriculture system is under tremendous pressure and its sustainability is essential for (i) the management of food security for increasing demand (ii) mitigation of adverse climatic effects and changes (iii) improvement in soil quality and nutrient (Lal, 2015).

Over the past decades, sustainable food and agriculture production (SFAP) has become one of the world’s most fundamental needs for food security. SFAP is an approach for the production of food and fiber in balance with a protected environment and public health. It includes the usage of resources more efficiently with minimized adverse effects on the environment, restore and preserve the quality of soil and water along with improved productivity. Per this definition, food provision to humans, enhanced natural resources with high resource use efficiency, and improved quality of life has been considered as the general goals of sustainable agriculture production (Walters et al., 2016).

Several strategies have been employed for enhancing the nutrient use efficiency (NUE) of crops and sustainable agriculture production. Of these approaches, the 4R strategy is more recently introduced that includes the use of the Right Source of nutrients at the Right Rate, Right Time, and in the Right Place. This strategy can be further expanded and made more comprehensive if we





include two major players in this interaction i.e., the plant which is using the nutrient and the rhizosphere where the nutrients are applied. In this scenario, the emphasis should be focused on the selection of low-fertilizer responsive crop germplasm and incorporation (inoculation) of the Right plant growth-promoting bacteria for rhizosphere engineering. Plant breeding or plant engineering is a separate branch that has been the subject of many comprehensive reviews published. The current review will focus on the rhizosphere engineering (RE) using PGPR with special reference to plant benefits, nutrient uptake, and ecological sustainability.

## RHIZOSPHERE ENGINEERING (RE)

### What Is RE

The rhizosphere is the narrow zone of soil that is in direct proximity to plant roots and the hotspot of various microbes. The plant influences the nearby soil through the release/secretion of different compounds known as rhizodeposits, which mainly consist of carbohydrates, secondary metabolites organic acids, and amino acids (Ahkami et al., 2017). As a consequence, rhizosphere soils favoring the growth of microbial populations are described as mesotrophic. The rhizosphere has been subdivided into three zones; endorhizosphere (the portion of endodermis, root cortex, and apoplastic space between cells); rhizoplane (the surface of the root); and ectorhizosphere (the zone extending from rhizoplane to bulk soil) (Mcneer, 2013). The rhizosphere harbors diverse microbial groups that perform various functions and exert numerous effects on plant growth. They are involved in nutrient cycling, protecting from phytopathogens as well as under biotic and abiotic stress conditions, and some may act as plant pathogens. These microbial activities in the rhizosphere lead to changes in the composition, quality, and quantity of root exudates released by the plants, which in turn affect the microbial component (Philippot et al., 2013). This phenomenon known as rhizosphere feedback proposes that plants, through rhizodeposition, shape the microbial community composition in the rhizosphere which subsequently influences plant growth and productivity (Dessaux et al., 2016). Such a relationship suggests that the rhizosphere can be exploited and/or engineered to promote the growth, nutrient uptake, and production of plants. Schmid et al. (2018) investigated the bacterial diversity in the rhizosphere of offsprings of eight plant species that were previously grown for 11 years in the field under monoculture and mixture planting. The study revealed that the rhizosphere community structure is determined by soil plantation history and plant species identity (Schmid et al., 2018). There is ample evidence that the overall growth, productivity, and health of the plant depends upon the plant-microbe relationship (Adesemoye and Egamberdieva, 2013; Htwe et al., 2019; Masood et al., 2020). Such an intricate relationship suggests that to improve the overall health, growth, productivity, and to protect the plants from biotic and abiotic stresses, the rhizosphere can be engineered which represents an eco-friendly approach for more sustainable agriculture production.

## Components of RE

Plants, microbes, and soil are three key components of the rhizosphere described in detail in **Figure 1**. All can be engineered (manipulated) to improve plant productivity.

The soil amendment (which has been practiced for two millennia) can influence the rhizosphere functioning for plant growth promotion. Soil amendments such as biochar, silicon (Villegas et al., 2017), zeolites (Jakkula and Wani, 2018), plant residues, coal fly ash, cattle manure, and sewage sludge have been used (Dessaux et al., 2016). Despite the recent progress in microbial ecology, soil analytical tools, and plant genetics, soil amendments remain an empirical technique providing descriptive information.

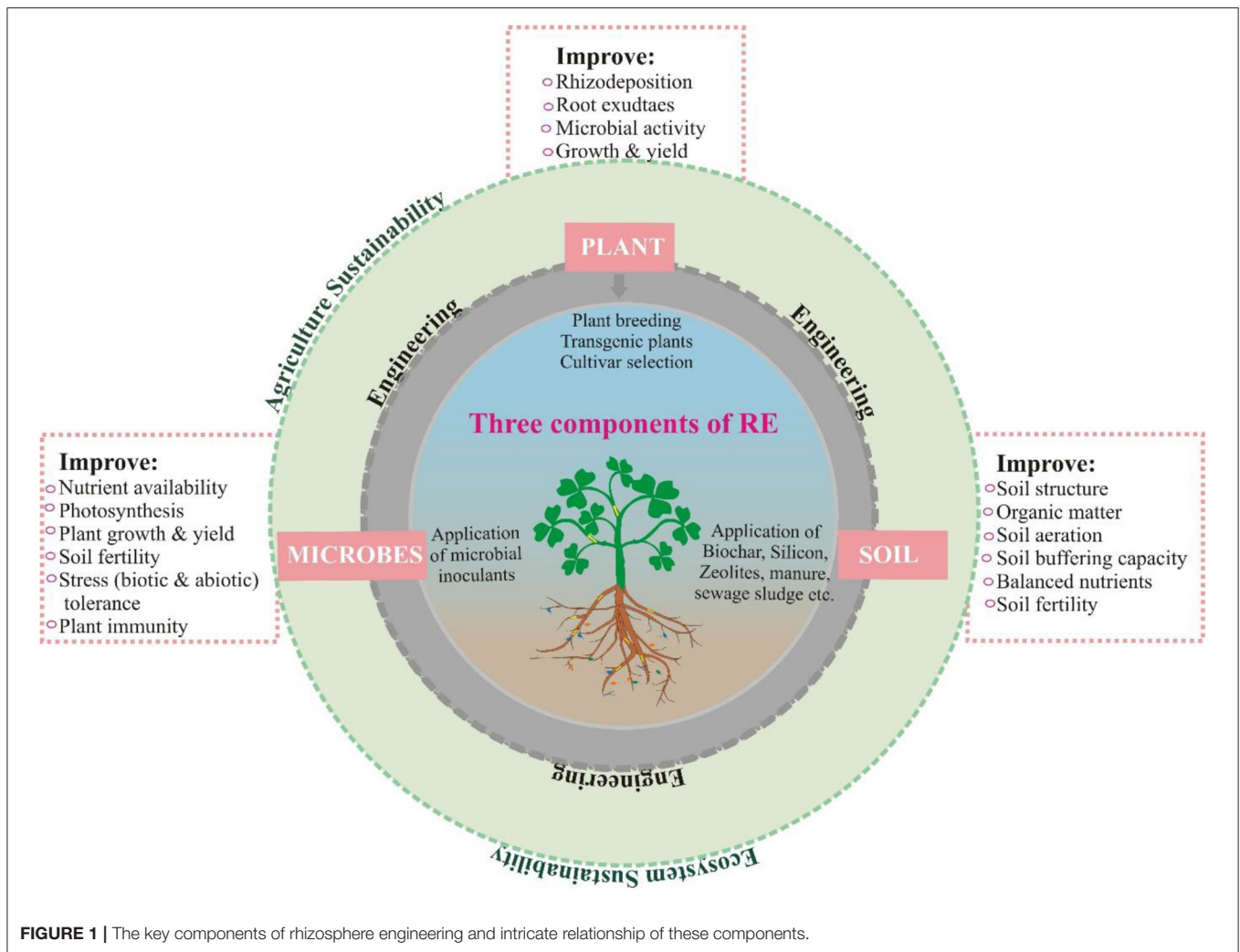
As the rhizosphere activity and functioning is shaped by the plant traits such as root architecture and root exudates, so these traits can be engineered through the genetic engineering of plants. Several plants have been engineered through breeding and gene editing techniques for the uptake of nutrients such as P, Fe, and Zn (Clemens, 2014; Wei et al., 2020), protection from diseases (Zhang T. et al., 2019) as well as the removal of heavy metals (Gunaratne et al., 2019). Although plant engineering brings favorable advantages to soil restoration, and plant growth, nutritional quality, and resistance to pathogens, but their application remain low due to the lack of social acceptance and concerns regarding human health and environmental sustainability.

## PLANT GROWTH PROMOTING MICROBES AS MAJOR TOOLS OF RE

One of the most important strategies to engineer the rhizosphere is the manipulation and engineering of the microbiome. Microbes can positively influence plant growth and counteract most of the problems of modern agriculture, thus represent a promising approach for agriculture sustainability. Due to the complexity of the microbiome, there is limited ability to manage and manipulate the whole rhizosphere microbiome, however, the most direct and eco-friendly way to alter the microbiome is the inoculation of artificially multiplied microbes. Various products containing one or several species of bacteria or fungi in the form of biofertilizers have been commercially synthesized and are available for the improvement of plant growth and sustainability.

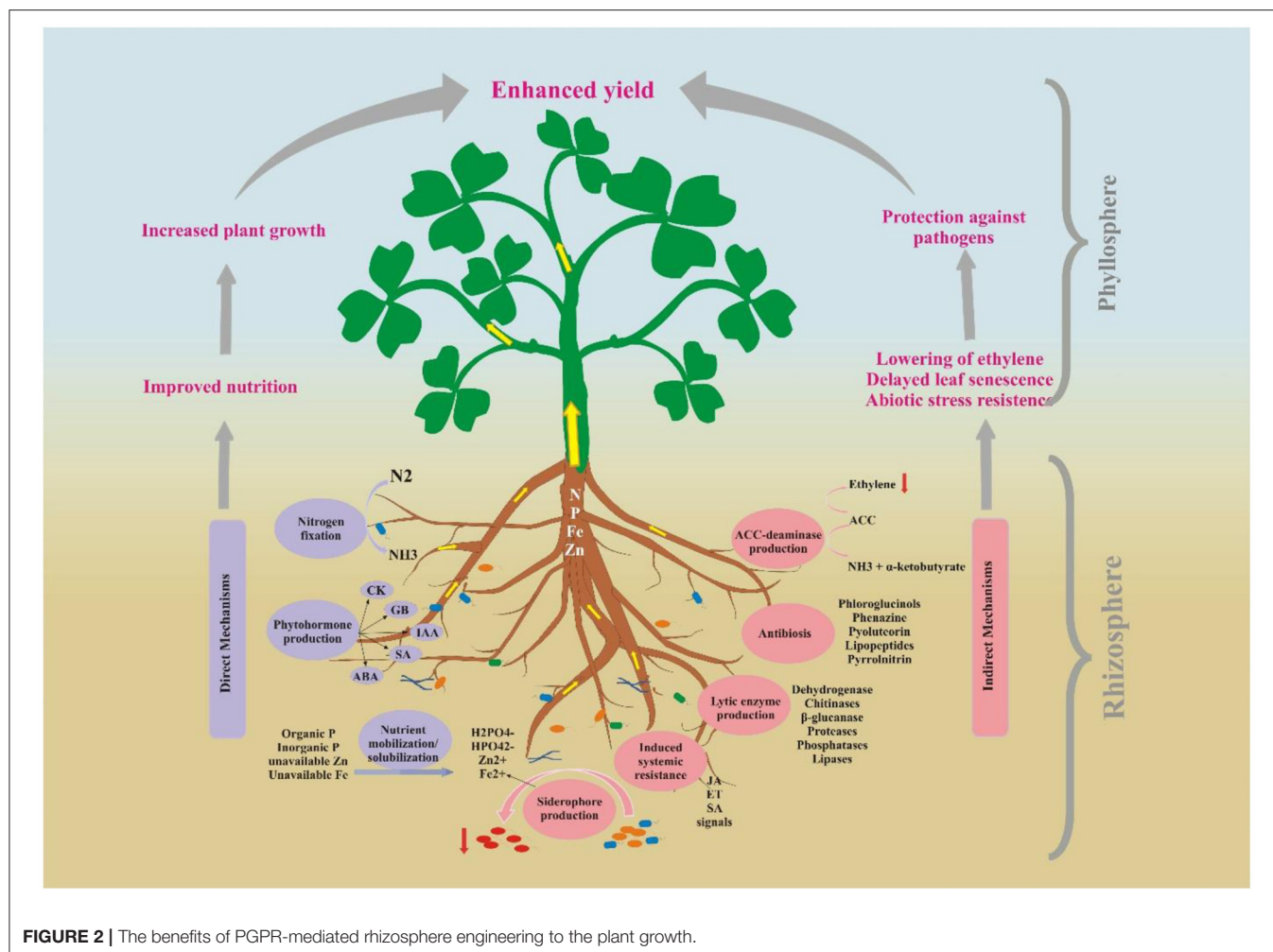
Among the rhizosphere microbiome, most of the bacteria known as plant growth-promoting rhizobacteria (PGPR) have positive interaction with the plant and promote their growth and survival, while only a few are found to be pathogenic for plants (Kumar and Dubey, 2020). These beneficial bacteria stimulate plant growth, make nutrients available to plants, suppress the growth of pathogens, and improve the soil structure, subsequently playing an essential role for sustainable crop production as shown in **Figure 2**. They also mineralize the organic pollutants and are used in bioremediation of polluted soils (Dessaux et al., 2016; Bibi et al., 2018).

Numerous symbiotic bacteria, termed intracellular PGPR, colonize the plant cells, produce special organs within the plant roots called nodules, and live inside



these nodular structures. A variety of bacteria such as *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Azorhizobium*, *Allorhizobium*, *Ochrobactrum*, and *Mesorhizobium* are included in this group and commonly used as inocula for legumes (Imran et al., 2010; Quiza et al., 2015; Hakim et al., 2018, 2020a). Along with rhizobia, several non-rhizobial endophytes like *Arthrobacter*, *Curtobacterium*, *Micromonospora*, *Microbacterium*, *Mycobacterium*, *Acinetobacter*, *Agrobacterium*, *Blastobacter*, *Bosea*, *Devosia*, *Enterobacter*, *Herbaspirillum*, *Pantoea*, *Pseudomonas*, *Ralstonia*, *Stenotrophomonas*, *Bacillus*, *Brevibacillus*, *Paenibacillus*, *Chryseobacterium*, and *Sphingobacterium* also colonize the interior of root nodules (Velázquez et al., 2013; De Meyer et al., 2015; Leite et al., 2017; Hakim et al., 2020b). Free-living rhizobacteria, known as extracellular PGPR (ePGPR), are present in soil and do not inhabit plant tissues. Some examples of ePGPR are *Arthrobacter*, *Bacillus*, *Azospirillum*, *Azotobacter*, *Micrococcus*, *Pseudomonas*, and *Serratia* (Adesemoye and Egamberdieva, 2013). Besides, actinomycetes are also a major group of microbes inhabiting the rhizosphere which display numerous beneficial traits to

improve plant productivity (Muleta and Assefa, 2018). However, culturable microbes represent 1–5% of the microbes present on the earth (Kumar and Dubey, 2020). Furthermore, there is limited information about the bacterial communities inhabiting the different plant compartments such as root nodules, leaves, stems (Kumar and Dubey, 2020). As a consequence, culture-independent approaches such as metagenomics has been used to investigating the rhizosphere microbiome. This method is also used to detect the functional diversity of specific microbes in different environments. The diversity of nitrogen fixers has been evaluated through the *nifH* gene amplified from diverse environments including forest and agricultural soils, marine environments, estuarine sediments, microbial mats, hydrothermal vents, termite guts, and Antarctic environments (Collavino et al., 2014; Jing et al., 2015). The diversity of denitrifying microbial populations in the sediment of boreal lakes has been evaluated through pyrosequencing of nitrite and nitrous oxide reductase genes (*nirK*, *nirS*, and *nosZ*) (Saarenheimo et al., 2015). The *mcrA* gene-based metagenomics was used for the assessment of methanogen



**FIGURE 2 |** The benefits of PGPR-mediated rhizosphere engineering to the plant growth.

diversity in anaerobic digesters (Wilkins et al., 2015). The microbiome-mediated rhizosphere engineering strategy requires culturing and inoculation of bacteria to increase the functional capacity of microbes in the rhizosphere. Bio-inoculants of *Acetobacter*, *Azospirillum*, *Bacillus*, *Micromonospora*, *Paenibacillus*, *Enterobacter*, *Pseudomonas*, *Herbaspirillum*, *Serratia*, *Rhodococcus*, and *Streptomyces* enhanced production in a range of crops (Martínez-Hidalgo et al., 2014; Zaheer et al., 2016; Htwe et al., 2018). Various studies revealed that plant inoculation with consortia of PGPR has a synergistic effect on the plant growth helps to alleviate abiotic and biotic stresses by producing various defense compounds. Co-inoculation of *Bacillus megaterium* and *Paenibacillus polymyxa* along with *Rhizobium* have shown enhanced plant biomass of *Phaseolus vulgaris* as compared to *Rhizobium* inoculation alone (Korir et al., 2017). Similarly, the application of *Rhizobium* and *Pseudomonas* increased the mung bean biomass and yield along with the improved concentration of nitrogen (N), potassium (K), and sodium (Na) in plants (Ahmad et al., 2012). Co-inoculation of *Bradyrhizobium* strain along with *Streptomyces griseoflavus* increased the nodulation, nitrogen fixation, and nutrients uptake of *Glycine max* (Htwe et al., 2018). Further, detailed studies are

required to investigate the functionality and persistence of the inoculants and their potential to form an association with nearby microbes when this strategy is used to engineer the rhizosphere.

## How PGPR Engineer the Rhizosphere

PGPR contain various traits which exert positive effects on plant growth through direct and indirect mechanisms. Major direct mechanisms of actions of PGPR include mobilization of nutrients (P, Zn, and Fe) nitrogen fixation, and production of phytohormone. Biocontrol of pathogens by the production of ACC-deaminase, siderophores, antibiotics, lytic enzymes, induced systemic resistance, and induction of resistance against abiotic stresses are described as indirect mechanisms of action of PGPR. **Figure 2** summarizes the direct and indirect growth-promoting effects exerted by PGPR on plants and the rhizosphere.

## Nutrient Mobilization in the Rhizosphere

### Phosphate solubilization

Phosphorus (P) is the 2nd most important macronutrient for plants that is involved in all major physiological and biochemical processes including cell division, photosynthesis, respiration,



root system development, and biosynthesis of macromolecules (Sharma et al., 2011). It also plays a major role in stem strength, crop maturity and production, and quality improvement of many fruits, vegetables, and grain crops (Sagervanshi et al., 2012). P is one of the important constituents of nucleic acid and phospholipids. At the cellular level, the most important function of phosphorus is energy storage and transfer through ATP (Kaviyarasi et al., 2011). It helps crops to survive in harsh winter conditions and improves the quality of the produce (fruits, vegetables, grains) (Sagervanshi et al., 2012). Phosphorus deficiency leads to stunted plant growth, chlorosis, and low productivity (Malhotra et al., 2018). In severe cases, symptoms include stunting, purpling, or browning of leaves, which are more pronounced at fast growth stages i.e., young plants (Ziadi et al., 2013). It is also important for biological nitrogen fixation in legumes.

Although P is abundantly present in both inorganic and organic forms in the soil, but a small fraction (0.1%) exists in the soluble (plant-available) form ( $\text{HPO}_4^{2-}$  or  $\text{H}_2\text{PO}_4$ ) (Sharma et al., 2013). Organic phosphorus constitutes about 20–80% of the total phosphorus pool and exists as soil humus and represent an important reservoir of immobilized P. Most of the organic P exists in the form of inositol phosphate (10–50%), sugar phosphates, phospholipids (1–5%), nucleotides (0.2–2.5%), phosphoprotein and phosphonates (Khan et al., 2014). The inorganic form of phosphate is produced by weathering of rock and sequestered in soil either by adsorption to the soil mineral surfaces or through precipitation (Richardson and Simpson, 2011). P is fixed by free oxides and hydroxides of aluminum and iron in acidic soil while by calcium in alkaline soils (Li et al., 2016). Due to the insufficient available phosphorus in cultivable lands, high doses of chemical phosphate fertilizers are added to the soil to support crop production. But only a small fraction of the applied fertilizer is taken up by the plant while the remaining is converted into an insoluble form; unavailable for the plant (Cabeza et al., 2019). This high accumulation causes eutrophication which is harmful to aquatic life as well as humans. The inaccessible inorganic P is hydrolyzed through the P solubilization process, while the insoluble organic P is converted to soluble form through mineralization. Several microorganisms i.e., bacteria, fungi, and actinomycetes are an integral component of the natural P-cycle and involved in the transformation of phosphate in soil. Among the total microbial population in the soil, 1–50% of bacteria and only 0.1–0.5% of fungi have the potential for P solubilization. They convert the insoluble inorganic phosphate to plant-available form through  $\text{H}^+$  excretion and organic acid production. Different types of organic acids like acetate, citrate, lactate, ketogluconate, gluconate, succinate, malate, oxalate, etc. are produced that can form complex with cations bound to phosphate and convert the P into a soluble form (Kalayu, 2019). Gluconic acid and ketogluconic acids are major acids involved in P-solubilization (Alori et al., 2017). The bacteria having this ability are known as P-solubilizing bacteria (PSB). Organic acids may chelate with cations present on the mineral surface of the soil, thus blocking phosphate adsorption sites on the soil particles and subsequently increasing the phosphate

availability (Bianco and Defez, 2010). Production and release of organic acids and protons by the PSB lead to a decrease in the pH of rhizospheric soil. Several PSBs related to genera *Arthrobacter*, *Bacillus*, *Brevundimonas*, *Delftia*, *Enterobacter*, *Gordonia*, *Klebsiella*, *Phyllobacterium*, *Pseudomonas*, *Serratia*, and *Xanthomonas* have been reported to solubilize P through proton excretion and organic acids production (Sharma et al., 2013; Hanif et al., 2020; Naqqash et al., 2020). Other mechanisms of inorganic P solubilization by PSB include the production of chelating agents and inorganic acids such as nitric, sulfuric, and carbonic acids (Khan et al., 2014). However, these compounds have been reported to be less effective for the release of P in the soil as compared to the organic acids (Alori et al., 2017). The soil available P was found to be increased due to these PSB, for instance, a 16% increase in soil P availability has been found in the rhizosphere of wheat by inoculation with *Pseudomonas* (Suleman et al., 2018). Similarly, *Acinetobacter* and *Pseudomonas* strain enhanced the soil available P to about 3.9 and 3.11  $\text{mg kg}^{-1}$  as compared to non-inoculated control (2.33  $\text{mg kg}^{-1}$ ) (Rasul et al., 2019).

The solubilization of organic P to soluble form is referred to as P-mineralization. Various soil microbes possess P mineralization ability. This process involves the liberation of different enzymes such as (i) phosphatases which are involved in the dephosphorylation of phosphor-ester or phospho anhydride bond of organic compounds, and (ii) phytases that release the P stored in plant materials in the form of phytate (Khan et al., 2014). The phosphatases released by the microbes can either be acid or alkaline. These phosphatases are non-specific, possess a greater affinity for organic P compounds, and convert them into a soluble form. Several bacteria from the genera *Aneurinibacillus*, *Bacillus*, *Burkholderia*, *Flavobacterium*, *Lysinibacillus*, *Pseudomonas*, and *Serratia* exhibit phosphatase activity and improve the growth of various crops including wheat and maize (Iqbal Hussain et al., 2013; Schoebitz et al., 2013; Behera et al., 2017; Matos et al., 2017). Phytases have been detected in many bacteria such as *Bacillus* sp., *Citrobacter braakii*, *Escherichia coli*, *Enterobacter*, *Pseudomonas* sp. *Raoultella* sp. and anaerobic rumen bacteria, particularly in *Megasphaera elsdenii*, *Mitsuokella* spp. *Prevotella* sp. and *Selenomonas ruminantium* (Azeem et al., 2015). Phytase-producing *Tetrathiodibacter* and *Bacillus* strains enhanced the shoot and root dry weight and thus improved the plant growth and P contents of *Brassica juncea* (Kumar et al., 2013).

### Zn-mobilization

Zinc is an indispensable micronutrient, required in a small amount for crops to play numerous important functions in their life cycle (Hafeez et al., 2013). It is involved in various physiological and biochemical functions of plants (Kumar et al., 2019) and performs a pivotal role in DNA transcription as it is found in DNA-binding proteins. Zn-finger proteins play a central role in several developmental processes as well as responses to environmental stresses (Noguero et al., 2013). Zn is described as the only element found in all six enzyme classes (hydrolases, isomerases, ligases, lyases, oxidoreductases, and transferases) playing an essential role in the proper functioning of enzymes



(Saravanan et al., 2011). It plays a vital role in the metabolism of carbohydrates, proteins, and phytohormone i.e., auxin, as well as involved in membrane integrity and reproduction, thus affecting the growth, development, vigor, maturity, and yield of the plants. The deficiency of zinc in plants manifested as a remarkable reduction in height and development of whitish brown patches that subsequently turn to necrotic spots. It also leads to chlorosis, retarded leaf and shoot growth, affect the uptake and transport of water, root development, pollen formation, and grain yield as well as causing susceptibility to heat, light, and fungal infections resulting in a major loss in crop production (Kumar et al., 2019). Zn deficiency in human results in impaired brain function, anemia, retarded growth, hypogeusia in children, anorexia, poor mental development, and several chronic diseases in humans (Liu D. et al., 2017). Zn-deficiency is prevalent in most developing countries and affected more than three billion people worldwide. Therefore, Zn-biofortification is recommended to prevent Zn-deficiency in humans.

FAO reports that 50% of the soils have inadequate Zinc (Shaikh and Saraf, 2017) or Zn is fixed in compounds which are unavailable to plants. In soil most of the zinc is present as sphalerite (ZnS), zincite (ZnO), hopeite  $[\text{Zn}_3(\text{PO}_4)_2 \cdot 4\text{H}_2\text{O}]$ , zinkosite ( $\text{ZnSO}_4$ ), franklinite ( $\text{ZnFe}_2\text{O}_4$ ), and smithsonite ( $\text{ZnCO}_3$ ) (Shaikh and Saraf, 2017). Plants can uptake zinc in the form of  $\text{Zn}^{2+}$  cation, but soil solution contains a minor fraction of it. The unavailability of zinc soluble form in the soil directly affects the plant growth and its grain quality and thus human health. Zinc fertilizers have been used to overcome its deficiency, but it leads to increased economic burden as well as threatened the public health and environment. Therefore, farmers are encouraged to use alternative eco-friendly approaches for sustainable agriculture.

PGPR can increase Zn-availability to plants by solubilizing complex Zn compounds and thus alleviate Zn deficiency in plants (Saravanan et al., 2011). Zinc biofertilizers containing effective zinc solubilizing bacterial strains (ZSB) help to enhance the availability of zinc and improve plant growth and development. A pioneering study in this area reported the solubilization of ZnO, ZnS, and  $\text{ZnCO}_3$  by *Bacillus* sp. and *Pseudomonas fluorescens* isolated from the garden and paddy soil (Saravanan et al., 2011). Later on, several efficient PGPR strains including *Acinetobacter*, *Bacillus thuringiensis*, *Burkholderia cenocepacia*, *Gluconacetobacter diazotrophicus*, *Pseudomonas aeruginosa*, *P. striata*, *Serratia liquefaciens*, and *S. marcescens* have been reported with the ability to solubilize zinc and improved the plant growth, zinc content, and yield of various crops. The Zn-solubilization mechanism is similar to the P-solubilization phenomenon where solubilization is done by acidification or chelation (Saravanan et al., 2011). ZSB produce organic acids such as acetic acid, formic acid, gluconic acid, citric acid, 2-ketogluconic acid, lactic acid, malic acid, and oxalic acids in the rhizosphere subsequently acidifying the surrounding areas. These organic acids chelate with the cations bound to the zinc compound and enhance zinc solubility.

Studies have documented the mitigation of Zn deficiency with subsequent increase in yield through the inoculation of ZSB in rice (Vaid et al., 2014), wheat (Kamran et al., 2017), maize

(Goteti et al., 2013), mung bean (Iqbal et al., 2010), and soybean (Ramesh et al., 2014). A *Rhizobium* strain RL9 also showed Zn solubilization ability and improved the nodulation, dry matter, yield, leghemoglobin, and grain protein of lentil (Kumar et al., 2019).

### Fe-sequestering

Iron (Fe) one of the dominant micronutrients, plays an important role in regulating the cellular processes essential for the growth and development of plants. It is an important cofactor in enzymes, involved in photosynthesis, regulation of respiration, synthesis, and protection of DNA, and metal homeostasis (Zhang X. et al., 2019). Although iron is the fourth most abundant element on earth, its availability for plants is low, particularly in calcareous soils. At neutral and alkaline pH, it exists in oxidized ferric ( $\text{Fe}^{3+}$ ) form which is highly insoluble and inaccessible for plants (Kramer et al., 2019). The low uptake of Fe in plants from iron-deficient soil leads to decreased photosynthesis, chlorosis, yield, and quality of crops (Zhang X. et al., 2019). Moreover, over 30% of the world's population is severely affected by iron deficiency leading to anemia which is a nutritional disorder in humans.

Plants use different strategies to obtain this essential trace element from the environment. One most important strategy is microbe-mediated iron uptake in plants. PGPR produce low molecular weight, high-affinity ferric iron chelator, known as siderophores which enable iron acquisition through specific uptake systems. The siderophores are divided into three main groups i.e., hydroxamates, catecholates, and carboxylates depending upon the functional group used to form complexes with the ferric (Shameer and Prasad, 2018). The siderophores are strongly bound with the insoluble ferric ion ( $\text{Fe}^{3+}$ ) through the ligands and form a soluble complex in soil. The siderophore receptors present at the cell membrane of plant roots recognize the Fe-siderophore complex and are taken up by the cell (Singh, 2020). Besides the acquisition of iron from insoluble hydroxide, siderophore can also acquire iron from ferric phosphate, ferric citrate, ferric transferring iron bound to plant flavones pigment, glycosides, and sugar (Ghosh et al., 2020). Several bacteria such as *Agrobacterium tumefaciens*, *Azospirillum*, *Azotobacter*, *Bacillus*, *E. coli*, *Enterobacter*, *Mycobacterium*, *Neisseria gonorrhoeae*, *Paracoccus denitrificans*, *P. fluorescens*, *Rhizobium meliloti*, *Serratia*, and *Streptomyces*, etc. have been reported to synthesize siderophores (Ghosh et al., 2020; Singh, 2020). Application of siderophore-producing bacteria have shown the improvement in growth and productivity of various crop plants such as rice (Karnwal, 2017), wheat (Gull and Hafeez, 2012), maize (Sah et al., 2017), and chickpea (Khalid et al., 2015) by increased availability of Fe. It has been reported that siderophores increased the chlorophyll contents and plant biomass along with an increase in iron concentration in sunflower under water stress conditions inoculated with *Pseudomonas* spp., *Enterobacter* spp., and *Bacillus sporothermodurans* (Pourbabaee et al., 2018). Furthermore, the PGPR strains *Agrobacterium* sp., *Alcaligenes* sp., *Bacillus* sp. *Pantoea* sp., and *Staphylococcus* sp., improved the organic acids in leaf and the Fe contents in leaf, root, and soil, as well as enhanced the activity of ferric chelate-reductase

(FC-R) enzyme in pear grown under calcareous soil conditions (Ipek et al., 2017).

## Nitrogen Fixation

Nitrogen (N) constitutes about 2% of the total dry matter of a plant and is essentially required for plant growth. Plants need N for the synthesis of nucleic acid, proteins, and enzymes (Bano and Iqbal, 2016). Its deficiency leads to reduced growth, yellowing of leaves, and reduced branching in legumes. The dinitrogen gas ( $N_2$ ) that represents about 80% of the atmosphere is not accessible to plants. Plants can only take up soil-available in the form of ammonia and nitrates through their roots. The ammonium form is directly assimilated into amino acids and stimulates root branching to increase the surface for the uptake of nutrients as well as results in higher amino acid, chlorophyll contents, sugar, and starch. While the nitrate has to be converted into ammonium before it can be used (Beeckman et al., 2018). Nitrate improves the uptake of more nutrients through lateral root elongation and has a more direct effect on different signaling pathways (O'Brien et al., 2016). Thus, both nitrogen sources have significance for plant growth.

The process in which inert  $N_2$  gas is converted to a metabolically tractable form in the soil is called nitrogen fixation. The manufacturing of synthetic N fertilizer through the industrial process is an expensive process as it needs six times more energy than required to produce either phosphorus (P) or potassium (K) fertilizers. During the last 45 years, the demand for nitrogen fertilizers has enhanced from 12 to 107 Tg year<sup>-1</sup> and is expected to increase to 111.5 Tg year<sup>-1</sup> in the year 2022 (FAO, 2019). On the other hand, the yield has been significantly decreased due to the poverty of farmers who are unable to apply costly synthetic fertilizer demands to the crops.

Biological Nitrogen Fixation (BNF), the process in which elemental nitrogen is converted to ammonia by bacteria is an alternative source of N for plants. These nitrogen-fixing bacteria are ubiquitous in nature and function under different environmental conditions. The input of nitrogen into soil through BNF ranges from 0 to 60 kg ha<sup>-1</sup> year<sup>-1</sup> (Reghuvaran et al., 2012) with an estimated contribution of 175 million metric tons annually covering 70% of all annual fixed nitrogen on the Earth (Lodewyckx et al., 2002). There are generally two categories of nitrogen-fixing microorganisms; (a) symbiotic and (b) non-symbiotic bacteria. The most common symbiotic  $N_2$  fixing bacteria able to infect legumes include *Rhizobium*, *Bradyrhizobium*, *Ensifer*, *Azorhizobium*, and *Mesorhizobium*, etc. while *Frankia* nodulates non-leguminous trees and shrubs. These rhizobia infect the root hair, stimulate root hair curling, and leads to the formation of infection thread. The bacterial cells enter the plant cells through infection threads and result in the formation of a nodule wherein the rhizobia reside and fix nitrogen for plants (Andrews and Andrews, 2017). The non-symbiotic diazotrophic bacteria include *Arthrobacter*, *Azoarcus*, *Azospirillum*, *Azotobacter*, *Enterobacter*, *Mitsuaria*, *Pseudomonas*, etc. which fix atmospheric nitrogen in the free-living form (Gupta et al., 2015). The nitrogen-fixing bacteria improve the soil  $NH_4^+$  concentrations, rhizobacterial population levels, soil nitrogenase activity as well as the growth and N

uptake in plants (Masood et al., 2020). Similarly, the application of biofertilizer produced from rhizobia (*Bradyrhizobium* strains) and free-living PGPR (*Streptomyces griseoflavus*) improved the growth and yield of several plants such as mung bean, cowpea, and soybean (Htwe et al., 2019).

BNF is carried out by a nitrogenase complex that consists of two components (i) dinitrogenase reductase (an iron protein) which provides high reducing power electrons and (ii) dinitrogenase which uses the electrons to reduce nitrogen to ammonia and has a metal cofactor. There are three different forms of nitrogenase classified based on metal cofactor. (a) Mo-nitrogenase whose cofactor contains molybdenum, (b) V-nitrogenase which contains a prosthetic group with vanadium, and (c) Fe-nitrogenase contains only iron (Ahemad and Kibret, 2014). Symbiotic as well as free-living diazotrophs use *nif* genes for the fixation which include structural genes that are involved in the biosynthesis of iron-molybdenum cofactor, iron protein activation, donation of electrons, and regulatory genes required for the functioning of the enzyme. The *nif* genes are found in the form of 20–24 kb cluster with seven operons that encode 20 different proteins in diazotrophs (Glick, 2012). The amino acid sequence of *nifH* is highly conserved and generally used to study the evolution of nitrogen-fixing bacteria. It is also widely used to analyze the diversity of diazotrophs in the soil (Gaby et al., 2018). Investigation of diazotrophs community structure using metagenomics based on 185 nitrogenase sequences identified a key group of diazotrophs including *Anaeromyxobacter*, *Azoarcus*, *Bradyrhizobium*, *Frankia*, *Geobacter*, *Nostoc*, and *Pelobacter* based on *nifH* phylogeny (Nash et al., 2018). In the Amazon rainforest, a shift in community composition and abundance of diazotrophs in response to deforestation (forest-to-pasture conversion) was evaluated using sequence analysis of the marker gene *nifH* (Mirza et al., 2014). This change in community composition of diazotrophs is a consequence of higher demand for N of pasture plant communities.

Besides the ability of PGPR to fix  $N_2$  to ammonia, they also have a great impact on nitrogen nutrition of plant by increasing the uptake of nitrate ( $NO_3^-$ ) (Beeckman et al., 2018). The PGPR can increase the uptake of nitrate directly by stimulating  $NO_3^-$  transport systems or indirectly as a consequence of stimulated lateral root development. It has been reported that two putative nitrate transporter genes i.e., *NRT2.5* and *NRT2.6* appeared to be strongly upregulated in response to the inoculation of *Phyllobacterium brassicacearum* in *Arabidopsis thaliana* (Kechid et al., 2013). Similarly, the nitrate transporter genes were found to be upregulated in rice roots during interactions with *Azospirillum brasilense* (Thomas et al., 2019). Calvo et al. (2019) reported that inoculation of PGPR consortia of *Bacillus*, *Lysinibacillus*, and *Paenibacillus* strains upregulated the four ammonium and five nitrate uptake genes along with a significant increase in plant biomass, chlorophyll content, and nutrient uptake in *A. thaliana* (Calvo et al., 2019).

Although, nitrate is a plant-available form, in the soil it is highly prone to leaching due to no adherence with soil particles, and thus becomes inaccessible for plants. Therefore, ammonium is the more efficient form of nitrogen under field conditions, but it is also oxidized to nitrate. Plants can

produce nitrification-inhibiting compounds which are known as biological nitrification inhibitors (BNIs) (Beeckman et al., 2018). The exposure of pasture grasses, sorghum, and wheat to more  $\text{NH}_4^+$  showed an increase in the synthesis and release of BNIs. Application of BNI promoted the root branching along with increased nutrient uptake providing a dual strategy to enhance fertilizer efficiencies (Liu et al., 2016b).

### Phytohormone Production

Phytohormones influence the physiological functions of plants at very low concentrations as chemical messengers. The phytohormones are key determinants of plant behavior and play a leading role in various physiological and developmental processes. Traditionally, plant hormones have been divided into five different classes: auxin, cytokinins, gibberellins, abscisic acid, and ethylene (Oleńska et al., 2020). Besides, several phytohormones such as jasmonate, brassinosteroids, and salicylic acid also play significant roles in plant growth and development particularly under biotic and abiotic stress conditions (Wong et al., 2015; Kang et al., 2016). A wide range of rhizosphere inhabiting bacteria can produce phytohormone for facilitating plant growth and development. The phytohormones produced by plants and rhizobacteria are involved in all the communication in plant cells (Maheshwari et al., 2015).

#### Auxin

Auxin is the most imperative phytohormone which controls nearly all aspects of plant development. Indole-acetic acid (IAA) is the most common, well-characterized auxin produced by bacteria and plants. In plants, IAA plays an important role in apical dominance, division, and cell differentiation, seed germination, and the development of roots. It also contributes to processes like photosynthesis, biosynthesis of metabolites, and stress resistance (Maheshwari et al., 2015). Bacteria-produced IAA promotes the root length and surface area for enhanced uptake of nutrients and water. The majority of the microbes (>80%) inhabiting the rhizosphere are capable of synthesizing and releasing auxin (Oleńska et al., 2020). Tryptophan has been identified as the main precursor of auxin biosynthesis. There are three main pathways involved in IAA synthesis by microbes: (1) Indole acetic acid synthesis via intermediates indole-3-pyruvic acid and indole-3-acetic aldehyde, is found in bacterial genera like *Erwinia*, *Agrobacterium*, *Pseudomonas*, *Azospirillum*, *Bradyrhizobium*, *Enterobacter*, *Klebsiella*, and *Rhizobium*. (2) IAA biosynthesis via tryptamine and indole-3-acetic aldehyde which has been reported in *Azospirillum* and *Pseudomonas*. (3) The IAA synthesis via indole-3-acetamide (IAM) formation, which operates in *Agrobacterium*, *Erwinia*, and *Pseudomonas* strains (Tahir and Sarwar, 2013). The indole-3-acetamide (IAM) pathway commonly found in bacteria, involves the conversion of tryptophan to indole-3-acetamide by the enzyme tryptophan-2-monooxygenase (IaaM), and then IAM is converted to IAA by the enzyme IAM hydrolase (iaaH). The two genes involved in the IAM pathway (iaaM and iaaH) have been identified in *Agrobacterium*, *Bradyrhizobium*, *Pantoea*, *Pseudomonas*, and *Rhizobium* strains. Genes involved in IAM pathway have been localized on the chromosome (*Pseudomonas* spp.) as well as

on plasmids (e.g., *Pantoea agglomerans*). These auxin-producing PGPR modulate the plant response as reported through cucumber-*Bacillus amyloliquefaciens* strain SQR9 system, which showed that inoculation of *Bacillus amyloliquefaciens* leads to the high amount of tryptophan secretion through roots of cucumber, subsequently increasing the IAA synthesis by bacteria inhabiting the rhizosphere (Liu et al., 2016a). Several studies reported the improvement in root formation, growth, and yield of various crops through auxin-producing PGPR (Ali et al., 2014; Imran et al., 2015; Majeed et al., 2015). Moreover, bacteria-derived auxins might be involve in the mitigation of deleterious effects of various abiotic stresses, like salinity, drought, and soil pollution (Kudoyarova et al., 2019). The application of auxin producing *Bacillus thuringiensis*, *B. amyloliquefaciens*, *B. simplex*, *Enterobacter aerogenes*, *Moraxella pluranimalium*, and *Pseudomonas stutzeri* strains showed positive effect growth and yield parameters of wheat grown under drought condition and suggested to be used for rhizosphere engineering in drylands (Raheem et al., 2018).

#### Cytokinin

Cytokinins (CK) are important signaling molecules that are involved in the regulation of plant growth and development. This plant hormone plays a crucial role in various physiological and developmental processes including apical dominance, seed germination, nodule formation, flower and fruit development, root elongation, vascular development, and plant-pathogen-interactions (Osugi and Sakakibara, 2015). The bacterial genera such as *Bacillus*, *Escherichia*, *Agrobacterium*, *Methylobacterium*, *Proteus*, *Pseudomonas*, and *Klebsiella* can produce cytokinins (Maheshwari et al., 2015). The pathway of cytokinin biosynthesis involves the formation of N<sup>6</sup>-isopentenyl adenosine monophosphate from adenosine monophosphate (AMP) and dimethylallyl pyrophosphate (DMAPP). While in bacteria, the synthesis involves the transfer of isopentenyl moiety from hydroxyl dimethyl butenyl diphosphate (HMBDP) to AMP (Wong et al., 2015). Bacteria synthesize and release cytokinin in the rhizosphere, subsequently increasing the contents of cytokinin in the soil solution and growing plants which leads to stimulation of the plant growth. Furthermore, these PGPR mitigate the effect of stresses as cytokinin-producing *Bacillus subtilis* alleviated the drought stress and increased the plant growth of *Platycladus orientalis* (Liu et al., 2013). The bacterial cytokinin also reported to be involved in regulating the defense system of the host plant against pathogens. For instance, cytokinins produced by *P. fluorescens* efficiently controlled the *P. syringae* infection, maintained the tissue integrity and yield in *Arabidopsis* (Großkinsky et al., 2016).

#### Gibberellin

Gibberellins (GAs) are plant hormones that are involved in nearly all stages of plant growth and development, including embryogenesis, stem elongation, flowering, leaf expansion, and ripening of fruits (Binenbaum et al., 2018). Natural GAs are the conjugates of  $\beta$ -D-glucose, but they are also found in free and bound states. Like auxins and cytokinin, bacteria also possess the ability to synthesize GAs. Among bacteria,



the characterization of gibberellins (GA1, GA4, GA9, and GA20) was first reported in *Rhizobium meliloti* (Atzorn et al., 1988). Since then, several bacterial genera such as *Acetobacter*, *Azospirillum*, *Herbaspirillum*, *Bacillus*, and *Pseudomonas* have been reported for GAs production (Maheshwari et al., 2015). The function of GAs in bacteria is still not known but they might act as signaling molecules toward plants. However, the stimulation of plant growth and yield is evident through various studies. For instance, inoculation of *Solanum lycopersicum* with GAs producing *Promicro monospora* strain upregulated the GA biosynthesis pathway while downregulated the synthesis of abscisic acid in the plant (Kang et al., 2012). A rhizobacterium *Leifsonia soli* showed GAs production ability and stimulated the growth of cucumber, radish, and tomato plants (Kang et al., 2014a). Another GA producing *Leifsonia xyli* strain maintained the growth of *Solanum lycopersicum* under copper (Cu) stress condition by modulating the endogenous polyphenol, flavonoid, and amino acids (arginine, proline, glycine, phenylalanine, threonine, and glutamic acid) reduced superoxide dismutase activity (Kang et al., 2017). The role of GAs in the alleviation of temperature stress has also been reported. *Serratia nematodiphila* enhanced the endogenous GA4 and ABA while reduced the salicylic acid and jasmonic acid contents of the host plant to mitigate the deleterious effects of low temperature on *Capsicum annuum* (Kang et al., 2015). Similarly, *Bacillus tequilensis* improved the plant biomass of the soybean by producing GA1, GA3, GA5, GA8, GA19, GA24, and GA53 under the high-temperature stress (Kang et al., 2019b).

### Biocontrol of Plant Pathogens

The phytopathogenic microorganisms and abiotic stresses are threatening global crop production. The vigorous application of pesticides and fungicides to prevent pathogenesis and growth of pathogenic microorganisms has degraded and contaminated the soil quality (Gopalakrishnan et al., 2015). The application of PGPR is an effective and environmentally safe strategy to achieve sustainable plant growth and soil fertility. This approach encourages the manipulation of wide-ranging PGPR to suppress pathogen growth. The use of biocontrol agents as pesticides has reduced the demand for agrochemicals because they use various mechanisms to kill the phytopathogens such as ACC-deaminase activity, production siderophores, lytic enzymes, quorum sensing (QS), induced systemic resistance (ISR) etc. (Torres-Cortés et al., 2018; Ali et al., 2020; Chen et al., 2020; Rodríguez et al., 2020).

In recent years, biocontrol of phytopathogenic microbes through other microorganisms has emerged for sustainable agriculture (Karthika et al., 2020). The antagonistic characteristics of PGPR play a key role in managing plant diseases while improving soil fertility and crop production (Liu Y. et al., 2017). The mechanisms utilized by PGPR for biocontrol are as follows.

#### Production of ACC-deaminase

In response to pathogenic infection, plants accumulate several hormones, including ethylene, which activates secondary stresses like root hair curling, oxidative stress, and nutrient deficiency, leading to decreased metabolism, growth rate, crop

productivity, development, and eventually results in plant death (Premachandra et al., 2016). PGPR mitigate the harmful effects of phytopathogens on the development and growth by interacting with host plants.

The ability of soil microbes for producing ACC deaminase enzyme is the key feature that decreases the level of pathogen-induced ethylene and its subsequent deleterious effects. A variety of microorganisms are reported that can produce ACC-deaminase and help plants in overcoming the stressed condition such as *Bacillus*, *Pseudomonas*, *Azospirillum lipoferum*, *Rhizobium*, and *Ralstonia solanacearum* (Ali et al., 2020). During stress, the concentration of ACC increases within the roots of the plant. The microbial populations producing ACC deaminase hydrolyses the exuded ACC into ammonia and  $\alpha$ -ketobutyrate, which ultimately reduces the stress induced by ethylene and its related growth inhibition (Saraf et al., 2010).

These rhizospheric microbes also possess the potential to improve the efflux of ACC from roots. The plant secretes a significant quantity of ACC for maintaining equilibrium both within and outside the roots. Consequently, the growth of ACC-deaminase producing beneficial microbes in the rhizosphere is accelerated, thus reducing the concentration of ACC in the roots of the plant and resulting in the decrease in ethylene production and improved plant growth (Nadeem et al., 2010; Gamalero and Glick, 2015). Furthermore, the microorganisms use this secreted ACC as the source of nitrogen. The activity of microbial ACC deaminase converts it again into ammonia &  $\alpha$ -ketobutyrate, which controls the ethylene production. Thus, PGPR having the potential to produce ACC deaminase serves as a soldier for the detrimental impact of ethylene and acts as biocontrol agents against different pathogenic attacks such as *Botrytis cinerea*, *Rhizoctonia solani*, *Fusarium oxysporum*, *Xanthomonas oryzae*, *Pythium ultimum*, *Phytophthora* sp., and *Sclerotium rolfsii* etc. However, it is crucial to decipher the precise mechanism of ACC deaminase activity. Such ACC deaminase producing-PGPR is fascinating scientists to manipulate them at the molecular level and thus establish a vision for a more specific use.

#### Production of siderophores

In soil, iron is present in an abundant amount, but it is not frequently accessible for soil microorganisms or plants. The oxidized form ( $\text{Fe}^{3+}$ ) reacts to produce hydroxides and insoluble oxides like  $\text{Fe}(\text{OH})_3$  that are not easily used by both microorganisms and the plants (Mehnaz, 2013). To solve this issue, PGPR produces siderophores. Siderophores are iron-chelating, high affinity low molecular weight (<1 kDa) compounds having the potential to provide iron to the cell or tissues of a plant (Hider and Kong, 2010). They enhance plant development and growth by improving the chances of iron availability in the rhizospheric region (Subramaniam and Sundaram, 2020).

The siderophores reduces pathogenic microbes by creating iron competition in the rhizospheric region (Reed et al., 2015) and improves plant growth. PGPR-synthesized siderophores show a high affinity for rhizospheric  $\text{Fe}^{3+}$  and retain almost all of the free iron, increasing plant uptake of iron and inhibiting the growth of pathogenic microbiota. Bacterial siderophores are



divided into four major classes (phenol catecholate, carboxylate, pyoverdines, and hydroxamates) depending on their ligands types, iron-directing functional groups, and structural features. Siderophores producing rhizobacteria colonize plant roots and eliminates all other microflora from this natural habitat. During extremely competitive circumstances, the acquisition of iron through siderophores determines the competition outcomes for various carbon sources available as the consequence of rhizodeposition or root exudation. In green gram, *Pseudomonas* sp. produced siderophores improved plant growth and inhibited disease progression (Sahu and Sindhu, 2011). *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* produces pyoverdine and pyochelin form of siderophores. These microorganism-produced siderophores increase the uptake of Fe and inhibit the growth and development of pathogenic microbes due to competition for iron scavenging (Shen et al., 2013). Studies have reported several siderophores producing rhizobacteria such as *Klebsiella*, *Bacillus*, *Bradyrhizobium*, *Streptomyces*, *Serratia*, and *Rhizobium* (Mustafa et al., 2019). Many studies related to the research of PGPR have focused mainly on the potential of PGPR to produce metabolites and siderophores that lead to antibiosis (Maksimov et al., 2011).

### Antibiosis

One of the most studied and powerful mechanisms against pathogenic microbes is antibiotic production by rhizobacterial strains. Antibiotic production is an extremely efficient mechanism of rhizobacteria for inhibiting pathogenic infections in various plants (Islam et al., 2016). Studies reported six different groups of antibiotics including phloroglucinols, phenazines, pyoluteorin, cyclic lipopeptides, and pyrrolnitrin. All these antibiotics are hydrogen cyanide, volatile, and diffusible, mostly associated with the biological control of various root infections. Lipopeptide bio-surfactants have been investigated in the biological control of various pathogenic microbes synthesized by *Bacillus* and *Pseudomonas* species (Ali et al., 2014, 2020). These bio-surfactants are potentially useful for competitive interactions against organisms such as fungi, bacteria, oomycetes, nematodes, and protozoa. *Stenotrophomonas*, *Streptomyces*, and *Bacillus* sp. produce different antimicrobial compounds like kanosamine, zwittermicin A, and oligomycin A, and xanthobaccin that inhibit the growth of phytopathogens on a large scale (Liu et al., 2018a).

One of the extensively studied and efficient antibiotic is 2,4-Diacetylphloroglucinol (DAPG), mainly synthesized by *Pseudomonads*. DAPG damages membrane and inhibit zoospores formation of *Pythium* sp. and also biocontrol bacterial canker disease of tomato plants (Bhattacharyya and Jha, 2012; Lanteigne et al., 2012). *Pseudomonads* also produce phenazine antibiotic which has redox activity having the potential to suppress phytopathogens, including *Gaeumannomyces graminis* and *Fusarium oxysporum*. *Bacillus* sp. produces several antibiotics such as polymyxin, circulin, and colistin that are vibrant and effective against phytopathogens (Maksimov et al., 2011; Liu et al., 2018a). Excessive application of antibiotic-producing PGPR for growth promotion and biocontrol of pathogenic microbes has developed the ISR mechanism in several phytopathogens causing resistance against specific

antibiotics because of the increased dependency of these strains. Different studies exploited specific biocontrol isolates that produce several antibiotics to avoid this kind of popularity.

### Lytic enzyme production

Lytic enzyme secretion and production are the essential characteristics of biological control agents in preventing the development of pathogenic microbes (Xie et al., 2016). The mechanism of action of lytic enzymes is to disrupt the structural stability and integrity of cell walls of target pathogens (Budi et al., 2000; Tariq et al., 2017). PGPR produces different lytic enzymes such as dehydrogenase, chitinases,  $\beta$ -glucanase, proteases, phosphatases, lipases (Lanteigne et al., 2012; Joshi et al., 2015). PGPR through the action of these lytic enzymes play a significant role plant growth mainly by protecting plants against several pathogenic fungi such as *Sclerotium rolfsii*, *Botrytis cinerea*, *Fusarium oxysporum*, *Pythium ultimum*, *Phytophthora* sp., and *Rhizoctonia solani* (Yadav et al., 2016; Yasmin et al., 2016; Chen et al., 2020).

Studies reported the antifungal and chitinolytic actions of *Serratia marcescens* to inhibit pathogenic fungi such as *Fusarium oxysporum* and *Rhizoctonia solani* (Karthika et al., 2020). The application of *Serratia marcescens* on mycelia of pathogenic fungi inhibits fungal growth by hyphae curling, rupture of hyphae tip, and partial hyphae swelling. *Streptomyces* sp. and *Paenibacillus* strains produce cellulase and  $\beta$ -1,3-glucanase that cause degradation of pathogenic fungi cell walls, including *Sclerotinia sclerotiorum* and *Fusarium oxysporum* (Mun et al., 2020). Moreover, *Bacillus cereus* and *Bacillus cepacia* produce amylase,  $\beta$ -1,3-glucanase, cellulase, protease, xylanase, and lipase, which ruptures the cell walls of several soil-borne pathogenic microbes (Karthika et al., 2020).

### Induced systemic resistance

Induced resistance is the physiological state of improved defensive ability elicited due to particular environmental stimuli and leads to strengthen and stimulate the innate defense system of the plant against subsequent pathogenic attacks. Bio-priming the plants using PGPR induce systemic resistance to a wide range of plant pathogens (Naznin et al., 2013). In plants, plant-growth-promoting fungi, and bacteria in their rhizosphere play a role in triggering ISR (Pieterse et al., 2014). Beneficial microbes that elicit ISR can suppress the immune response in roots locally. The exudation pattern in roots are altered due to the pathogen attack that in turn employs some selective microbiota for ISR induction (Berendsen et al., 2018; Chialva et al., 2018). For example, in tomato plants, the heterogeneous communities of microorganisms residing in the endosphere and episphere are involved in the modulation of phenylpropanoid metabolism that results in fortification of the cell wall and provides protection against *Fusarium oxysporum* f. sp. *Lycopersici* (Cha et al., 2016). This fortification of the cell wall is not only involved in the protection of plants against biotic stress but also the abiotic challenges like saline conditions, suggesting cross-talk between immunity and stress tolerance in plants (Kesten et al., 2019). In ISR, ethylene and jasmonate signaling is involved, both of these hormones play a significant role in enhancing the defense

response of plants against a wide variety of phytopathogens (Bukhat et al., 2020).

The recruitment of plant defense and jasmonate signaling modulated due to different microbial communities in the rhizosphere also affects the herbivory of insects above the ground (Yuan et al., 2018). Several bacterial components are involved in inducing ISR such as 2, 3-butanediol, 2,4-diacetylphloroglucinol, LPS, acetoin, cyclic lipopeptides, homoserine lactones, siderophores, and flagella (Torres-Cortés et al., 2018). Rhizobacteria mediated ISR induction in plants stimulates the release of some antimicrobial components such as benzoxazinoids and coumarin, which further increase the induction of ISR triggering strains (Hu et al., 2018; Stringlis et al., 2018). Thus, the immune responses of the plant can be engineered to recruit microbes for providing plant resilience over thousands of generations.

### Rhizosphere Manipulation Through Quorum Sensing Signals

Another effective agricultural approach is the intervention of QS systems in phytopathogens or biocontrol agents. QS is the inter-cellular communication mechanism in which microbial gene expression is coupled with the concentration of bacterial cells, is regulated through the diffusion of particular signaling components such as AHLs (Awan et al., 2011). This mechanism controls the expression of various phenotypic traits, and most of them are reported to be involved in the microbial pathogenesis of several economically significant agricultural pathogens (Liu et al., 2018b). For example, QS regulates various phenotypes in *P. atrosepticum*, *Pectobacterium carotovorum*, *Ochrobactrum*, *Agrobacterium tumefaciens*, *Pseudomonas syringae*, *Ralstonia solanacearum*, *Dickeya solani*, and *Erwinia amylovora* (Imran et al., 2014). The interruption of this sensing system is another interesting approach in agriculture to combat the pathogenicity of pathogenic strains (Grandclément et al., 2016).

Quorum quenching (QQ) is a well-known interrupting strategy of a sensing system that is involved in the enzymatic hydrolysis or/degradation of signaling molecules (AHLs). Oxidoreductases, lactonases, and acylases enzymes are involved in the modification and the degradation of AHLs (Fetzner, 2015). Studies showed that this approach produces promising results in decreasing the pathogenicity of many phytopathogens (Chane et al., 2019; Rodríguez et al., 2020; Ye et al., 2020). Instead of killing or suppressing the growth of infectious agents by antibiotics, QS-mediated defense decreases the virulence and infection without compromising the growth of bacterial pathogens (Defoirdt, 2018; Torres et al., 2019; Rodríguez et al., 2020; Ye et al., 2020). This strategy neither disrupts the essential genes of microbes nor results in resistance development; thus this approach is more efficient in the long term as compared to antibiotics, though further studies are needed to confirm this claim (Bjarnsholt et al., 2010; Defoirdt, 2018). Several studies have reported resistance development against QS interference, and they also proposed that this QS interfering strategy might be less likely to transfer resistance in other microorganisms (García-Contreras et al., 2016). Remarkably, some QS bacterial strains were isolated from the aquatic environment (Romero et al.,

2012), while some are indigenous isolates of plants but their effect on growth and other parameters of plants is still not explored.

### Rhizosphere Intervention Against Abiotic Stress

Various studies have also reported rhizobacteria mediated tolerance in plants against various abiotic stresses (Rajput et al., 2018; Asghari et al., 2020; Khan et al., 2020). However, the tolerance levels depend on the ability of these soil microbes to stimulate plant systems for expressing stress-responsive EPS, transcription factors, scavenging of ROS species, proline synthesis, and biomass stabilization (Habib et al., 2016; Chatterjee et al., 2020; Mukhtar et al., 2020). Understanding and exploration of these pathways help in better utilization of these microorganisms to alleviate different abiotic stresses. **Table 1** summarizes some of the studies published on the induction of biotic or abiotic stress tolerance by PGPR inoculation. The induction of abiotic stress tolerance is discussed in the sections below.

#### Drought

Studies proposed that drought-tolerant rhizobacterial strains enhance plant development and growth during water deficit conditions. PGPR exhibit various tolerance and adaptation mechanisms to overcome reduced water potential, including thickening of walls, maintaining dormant stage, osmolyte accumulation, spore formation, and EPS production (Kour et al., 2019; Asghari et al., 2020). Besides supplying water content, PGPR also provides an optimum environment and nutrition for the persistent growth of plants (Kang et al., 2014b; Staudinger et al., 2016). The possible PGPR mechanisms involved in inducing drought tolerance include the production of phytohormones (IAA, ABA, and cytokinins), ACC deaminase activity, ISR, and EPS. The application of PGPR during drought stress produces IAA, which regulates shoot growth, cell division, differentiation of lateral and adventitious roots, and vascular tissues (Khan N. et al., 2019). Seed priming or co-inoculation with rhizobacterial strains triggers ABA production, which improves the molecular and physiological characteristics of plants by regulating drought stress-related transcription factors and hydraulic conductivity of roots to induce tolerance in a water deficit environment (Jiang et al., 2013). *Azospirillum brasilense* improved the negative effects of drought stress in *Arabidopsis thaliana* due to the increased production of ABA (Cohen et al., 2015).

In drought stress, accumulation of proline, trehalose, and glycine betaine helps bacteria to retain their membrane permeability, integrity, and functional proteins (Asghari et al., 2020). Plant biomass, nutrient uptake, growth, survival, and relative water content are improved after inoculation with drought-tolerant microbiota (Dai et al., 2019). In *Trifolium*, co-inoculation with *P. putida* and *B. thuringiensis* accumulates proline content, resulting in reduced stomatal conductance and electrolyte leakage (Ortiz et al., 2015). The inoculation of beneficial microbes reduces the activity of antioxidants while increasing the production of sugar and free amino acids for stimulating abiotic stress tolerance (Vardharajula et al., 2011). Drought-tolerant rhizobacterial

**TABLE 1 |** Role of rhizosphere engineering with PGPR on plant growth and stress tolerance.

Rhizosphere/Plant host	Microbes used	Effect on plant/soil	References	
Growth stimulation under normal conditions in rhizosphere				
Wheat	<i>Enterobacter</i> sp., <i>A. chlorophenolicus</i> , <i>S. marcescens</i> , <i>B. megaterium</i>	P-solubilization, IAA, HCN, N-fixation, Gibberellin, Siderophores	Kumar et al., 2015	
Potato	<i>Azospirillum</i> sp.	N-fixation, IAA production	Naqqash et al., 2016	
Soybean	<i>Bacillus cereus</i>	Sideophore, IAA, P-solubilization, EPS	Arif et al., 2017	
<i>Arabidopsis thaliana</i>	<i>Bacillus amyloliquefaciens</i>	Phytohormone production, Lipopeptide	Asari et al., 2017	
Tomato	<i>Bacillus pumilus</i> , <i>Bacillus amyloliquefaciens</i>	HCN, Siderophore, N-fixation, IAA, P-solubilization	Xiaohui et al., 2017	
Potato	<i>Brevundimonas</i> spp.	P-solubilization, N-fixation	Naqqash et al., 2020	
Potato, Rice, wheat, maize, soybean	<i>Serratia</i> spp.	AHLs, IAA production, Phytase activity	Hanif et al., 2020	
Pea	<i>Azospirillum</i> , <i>Agrobacterium tumefaciens</i>	N-fixation, P-solubilization	Ejaz et al., 2020	
Rice	<i>Acinetobacter soli</i> , <i>Bacillus</i> sp., <i>Pseudomonas putida</i> , <i>Pseudomonas mosselii</i> , <i>Arthrobacter woluwensis</i>	P solubilization, ACC deaminase activity, Siderophores production	Aw et al., 2019; Khan M. A. et al., 2019; Rasul et al., 2019	
Maize	<i>Achromobacter xylosoxidans</i> , <i>Azospirillum brasilense</i> , <i>Bacillus subtilis</i> , <i>Bacillus megaterium</i> , <i>Pseudomonas stutzeri</i> , <i>Rhodococcus rhodochrous</i>	IAA production, P solubilization, Zn solubilization	Goteti et al., 2013; Qaisrani et al., 2014; Zahid, 2015	
Chickpea	<i>Mesorhizobium ciceri</i> , <i>Ochrobactrum ciceri</i> , <i>Serratia marcescens</i>	P solubilization, IAA production, Nitrogen fixation	Imran et al., 2015; Zaheer et al., 2016	
Cotton	<i>Bacillus amyloliquefaciens</i> , <i>Bacillus</i> spp.	Production of phytohormones, N-fixation, P solubilization, and antibiotic activity	Fahimi et al., 2014	
Mungbean	<i>Rhizobium</i> , <i>Bradyrhizobium</i> , <i>Bacillus cereus</i> , <i>B. drentensis</i> , <i>B. pumilus</i> , <i>B. subtilis</i> , <i>Enterobacter cloacae</i> , <i>Pseudomonas putida</i> , <i>Ochrobactrum</i>	N-fixation, P solubilization, IAA production, Siderophore production,ACC-deaminase activity	Akhtar and Ali, 2011; Tariq et al., 2012; Mahmood et al., 2016	
Cabbage	<i>B. subtilis</i>	Gibberellins production	Kang et al., 2019a	
Disease infested soils				
Rhizosphere/Plant host	Microbes used	Pathogen	PGPR characteristics	References
Bacterial pathogen				
Tomato	<i>P. fluorescens</i>	<i>Ralstonia solanacearum</i>	Antibiosis activity	Seleim et al., 2011
	<i>Bacillus velezensis</i>		Lipopeptides production	Chen et al., 2020
Wild cabbage	<i>Paenibacillus</i> sp.	<i>Xanthomonas campestris</i>	ISR	Ghazalibiglar et al., 2016
	<i>Bacillus velezensis</i> sp.		Antibiosis activity	Liu et al., 2016
Rice	<i>Pseudomonas</i> sp.	<i>Xanthomonas oryzae</i>	Peroxidase activity, polyphenol-oxidase & phenylalanine-ammonia lyase	Yasmin et al., 2016
Potato	<i>Lysinibacillus</i> sp., <i>Pseudomonas fluorescens</i> , <i>Bacillus subtilis</i> ,	<i>Ralstonia solanacearum</i>	Antibiosis	Djaya et al., 2019
Fungal pathogen				
Peanut	<i>P. fluorescens</i>	<i>Sclerotium rolfsii</i>	Antimicrobial compound	Lohitha et al., 2016
Cucumber	<i>Bacillus amyloliquefaciens</i>	<i>Fusarium oxysporum</i>	Secondary metabolites, ISR, Phytohormones	Liu Y. et al., 2017
Tomato	<i>Paenibacillus lentimorbis</i>	<i>Sclerotium rolfsii</i>	Expression of defense-related & autophagy-related genes	Dixit et al., 2018
	<i>Bacillus cereus</i>	<i>Alternaria solani</i> , <i>Fusarium oxysporum</i>	IAA, Siderophore, Ammonia, ACC deaminase, Catalase, N-fixation	Karthika et al., 2020

(Continued)

TABLE 1 | Continued

Rhizosphere/Plant host	Microbes used	Effect on plant/soil		References
Chili	<i>Talaromyces funiculosus</i>	<i>Colletotrichum capsica</i>	ISR, Callose & Lignin deposition	Naziya et al., 2020
Viral Pathogen				
Cucumber	<i>B. subtilis</i> , <i>Azotobacter chroococcum</i> , <i>P. fluorescens</i>	<i>Cucumber mosaic</i> , <i>Cucumovirus</i>	PR protein, Peroxidase activities, 1, 3-glucanase	El-Borollosy and Oraby, 2012
Black Mustard	<i>Bacillus subtilis</i> , <i>Pseudomonas fluorescens</i>	<i>Turnip mosaic virus</i>	Catalase activity, ISR, Phenol production	Diyansah et al., 2017
Periwinkle	<i>B. pumilus</i> , <i>B. subtilis</i>	<i>Cucumber mosaic virus</i>	Phenol, alkaloid and flavonoid production	Al-Zahrani et al., 2018
Tomato	<i>Azospirillum brasilense</i>	<i>Potato virus X</i>	ISR, Protein accumulation	Lade et al., 2019
Chili	<i>Bacillus amyloliquefaciens</i>	<i>Groundnut-bud necrosis virus</i>	Flagellin, ISR, Phytohormone	Rajamanickam and Nakkeeran, 2020
Abiotic stress				
Plant	PGPR	PGPR characteristics		References
Drought stress				
Soybean	<i>P. putida</i>	JA production, Flavonoids, Antioxidant activity		Kang et al., 2014b
Medick	<i>Sinorhizobium medicae</i>	Nodulation, Nutrient uptake		Staudinger et al., 2016
Maize	<i>Azospirillum</i> sp.	Siderophore, N-fixation, IAA, P-solubilization		García et al., 2017
Chickpea	<i>Bacillus</i> sp.	IAA, HCN, EPS, Ammonia production		Khan N. et al., 2019
wheat	<i>Pseudomonas libanensis</i>	Siderophore, Ammonia, IAA, P-solubilization, ACC deaminase		Kour et al., 2019
Mint	<i>Azospirillum brasilense</i> , <i>Azotobacter chroococcum</i>	Osmolyte accumulation, Phenol production, Antioxidant activity, ABA		Asghari et al., 2020
Salt stress				
Wheat	<i>Planococcus rifietoensis</i>	ACC-deaminase, P solubilization, IAA production,		Rajput et al., 2013, 2018
	<i>Aeromonas</i> spp.	ACC-deaminase, Zn-/P solubilization, IAA production		
Okra	<i>Enterobacter</i> sp.	ACC deaminase, Antioxidant activity		Habib et al., 2016
Sweet and chili pepper	<i>Rhizobium massiliae</i> , <i>Brevibacterium iodinum</i> , <i>Microbacterium oleivorans</i>	Proline, Antioxidant enzyme, Sugar		Hahm et al., 2017
False Flax	<i>P. migulae</i>	ACC activity, Phytohormone production, ABA		Heydarian et al., 2018
Rice	<i>Glutamicibacter</i> sp.	ACC deaminase activity, IAA production		Ji et al., 2020
Heat stress				
Wheat	<i>P. putida</i>	Antioxidant activity, Proline, Total proteins, Sugars, Amino acids		Ali et al., 2011
	<i>Azospirillum brasilense</i> , <i>Bacillus amyloliquefaciens</i>	Secondary metabolites, Antioxidant enzymes		Abd El-Daim et al., 2014
	<i>B. safensis</i>	Antioxidant enzymes, osmolyte accumulation		Sarkar et al., 2018
Soybean	<i>B. aryabhattai</i>	JA, IAA, Antioxidant enzymes		Park et al., 2017
Eucalyptus	<i>Brevibacterium linens</i>	ACC deaminase activity, Volatile compounds		Chatterjee et al., 2020
Tomato	<i>Bacillus cereus</i>	ACC deaminase activity, EPS		Mukhtar et al., 2020

strains such as *P. aeruginosa*, *Proteus penneri*, and *Alcaligenes faecalis* produce sugar, proline, and protein content in maize plants, which helps to improve water potential, growth, and reduces water loss for inducing tolerance against drought conditions (Naseem and Bano, 2014). Drought stress decreased

photosynthetic activity and chlorophyll content in soya bean plants while the application of *P. putida* mitigates water deficit conditions by improving biomass and enhancing photosynthetic machinery (Tiwari et al., 2016; Etesami, 2018).



## Salinity

Halo-tolerant rhizobacterial strains can survive in high saline soils and possess certain properties that help plants to thrive in a saline environment (Abbas et al., 2019). Different studies reported the mitigating role of PGPR against the detrimental effects of high saline conditions on various plants utilizing several mechanisms such as biofilm formation, osmolyte accumulation within the cytoplasm of the plant cell, retaining turgor pressure of salt-stressed cell, and production of EPS that binds with cations, limiting their accessibility for plant cells (Kang et al., 2014b; Egamberdieva et al., 2015; Heydarian et al., 2018; Rajput et al., 2018; Nawaz et al., 2020). PGPR also alleviate salt stress-induced secondary stresses such as osmotic and oxidative stress by producing antioxidants, phytohormones, and ISR signaling (Hahm et al., 2017; Abbas et al., 2019). The isolated *B. subtilis* and *B. pumilus* strains from saline soil showed various PGPR features such as phosphate solubilization, hydrogen cyanide, ammonia, and IAA production, and salt stress tolerance (Damodaran et al., 2013). In rice, co-inoculation with *P. pseudoalcaligenes* and *B. pumilus* results in increased glycine betaine levels and improve tolerance against salinity stress (Jha and Saraf, 2015). *Pseudomonas* sp. and *Acinetobacter* spp. accumulate ACC deaminase and IAA during saline stress to improve the growth of oats and barley plants (Chang et al., 2014).

PGPR induce chemical and physical changes during salinity that lead to increased growth and ISR signaling (Ji et al., 2020). Salt-tolerant strains of *Azospirillum* improved grain weight and growth of salt-stressed wheat plants (Nia et al., 2012). ABA-mediated apoplastic acidification plays a major role in inducing salt tolerance in maize plants. Bio-priming of lettuce seeds with *Azospirillum* improved product quality, shelf life, and growth, during stressed conditions (Fasciglione et al., 2015). *P. stutzeri*, *P. fluorescens*, and *P. aeruginosa* strain isolated from the rhizosphere of tomato plant showed high salt levels and improves tri-carboxylic acid cycle, accumulation of phytohormones and ACC deaminase resulting in enhanced salt tolerance (Tank and Saraf, 2010; De La Torre-González et al., 2018). In rice, inoculation with *B. amyloliquefaciens* enhances salinity tolerance and improves growth by producing auxin, ABA and regulating the expression of several salt-stressed genes (Nautiyal et al., 2013; Shahzad et al., 2017).

## Heat

Global warming is a serious threat to living species, which is becoming an alarming issue worldwide. According to the report of IPCC (2007), the temperature is expected to rise by 1.8 to 3.6°C globally at the end of this century due to changes in climatic conditions. The increase in temperature is considered the biggest challenge in microbial colonization and crop production, resulting in protein degradation and severe cell damage. For instance, heat-shock proteins (HSPs) are specific polypeptides, which an organism can produce due to an increase in temperature. In microorganisms, different genes are implicated in the mechanism of responding to stress because PGPR possesses several mechanisms in inducing tolerance against heat stress. Numerous studies have reported that PGPR inoculation mitigated the detrimental impacts of heat stress in

different plants including sorghum, wheat, chickpea, and tomato (Ali et al., 2009, 2011; Abd El-Daim et al., 2014; Issa et al., 2018). These PGPR induce tolerance by synthesizing different phytohormones, improving biofilm formations, and enhancing levels of HSPs (Ali et al., 2011; Park et al., 2017; Sarkar et al., 2018). In the chickpea plant, the application of *P. putida* showed thermotolerance by the formation of thick biofilm and expression of stress-related transcription factors.

The accumulation of proteins in heat-stressed sorghum plants is another effective mechanism of PGPR (*Pseudomonas* AKM-P6) against abiotic stress (Ali et al., 2009). The potential of *Bacillus cereus* to produce several metabolites such as organic acids, IAA, GAs, essential amino acids, and HSPs enhances thermotolerance in tomato and sorghum plants (Khan et al., 2020; Mukhtar et al., 2020). However, further studies are needed to decipher the function of PGPR mediated expression of HSPs for inducing thermotolerance.

## PGPR-MEDIATED RHIZOSPHERE ENGINEERING FOR AGRICULTURE AND ECOSYSTEM SUSTAINABILITY

In agriculture, it is difficult to apply sustainable and economical systems of farming, which can accommodate the new and advanced technologies (Bhat et al., 2019; Disi et al., 2019; Guo et al., 2019; Hassan et al., 2019b). The rhizosphere determines the plant's health and helps in inducing tolerance against environmental stresses by enhancing nutrient uptake, water availability, and buffering capacity. The knowledge of these plant-microbe interactions can help in developing new sustainable, eco-friendly, and economical systems for agriculture (Kumar et al., 2015; Rakshit et al., 2015; Naqqash et al., 2016). The selective aggregation of microbial populations against abiotic and biotic stresses affects plant defense and immunity in successive generations through legacy effects and soil-plant feedback (Kostenko and Bezemer, 2020). The microbes responsible for disease resistance in plants could be manipulated or engineered, but it requires understanding their interaction with the environment and plant for sustainable agriculture. **Table 1** summarizes different examples of PGPR involved in sustainable agriculture by stimulating growth and mitigating abiotic and biotic stresses in different crops.

The literature advocates that PGPR inoculation leads to sustainable and efficient crop production to feed a growing world population during that time when agriculture faces different environmental constraints. It is essential to fully understand the important characteristics of effective PGPR inoculants before applying them to intensive farming practices. The engineering of biopesticides and biofertilizers with improved performance for sustainable agriculture requires identification and exploration of useful genes associated with complex pathways of rhizospheric colonization, production of secondary metabolites, and specific promoters which are expressed in a particular rhizospheric environment (Hassan et al., 2019a; Hanif et al., 2020). In the future, the investigation for elucidating the role of those genes

involved in microbe-plant interaction will help develop new tools for improving plant and soil health.

## CURRENT CHALLENGES AND FUTURE PERSPECTIVE

This review has explicitly described the heterogeneous group of PGPR present in the rhizosphere with significant potential to transiently or permanently alter the rhizosphere. PGPR inoculants possess the potential for sustainable agriculture as they are one of the accepted and validated substitutes for chemical fertilizers, fungicides, and other chemical-based simulators. Over the past few decades, PGPR have started replacing chemical compounds in silviculture, horticulture, agriculture, and environmental remediation procedure. In PGPR studies, most of the research is limited to the greenhouse and laboratory level; therefore, it is necessary to take it toward the field to explore the PGPR role in the natural environment. Another critical challenge in implementing microbial inoculants includes microbes screening, their production, marketing, and commercialization. At the field and laboratory level, identification of the microbiome interactions, their diversity, impact on environmental stresses, including their mode of action is necessary.

In the future, recent and advanced technologies such as meta-proteomics, nanotechnology, and rhizoengineering must be utilized to produce eco-friendly and effective inoculants. Besides, the production of bio-formulations should focus on the type of PGPR inoculant and their ecological and physiological

acceptance. Inoculation with ice-nucleating PGPR can be a promising technology to improve plant tolerance and growth during chilling stress. Different contaminants and nutrients can be detected by developing smart biosensors, which leads to precision farming. The involvement of nanotechnology is promising, but nano-products still need to be of acceptable quality and cost-effective. The essential aspects of nanoparticles regarding their physical, biological, and chemical properties exhibit their potential for improving food quality, enhance plant defense, detect pathogenic infection, regulate plant growth, reduce waste and enhance food production and serve as nano-biofertilizers. The encapsulation of nano-biofertilizers can regulate the transfer of fertilizers to the specific cell and prevent unintentional loss. The main goal of farming in the future should be precision farming which is meant to reduce input and increase output by implementing targeted action and tracking environmental variables. Novel or superior PGPR strains can be developed by modifying their particular traits using genetic engineering. These manipulated PGPR can help to control plant stresses as sustainable, eco-friendly, and low-input technology.

## AUTHOR CONTRIBUTIONS

SH and TN wrote the initial draft and made figures. MN, IL, MS, and RZ helped in collection and review of literature. MM edited the manuscript. AI finalized the review. All authors contributed to the article and approved the submitted version.

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# Disentangling the Association of Corn Root Mycobiome With Plant Productivity and the Importance of Soil Physicochemical Balance in Shaping Their Relationship

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Soil bacteria and fungi are integral parts of healthy ecosystem functioning in production agriculture. The effects of fungal abundance and diversity on crop productivity is poorly understood. We sampled 10 corn farms at the V10 growth stage across southwestern Ontario, Canada, using aerial infrared imaging to identify zones of low and high productive corn plants. Roots and soils were sampled from low and high yield zones and soil physical and chemical properties were measured in conjunction with assessment of the root mycobiome communities using Illumina MiSeq sequencing of 4 rRNA amplicons. Higher crop yields were associated with sites having greater fungal phylogenetic diversity and Fisher's  $\alpha$  diversity. Indicator species associated with high and low yield sites within a farm could be identified but there were no shared fungal indicators of productivity differences across farms. Communities largely varied across locations despite crop genetics, demonstrating a major influence of soil texture and chemistry in shaping the mycobiome in a site-specific manner. Across all 4 primers, roots from high-yielding sites shared 35 major OTUs including *Penicillium* spp., *Trichoderma*, *Chalara fungorum*, and *Gibellulopsis*. Low-yielding sites shared 31 OTUs including *Fusarium* spp., *Pythium*, *Setophoma terrestris*, and *Neonectria*. Soil physical and chemical parameters that contributed to broad scale differences in yield and mycobiome diversity included: %clay, %sand, %phosphorus saturation, cation exchange capacity, aluminum, pH, iron, potassium, %moisture, organic matter, and chlorine. The results show the importance of physicochemical balance in shaping the relationship between root mycobiome and plant productivity.

**Keywords:** root mycobiome, soil quality, soil productivity, soil chemistry, sustainable agriculture, high throughput sequencing, rhizosphere

## INTRODUCTION

Sustainable agricultural production can be achieved through the development and maintenance of high soil quality. One of the main biological factors related to soil quality is its fungal and bacterial microbiome abundance and diversity (Liebig and Doran, 1999). Microbial diversity can directly influence plant productivity by influencing plant growth and development, plant competition,

nutrient and water uptake (Baliyarsingh et al., 2017), and indirectly through contributions to nutrient turnover and soil aggregation (Wilpiseski et al., 2019). Soil type, cropping, and tillage systems substantially influence microbial structure and its function (Suzuki et al., 2005). Many of our current agricultural techniques and production practices result in the physicochemical and biological depletion of the soil environment due to intensive and extractive crop production, resulting in increased soil degradation including losses of nutrients and soil organic matter (Dregne, 2002; Rashid et al., 2016).

Maintaining microbiome-friendly agricultural practices in conjunction with the integration of naturally occurring beneficial microbiomes is an essential part of sustainable agriculture in order to ensure food security (Blaser, 2016). Root associated microbiota play important roles in agricultural ecosystems by helping to improve soil and plant health, thereby improving plant fitness in various production systems. In addition, microbes aid in improving yield while protecting the environment by reducing the need for chemical agricultural inputs such as fertilizers and pesticides (Rodriguez et al., 2009). Bacteria and fungi grow in the rhizosphere and rhizoplane, with some moving systemically to colonize aboveground plant parts and develop symbiotic relationships with host plants by improving beneficial plant functional traits (Hardoim et al., 2015). Petrini (1986) reviewed 100 years of research and suggested that almost all the plants in natural ecosystems have symbiotic relationships with mycorrhizal fungi and/or fungal endophytes.

Ecological factors and plant genetics that regulate plant-microbe interactions are variable, and understanding these relationships is essential for the appropriate integration of beneficial microbiota in crop production (Siddikee et al., 2016). Proper agricultural production practices can positively influence the soil system and its microbial diversity. Practices such as no-till farming (Islam et al., 2015), intercropping (Bargaz et al., 2017), and rotational cropping (Cook, 2007; Peralta et al., 2018) help to increase soil organic matter and to build and conserve microbial communities with specific functional traits to improve crop production. Increased plant productivity, plant health, enhanced photosynthetic rates, and grain yield have been linked with the beneficial functional activities of rhizosphere/root associated microorganisms with no-till fence-row farming of maize and soybeans with yearly rotation (Islam et al., 2015).

The development of high throughput single gene sequencing allows for the in-depth analyses of microbial communities (Fierer et al., 2007; Rousk et al., 2010; Caporaso et al., 2011). In order to maximize crop yields, we must combine the knowledge of the root-associated microbial communities, soil health, and chemical factors. Crop productivity has been linked to deficiencies or toxicities in nutrients including N, P, K, Mn, S, Zn, Fe, B, Cl, Si, and several heavy metals that may affect plant physiology, enhance or reduce biotic stress, or both (Astolfi et al., 2004; Dordas, 2008; Hänsch and Mendel, 2009; Veresoglou et al., 2013; Jacoby et al., 2017).

Providing a suitable environment for the establishment of beneficial microorganisms will increase the nutrient availability to plants and allow for the development of environmentally friendly, sustainable agricultural practices and technologies

(Singh et al., 2011, 2017; Rashid et al., 2016). Plant root exudates promote rich microbial diversity around the root-zone, providing nutrition for bacteria and fungi in exchange for plant growth promotion through various mechanisms (Patel et al., 2015; Rashid et al., 2016; Singh et al., 2017). Beneficial microbes such as plant growth promoting rhizobacteria (PGPR) and plant growth promoting fungi (PGPF) are familiar targets for the development of agricultural technologies and practices that enhance plant productivity by reducing abiotic/biotic stress, providing pathogen protection, increasing nutrient solubilization (N and P), and producing bioactive compounds (Smith et al., 2003; Adesemoye et al., 2008; Behn, 2008; Gamalero et al., 2009; Nadeem et al., 2014; Singh et al., 2017; Bilal et al., 2018; Rocha et al., 2019), to list a few. Identifying potential PGPFs that have entomopathogenic, endophytic, and/or mycorrhizal (Bilal et al., 2018; Zhou L. et al., 2018) lifestyles for the development of inoculants or mycofertilizers (Hnatowich et al., 2012) is a major driver in increasing agricultural sustainability (Bashan, 1998; Berg, 2009).

The use of unmanned aerial vehicles (UAVs) with near-infrared imaging devices to construct a Normalized Difference Vegetation Index (NDVI) map allows researchers to identify areas of good or poor crop health (Mahajan and Bundel, 2017; Hassler and Baysal-Gurel, 2019; Shafi et al., 2019). Large variations in yield are common within a field, despite nearly identical levels of farm inputs (e.g., fertilizers, same genetic hybrid), and this variation can be related to interactions between plant, soil physical and/or chemical characteristics, and the interactions between soil- and plant- associated microbial communities. Our previous studies hypothesized that differences in plant productivity within a field are linked to the differences in abundance and diversity of microbial communities, and their impacts on plant growth and yield. However, we did not look for the contribution of soil physical and chemical characteristics on these interactions (Ali et al., 2020). A subsequent growth room study demonstrated the influence of key microbes on a soil's native microbial diversity and its plant productivity (Kandasamy et al., 2019). Disentangling, understanding, and manipulating these relationships may help to reduce unevenness in crop yields and to develop mycofertilizers.

Corn (*Zea mays*) is the major cereal crop grown in Ontario, and the province grows ~63% (7.5–9 million tons a year) of Canada's corn grain on 800,000 ha of farmland (OMAFRA: Field Crops, 2019). Corn production in Canada is predicted to be 14.1 million metric tons with the average yield of 10.33 t/ha (153.6 bu/ac) in 2020 (<https://www150.statcan.gc.ca/n1/en/daily-quotidien/190912/dq190912b-eng.pdf>). The basis for lower-than-average production and within field variability may be due to different soil nutritional and physical characteristics as well as biological components including fungi and bacteria. There are many studies that assess the association of bacterial communities on plant health and productivity, but fungal interactions are often harder to clarify. We hypothesized that root fungal communities would have strong correlations with crop productivity despite varying soil types and that there may be one or more indicator taxa present in high/low productive areas across all farms. In order to test the hypothesis: (1) we

sampled soil and plants from low and high yielding sites within 10 corn fields across southwestern Ontario by using NDVI maps to identify sites with or without plant stress, (2) sequenced corn root-associated mycobiome to identify taxa that may be contributing to or reducing plant health and yield, and to identify the key soil physicochemical factors that associate with low or high productivity, and (3) assessed the underlying relationship of these fungal communities with soil nutritional and physical characteristics and their impact on yield.

## MATERIALS AND METHODS

### Study Sites

Ten corn fields across southwestern Ontario, varying in cultivation practices but sharing similar eco-climatic factors, were sampled as a part of this study (Table 1). These regions represent various heat zones and fertility statuses. High and low yielding sites within each farm were determined by flying a remote sensing UAV to identify healthy and stressed corn plants, respectively (see Section UAV Data Collection and Construction of NDVI Maps; Figure 1). GPS coordinates of the stressed and healthy sites were then used to navigate to these locations during field sampling (Table 1).

### UAV Data Collection and Construction of NDVI Maps

A remote sensing unmanned aerial vehicle (UAV) was flown over fields in each location at the V10 stage of the crop cycle (~80 days after planting) to create a visual representation of healthy and stressed sites across the field through the Normalized Difference Vegetation Index (NDVI). The UAV is ~2.1 m wide and 1.2 m long and contains two cameras mounted on each wing; one camera collects full color imagery while the other camera collects infrared imagery. NDVI is calculated based on contrasting intense chlorophyll pigment absorption in red (R) visible light against the high reflectance of plant materials in the near infrared (NIR) (Moriondo et al., 2007):

$$NDVI = \frac{NIR - R}{NIR + R}$$

### Sampling

Selected high and low producing sample sites were ~15 × 15 m each. Ten corn plants were sampled randomly from each site. Approximately 450 g of rhizosphere soil was collected from every plant by shaking the roots of corn plants into a bag after carefully removing the soil away from the roots. We also sampled ~500 g of bulk soil from non-root zones. Afterwards, roots were separated from the stalk, washed under running tap water, blotted dry, chopped into fine pieces, mixed, and 200 mg samples were weighed out for microbial DNA extraction.

### Soil Chemical Analysis

All soil samples were sent to A&L Canada Inc. for chemical analysis which included general fertility index (GFI), organic matter (OM%), phosphorus (bicarbonate & Bray-P1; ppm),

potassium (ppm), magnesium (ppm), calcium (ppm), sodium (ppm), sulfur (ppm), zinc (ppm), manganese (ppm), iron (ppm), copper (ppm), boron (ppm), aluminum (ppm), nitrate-nitrogen (NO<sub>3</sub>-N; ppm), ammoniacal-nitrogen (NH<sub>4</sub>-N; ppm), chloride (ppm), pH, cation exchange capacity (CEC meq/100 g), percent base saturations (K, Mg, Ca, H, Na), soluble salts (ms/cm), % phosphorus saturation, K/Mg ratio, estimated nitrogen release (ENR), % moisture, and microbially active carbon (%MAC). All the soil chemical, physical, and textural parameters were measured using the A&L Canada Laboratories accredited soil testing protocols.

### Yield Measurements

Each sampling site was revisited in mid- to late- October for corn harvest. Fifty plants were randomly selected from high and low producing sampling sites, cobs were collected and threshed. The total kernel mass of the 50 plants was measured. Percent moisture was determined by drying a small subset of kernels from each site. We then calculated the yield of each site by adding 13% moisture to the kernel dry weight in order to balance the humidity difference across and between farms. The hand harvested yield was compared with the exact yield of sampling sites from a yield monitor attached to combine which can track yield to GPS coordinates of each sampling site.

### Fungal Community DNA Extraction and Sequencing

Exactly 200 mg of root tissue was extracted for DNA extraction using the Soil DNA Isolation Kit (Norgen Biotek Corporation) as per the manufacturer's instructions with minor modifications. DNA samples were PCR-amplified with the following four sets of primer combinations, with primer-specific PCR conditions. Four sets of primers were chosen to target the amplification of fungal nuclear ribosomal small subunit to cover wider fungal communities. General fungi (minus Ascomycota) 28S LSUBG: LSU200-F (5'-AAC KGC GAG TGA AGM GGG A-3') and LSU481-R (5'-TCT TTC CCT CAC GGT ACT TG-3') (Asemaninejad et al., 2016); Ascomycota 28S LSUA: LSU200A-F (5'-AAC KGC GAG TGA AGC RGY A-3') and LSU476A-R (5'-CSA TCA CTS TAC TTG TKC GC-3') (Asemaninejad et al., 2016); Glomeromycota 18S V4AM: AMV4.5N-F (5'-AAC KGC GAG TGA AGC RGY A-3') and AMDG-R (5'-CCC AAC TAT CCC TAT TAA TCA T-3') (Sato et al., 2005); Fungi ITS2: 5.8S\_Fun-F (5'-AAC TTT YRR CAA YGG ATC WCT-3') and ITS4\_Fun-R (5'-CCT CCG CTT ATT GAT ATG CTT AAR T-3') (Taylor et al., 2016). The four fungal primers (LSUBG, LSUA, V4AM, ITS2) were tagged with Illumina MiSeq (MiSeq) adaptors, a four bp linker (NNNN), and eight nucleotide barcode sequences starting at the 5' end of each forward and reverse primer (Gloor et al., 2010; Asemaninejad et al., 2016). Pooled amplicon libraries were sequenced through the MiSeq platform. Sample and sequence read data were deposited in the European Nucleotide Archive (<https://www.ebi.ac.uk/ena/browser/home>) with the project number PRJEB40800.



**TABLE 1** | Field sites, corn variety, sampling information and the cropping regimes.

Site	Location (Lat/Long)	Variety	Planting date	Sampling date	Cropping regimes	Yield (bu/ac)
Farm 1	43.656327, -80.534160	Dekalb 45-65	11-May-17	17-July-17	Corn monocropping	L: 173 (11.63 t/ha) H: 284 (19.1 t/ha)
Farm 2	43.638370, -80.399300	DKC 39-97RIB	18-May-17	17-July-17	Corn monocropping with fall cover cropping	L: 169 (11.37 t/ha) H: 186 (12.51 t/ha)
Farm 3	43.052629, -81.449286	PO496AMXT	16-May-17	19-July-17	Corn monocropping	L: 262 (17.62 t/ha) H: 307 (20.65 t/ha)
Farm 4	43.335260, -80.792026	DKC48-56RIB	17-May-17	24-July-17	Corn-Soybean rotation	L: 137 (9.21 t/ha) H: 228 (15.33 t/ha)
Farm 5	43.139408, -80.837053	DKC50-78RIB	18-May-17	24-July-17	Corn-Soybean-Wheat	L: 239 (16.07 t/ha) H: 320 (21.52 t/ha)
Farm 6	43.090045, -81.346837	PO496AMXT	14-May-17	24-July-17	Corn monocropping	L: 273 (18.36 t/ha) H: 322 (21.65 t/ha)
Farm 7	42.836740, -81.101202	Dekalb 48-56	17-May-17	25-July-17	Corn-Soybeans-Wheat	L: 247 (16.61 t/ha) H: 292 (19.64 t/ha)
Farm 8	43.289837, -80.545299	P9526YXR	20-May-17	26-July-17	Corn-Soybeans-Wheat	L: 223 (15.00 t/ha) H: 262 (17.62 t/ha)
Farm 9	42.705807, -81.316867	DKC 48-56RIB	09-Jun-17	01-August-17	Corn-Edible beans-Wheat	L: 172 (11.57 t/ha) H: 266 (15.2 t/ha)
Farm 10	42.736850, -81.443932	P0157AMX	02-Jun-17	08-August-17	Corn-Soybean rotation with fall cover cropping	L: 171 (11.50 t/ha) H: 299 (20.11 t/ha)

## Bioinformatic and Statistical Analyses

Raw FASTQ datasets were separately processed using a custom MiSeq data processing pipeline ([https://github.com/ggloor/miseq\\_bin/tree/Jean](https://github.com/ggloor/miseq_bin/tree/Jean)). PANDAsseq ([https://github.com/neufeld/panda\\_seq](https://github.com/neufeld/panda_seq)) (Masella et al., 2012) was used to overlap reads. Sequences containing ambiguous base calls (N) or mismatches to the primer sequence were removed, and barcodes and primers were trimmed prior to clustering. Individual reads were clustered into identical sequence units (ISUs) and chimera-checked using UCHIME (Edgar et al., 2011). ISUs were grouped into operational taxonomic units (OTUs) at 97% similarity around the most abundant centroid sequence using USEARCH v7.0.1090 (Edgar, 2010). A separate OTU table was made for each primer. Primary taxonomies were done using RDP Classifier and the Unite Fungal ITS trainset (07-04-2014) and the Fungal LSU training set 11 (Wang et al., 2007) for ITS2 and both LSU datasets, respectively. Taxonomic identifications were confirmed manually using BLASTn (Madden, 2002) for assignments with confidence scores lower than 70%. All VAM (Glomeromycota) reads were manually identified using the MaarjAM database (Öpik et al., 2010). Reference gene datasets were made for each primer set using the top BLASTn hits for each OTU, and phylogenetic trees were constructed using the reference sets and OTU sequences to confirm annotations by using Muscle v3.8.31 (Edgar et al., 2011) alignments and Neighbor Joining (NJ) and Maximum Likelihood (ML) in MEGA X v10.0.5 (Kumar et al., 2018).

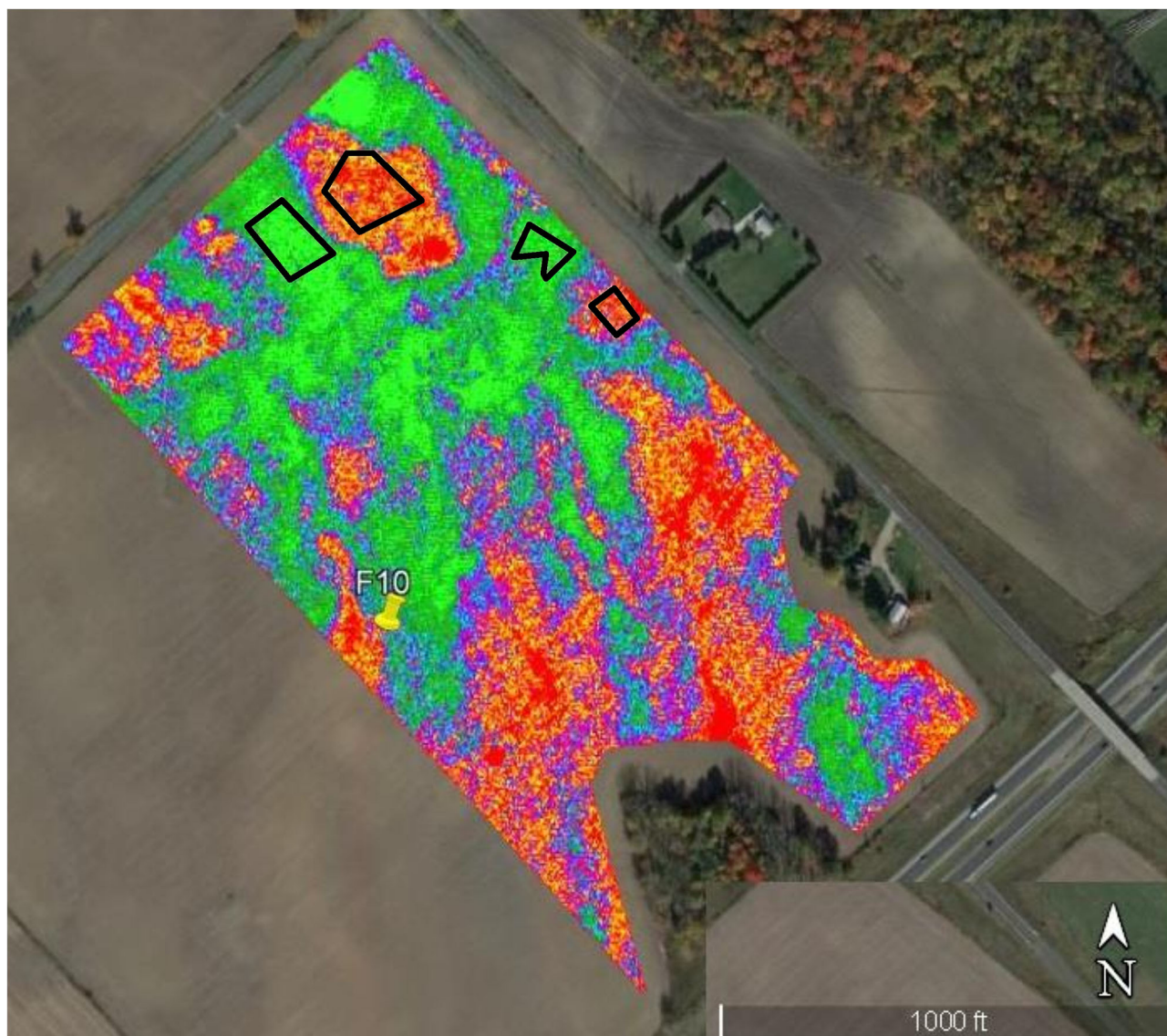
The programs R v3.6.1 within RStudio 1.2.1335 were used for statistical analyses and visualization. The vegan package was used to make NMDS ordinations with rhizosphere metadata (Oksanen et al., 2019), the factoextra package was used to make biplots (Kassambara and Mundt, 2017), the corrplot package was used to do variable correlation analyses (Wei and Simko,

2017), a custom variance inflation factor (VIF) function was used to do a stepwise VIF analysis using the fsmb package (Beck, 2015; Nakazawa, 2018), and the metacoder package was used to make the taxonomy heatmap (Foster et al., 2017). The phyloseq package was used to calculate diversity indices and create stacked barplots (McMurdie and Holmes, 2013), alongside the mblm package that was used to do a Kendall-Theil Sen Siegel non-parametric linear regression on rarefied OTU data ( $n = 500$  OTUs per sample, without replacement) (Theil, 1950; Sen, 1968; Siegel, 1982; Komsta, 2019), and btools to calculate Faith's Phylogenetic Distance (Battaglia, 2020). A PERMANOVA analysis was done using the "Adonis" function in R, starting with an analysis of variance using UniFrac distance matrices to highlight variables that fit linear models with yield and SIMPER was done to identify which taxonomic groups differed between yields and farms (vegan package) (Oksanen et al., 2019). The ALDEx2 package was used as an ANOVA-like univariate comparison tool incorporating the Bayesian estimate of taxon abundance into a compositional framework, useful for the differentiation of OTU abundances between two conditions to identify taxa that are significantly associated with each condition (Fernandes et al., 2013). The microbiomeSeq package was used to create a correlation matrix between ALDEx2 taxa and soil physicochemistry (Ssekagiri, 2020), and heatmaps were made with Complex Heatmap (Gu, 2016).

## RESULTS

### Soil Physicochemical Analysis

The complete metadata set, including 52 soil variables from both bulk and rhizosphere, and yields for each high and

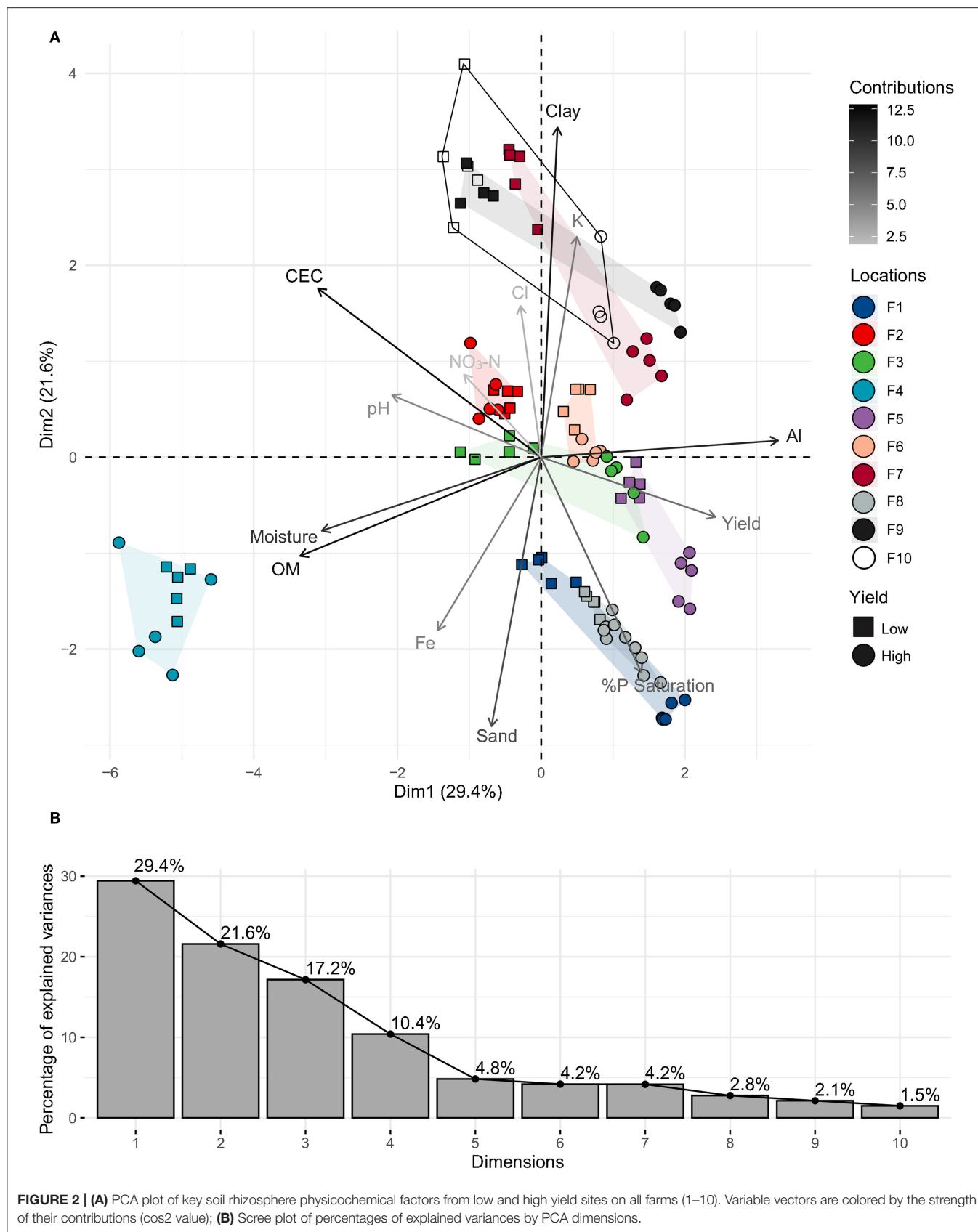


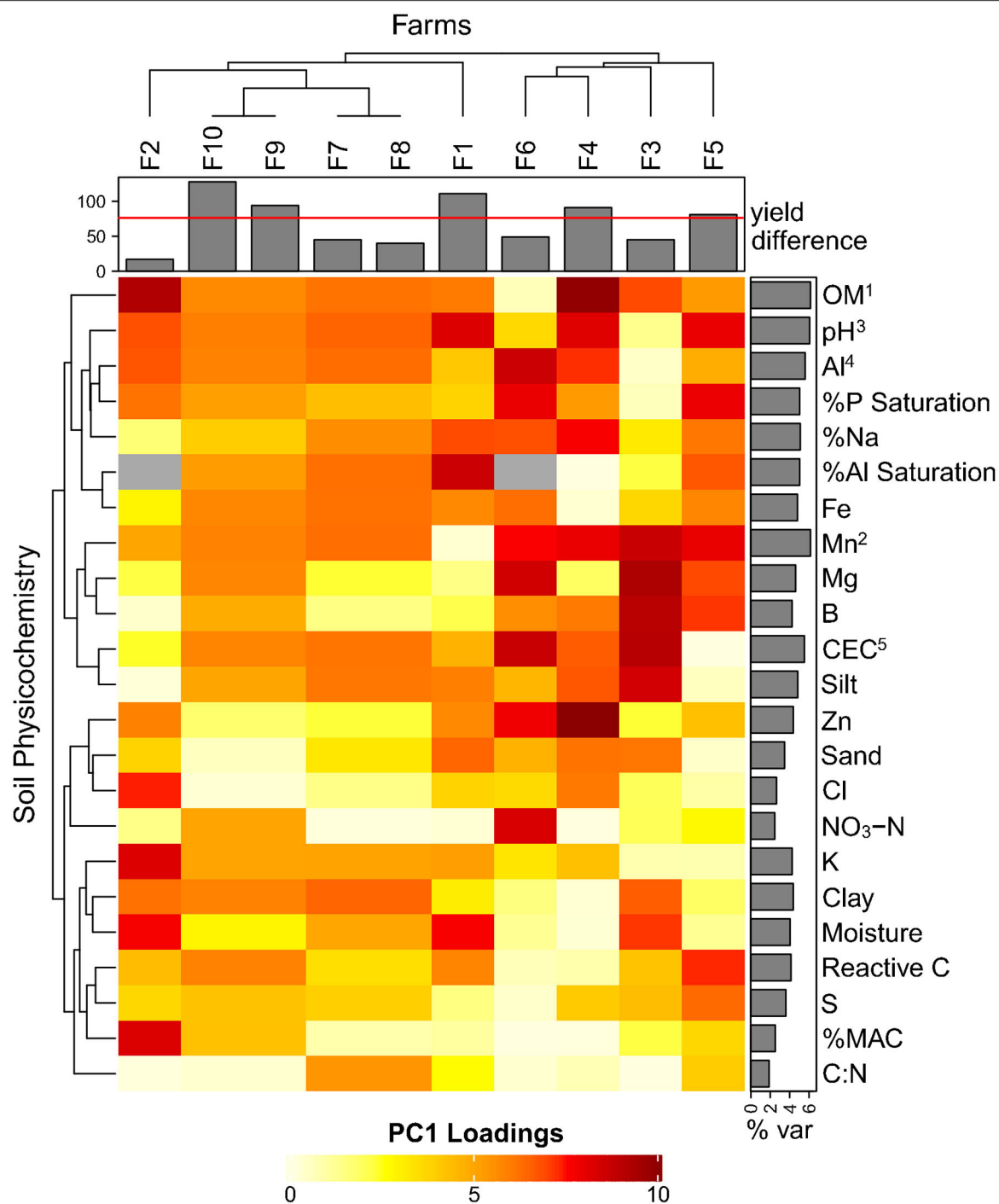
**FIGURE 1** | Infra-red aerial image taken by an Unmanned Aerial Vehicle (UAV) of corn at the V10 growth stage in Field 10. Black polygons outline stressed, low yield (red-orange) and healthy, high-yield (green-blue) corn.

low yielding site was analyzed using PCA to identify the top contributing soil factors. There was no significant difference in the factor contribution analysis between bulk and rhizosphere soil; therefore, we used rhizosphere soil for all analyses ( $n = 103$ ). PCA components with variable contributions  $> 10\%$  in each dimension were retained. PC1 was separated by OM (19.6%), Al (19.0%), CEC (16.8%), moisture (16.3%) and yield (10.2%); PC2 by clay (28.1%), sand (18.7%), K (12.5%), %P saturation (12.0%), CEC (10.6%); and PC3 by  $\text{NO}_3\text{-N}$  (22.6%), Cl (22.3%), (K, Fe, and %P saturation were also significant) (**Figure 2**).

There were slightly larger between site differences in soil (linked to differences in OM/moisture, Al, CEC) that were seen along PC1 (29.4% explained variance), whereas within-site factors (Clay, sand, K, %P saturation, CEC) separated most

high/low yield groups along PC2 (21.6% explained variance), and there was slight within-farm-yield separation seen in PC3 (17.2% explained variance) (influenced by K/Cl/ $\text{NO}_3\text{-N}$  on sample replicates in farms 1, 4, and 10) (data not shown). The farms clustered into five groups (defined by overlapping polygons): group 1 containing farms 7, 9, and 10 had high clay, CEC, and K influencing low yield sites; group 2 containing farms 1 and 8 had high %P saturation and sand influencing high yield sites; group 3 containing farms 3, 5, and 6 had low CEC and high %P saturation influencing high yield sites; group 4 containing the outlier farm 4 was characterized by low soil Al and high moisture and OM (due to high hog manure amendments), and group 5 containing farm 2 that did not have differences in final yield measurements (186 vs. 169 bu/ac), resulting in no site separation.





**FIGURE 3 |** Heatmap of individual farms with PCA-derived loading variables (% explained variation in PC1) for soil physicochemical components (data not shown). The % var row barplot averages each component's explained variation along the PC1 axis, superscript numbers show the order of the top 5 contributing vectors. The column barplot shows the yield differences for each farm (high—low) with a cut-off line at 80 bu/ac (5.38 t/ha) identifying substantial yield differences. N/A values are in gray.

In general, higher yields were seen in soils that had high %P saturation (H: 16 vs. L: 11), high sand (49 vs. 35%), low clay (13 vs. 20%), low CEC (11 vs. 19 meq/100 g), low pH (6.6 vs. 7.3) low Ca (1,900 vs. 2,700 ppm), slightly higher levels of %K

saturation (3.5 vs. 3.1), and NO<sub>3</sub>-N (59 vs. 51 ppm). This was true for high yield sites across all farms, except for farms 2 and 4, where there was no clear yield separation along PC1 or PC2.



A stepwise linear regression analysis with yield as the dependent variable and all twelve soil PCA vectors described a broad-scale linear model:  $\text{yield} \sim \text{sand} + \text{pH} + \% \text{ moisture} + \text{OM} + \text{CEC} + \% \text{P saturation}$  ( $F_{(6, 96)} = 17.62$ ,  $p < 0.001$ ,  $R^2 = 0.52$ , Adj.  $R^2 = 0.49$ ; continuous predictors are mean-centered and scaled by 2 S.D.). A collinearity check showed that % moisture and OM were 81% collinear, and nothing was highly collinear with yield. Despite the high collinearity between OM and moisture, both are important factors that help to correctly predict yield, so we retained both in the model.

Separate PCA analyses were done within individual farms using 22 soil chemical parameters to identify common factors leading to yield differences (Figure 3). Nine out of ten farms (except farm 6), cleanly separated high and low sites along PC1 (data not shown), allowing us to deconstruct the individual factor loading scores (explained % variation along PC1). OM explained most of the PC1 variation (between H/L separated sites averaged across farms), followed closely by Mn, pH, Al, and CEC (Figure 3). The factors that least explained H/L separation across farms were C:N,  $\text{NO}_3\text{-N}$ , %MAC, Cl, and sand. When considering only the farms with substantial yield differences > 80 bu/ac (5.38 t/ha) (farms 10, 1, 9, 4, and 5), we see distinctions between low and high yield sites with: pH (L: 7.51, H: 6.20), OM (L: 7.64, H: 10.28), %Na (L: 0.28, H: 0.40), Mn (L: 42 ppm, H: 17.5 ppm), Al (L: 575 ppm, H: 700 ppm), and %P saturation (L: 6.21, H: 19.17) (data not shown).

Soil texture, specifically clay content, shows a strong effect in separating study sites including farms 1, 4, 7, 9, and 10 (Figure 4). Low yield farm 1 soils were sandier than high yield, whereas higher yields in farm 4 showed the opposite trend, with low yield sites having a sand-loam or loam texture. Low yield sites in farms 7 and 10 had clay-loam or silt-clay-loam soils, high yield sites were in sand-loam/loam (farm 10) or silt-loam (farm 7) soils. High yield sites in farm 9 followed the opposite pattern, where high yield soils had more clay. Other farms (2, 3, 5 and 6) showed no clear textural distributions, with all of them evenly spread between loam and silt-loam categories, and both H/L sites in farms 2, 5 and 8 clustering closely (Figure 4).

## Root Mycobiome Analysis

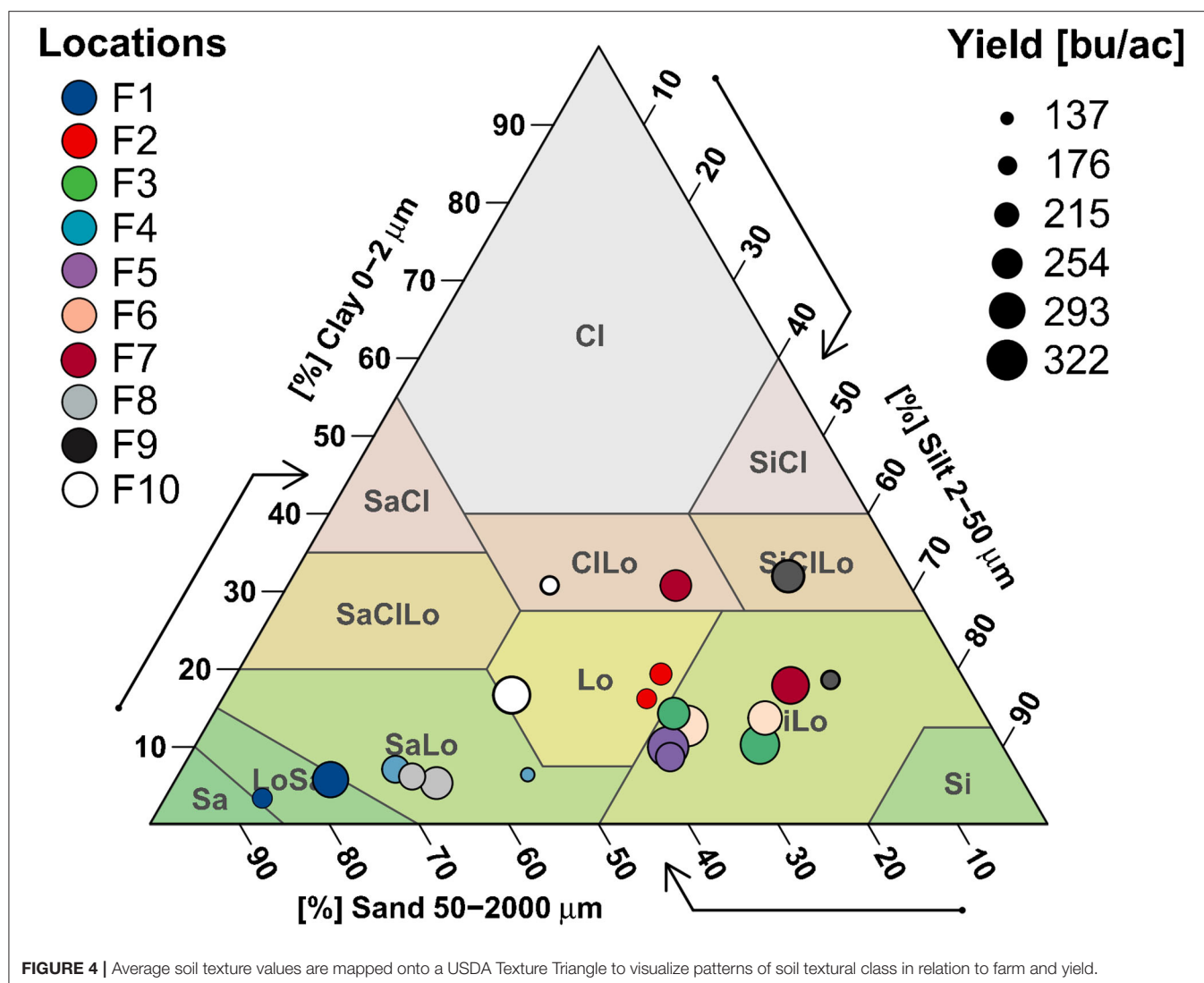
MiSeq outputs from the four fungal primers are outlined in Table 2. Raw demultiplexed reads ranged from 346,071 to 9,065,818 reads depending on the primer, however, the number of on-target taxa obtained from each primer set was not always reflective of the proportion raw reads. LSUBG and V4AM primers both had a similar number of target taxa (42 and 22) despite ~240-fold difference in raw reads (8.4 million vs. 32,232). The majority of reads from the fungal LSU Basidiomycota/Glomeromycota primers were 9 OTUs from *Zea mays* (7,220,937 reads) and 5 OTUs from unknown Animalia (949,092 reads). Other non-target OTUs included: Cercozoa, Animalia, Amoebozoa, other Streptophyta, and Ascomycota. ITS2 universal fungal primers yielded 529 target fungi (584,380 reads), spanning with 373 Ascomycota (518,425 reads); 76 Basidiomycota (20,518 reads); 5 Chytridiomycota (2,256 reads); 9 Glomeromycota (194 reads); and Zygomycota (36,101 reads), with few non-target Viridiplantae (15,961 reads)

(Figure 5A, Supplementary Table 1). V4AM primers yielded 22 target Glomeromycota (19,648 reads), 20 non-target Fungal OTUs (8,497 reads), Amoebozoa (2,404 reads), and a few others (Table 2). LSUA primers yielded 334 target Ascomycota (984,428 reads) (Figure 5B); 12 non-target Fungi (16,239 reads) including Basidiomycota, Chytridiomycota, Zygomycota, and Glomeromycota; Animalia (161,249 reads); Oomycota (6,621 reads; including *Pythium* and unknown Pythiales); Viridiplantae (200,247 reads of *Zea mays*); plus Protozoa (505 reads), Rhizaria, and others.

The ALDEx analysis is robust to noise in environmental metagenomic datasets. High ( $-1.0 \leq x \leq 1.0$ ) and moderate ( $-0.8 > x > -1.0$  and  $0.8 < x < 1.0$ ) effect sizes highlight important indicator taxa between high and low yield communities within farms. This within-site approach was necessitated due to the high degree of physicochemical variability between farms (and a possible batch effect) that did not allow the algorithm to identify any taxa of interest when considering all sites simultaneously. Instead, a narrowed within-farm analysis identified a total of 163 OTUs (including non-target taxa belonging to Amoebozoa and Platyhelminthes amplified by VAM and LSUBG primers; Supplementary Table 1), and those with relevant ecological strategies are visualized in a taxonomic heatmap (Figure 6).

There are a number of OTUs significantly associated with both high and low yield sites: the saprotrophs *Harpophora radicola* (= *Phialophora radicola*) and *Myrmecridium* spp., the potential plant pathogen/endophyte/saprotroph *Periconia pseudodigitata*, and the arbuscular mycorrhizal fungus *Claroideoglossum* sp.—amplified by three primers in three low yield farms—and *C. lamellosum*—amplified twice by two primers in two high yield farms. The plant pathogen *Pythium* spp.—amplified by two primers—was also detected in three low yield and one high yield farm. In *Setophoma terrestris*, a fungal pathogen/plant pathogen/saprotroph, three OTUs were significantly associated with low yield sites in farm 6 and one OTU with high yield in farm 4. Plant pathogens *Neonectria fuckeliana* and *N. candida* were amplified in high and low yield sites, respectively. *Fusarium* was represented by three species: four OTUs of *F. oxysporum* significantly associated with only high yield sites, and one OTU each of *F. equiseti/chlamydosporum* and *F. tricinctum/avenaceum/acuminatum* that were significantly associated with only low yield sites. *Penicillium* species were significantly associated with high yield sites in farms 1, 6, 8, and 10, with a large proportion of significant OTUs found in farm 10. We also observed that farms 2, 4, 8 and 9 had few to no taxa differentially associated with high or low yield sites, although farms 2 and 8 had low yield differences between H and L sites, along with farms 3, 6 and 7 (Table 1, Figure 3). Farms 6 and 10 both had 37 unique significant taxa, farm 7 with 21, farm 5 with 19, and farm 1 with 17 (Supplementary Table 1).

Selected OTUs with ecological strategies of interest (pathogenic and/or beneficial) were visualized by their percent relative abundance within each farm (Figure 7). Relative abundances were typically preserved between OTUs identified as the same species using two separate primers. For example, *Pythium* sp. (OTUs 49 and 5) captured by LSUA and



**FIGURE 4 |** Average soil texture values are mapped onto a USDA Texture Triangle to visualize patterns of soil textural class in relation to farm and yield.

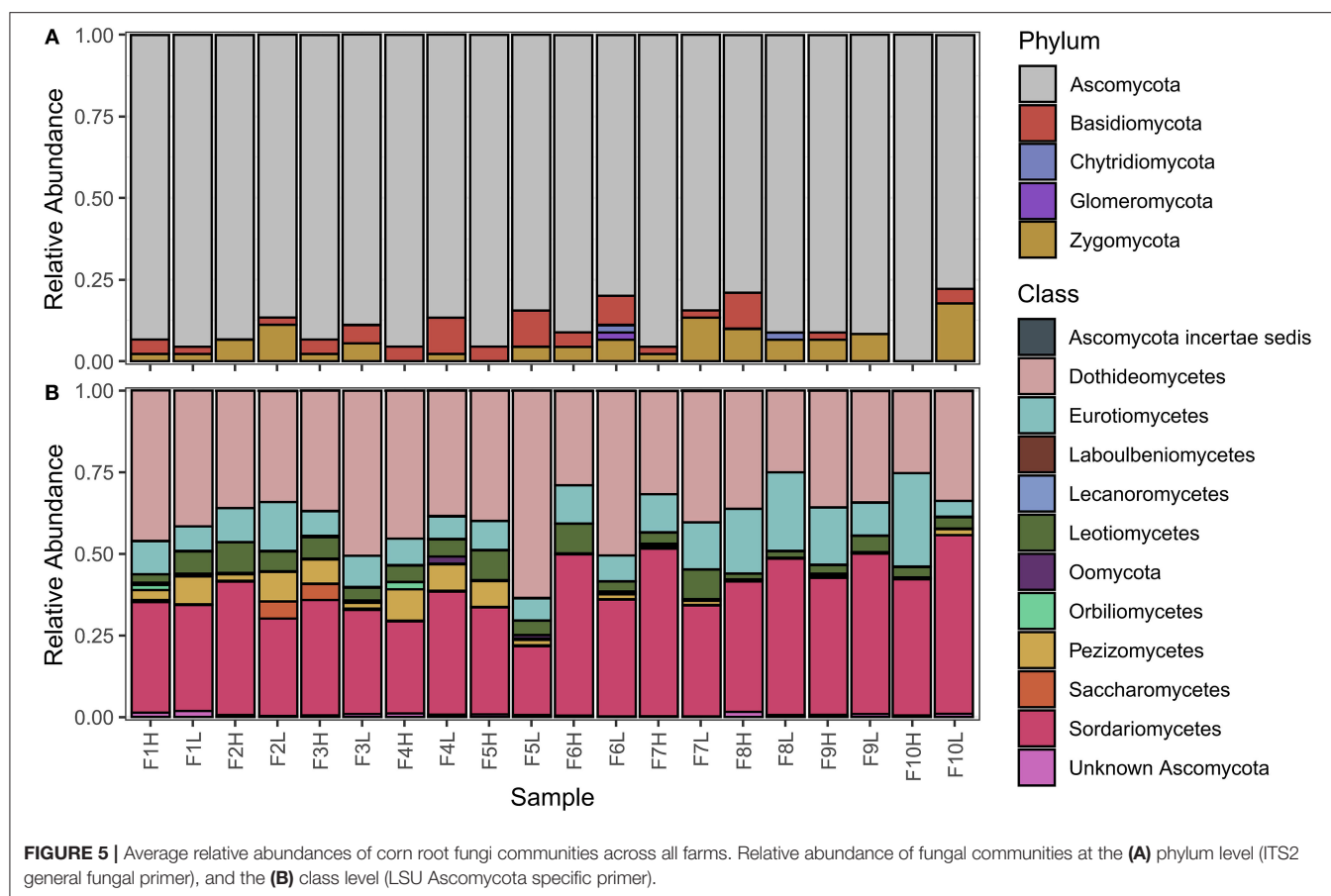
LSUBG primers had approximately the same range of relative abundances in each farm, although its differential abundance in LSUA-F7 was not reported as significant by ALDEx. In high-yield sites, *Fusarium oxysporum*, *Chalara fungorum*, *Talaromyces* sp., *T. diversus*, *Penicillium ochrochloron*, *P. janthinellum*, *P. paneum/chrysogenum*, *Gibellulopsis* spp., *Neonectria fuckeliana*, and *Mucor hiemalis* were consistently found, in 8 of 10 replicates in farm 6, 7 times in farm 10, and once in farm 1, 5 and 7. *Pythium* sp. was consistently found in low yield sites in farms 6 and 7, and *Setophoma terrestris* and *Talaromyces diversus* were seen in both high and low yield sites, depending on the location. A large percentage of reads in each farm belonged to *S. terrestris* or *C. fungorum*, with some having sites with >30% relative abundance. *Penicillium ochrochloron* was seen with >20% relative abundance in 6 low-yielding sites in farm 9, using both LSUA and ITS2 primers, and in low-yield sites in farm 8.

Based on the results of rarefied ( $n = 500$  OTUs in each sample) LSUA and ITS2 primers, there were significant trends in selected diversity and evenness indices. Non-parametric Kendall–Theil

Sen regressions modeled significant linear relationships in both LSUA and ITS2 data between yield and observed OTU richness, Fisher's  $\alpha$  (Fisher), and Phylogenetic Diversity (PD; Faith's PD-index) (Faith, 1992) (Figures 8A,B). No significance was seen in either Shannon's or Simpson's diversity indices, and the Abundance-based Coverage Estimator (ACE) was found significant for LSUA but not ITS2 data (data not shown). A strong batch effect was detected in each OTU dataset between the two NGS runs, which may have a stronger effect on mycobiome distribution than location, genetics, soil chemistry, or any other tested factors and partially confound OTU differences between the productivity sites.

### Key Soil Physicochemical and Fungal Factors Related to Yield

A correlation analysis between key fungi, soil physicochemical factors and yield identified three taxa that significantly correlate with soil chemistry (after a Benjamin–Hotchberg  $p$ -value adjustment) (Figure 9). *Rhodotorula* sp., a potential plant



**TABLE 2 |** Summary of demultiplexed Individual Sequence Units (ISUs) and clustered Operational Taxonomic Units (OTUs) from each of the 4 fungal primer sets used in this study.

Primer	Demulti-plexed reads <sup>1</sup>	Usearch/Uchime				Pipeline output OTU table		Final OTU table (>5 reads)		Target	
		ISUs	OTUs	Chimeras (%)	Single-tons/low abundance <sup>2</sup>	OTUs	Reads	OTUs	Reads	OTUs	Reads
LSUBG <sup>3</sup>	9,065,818	150,865	1,323	2,033 (1.35%)	1,231	92	8,422,920	92	8,417,761	42	213,176
LSUA <sup>4</sup>	4,113,604	117,784	1,208	6,813 (5.78%)	837	371	2,907,129	370	2,881,908	316	980,428
ITS2-F <sup>5</sup>	2,214,281	46,482	1,293	391 (0.08%)	542	751	108,144	530	600,265	529	584,380
V4AM <sup>6</sup>	346,071	3,439	261	62 (1.80%)	1	260	36,140	113	32,232	22	19,212

<sup>1</sup>Read counts were obtained by the rekeyed\_tab\_file.txt made through the workflow.sh custom pipeline (Greg Gloor).

<sup>2</sup>Low abundance OTUs (those that are <= 0.1% in any sample) were automatically removed by the custom pipeline.

<sup>3</sup>LSUBG primer targets included: Basidiomycota, Oomycetes (Heterokonta), Chytridiomycota, Glomeromycota, and Zygomycota. Non-target reads were primarily Viridiplantae, Metazoa, and Rhizaria.

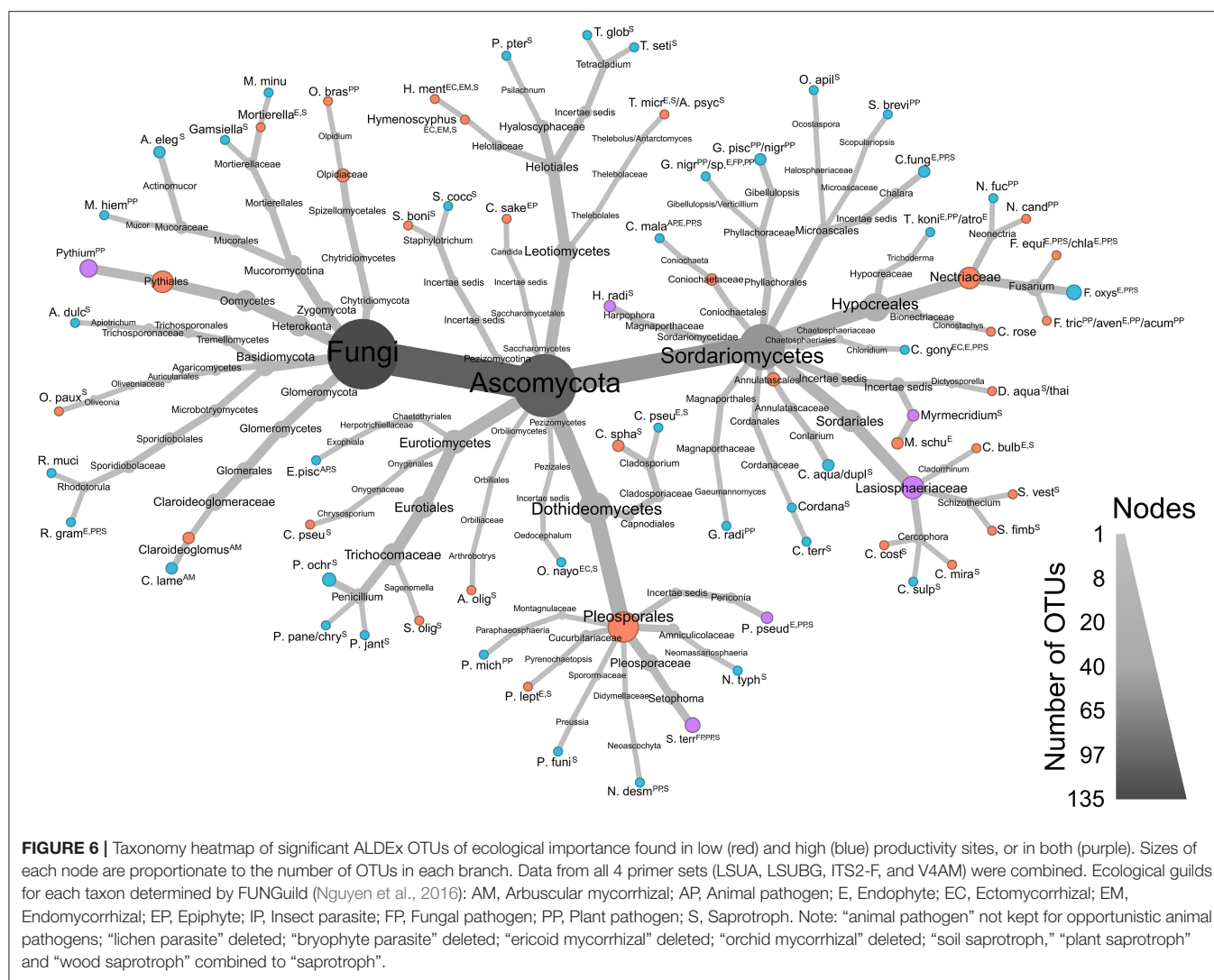
<sup>4</sup>LSUA non-target reads included: Annelida, Chytridiomycota, Basidiomycota, Zygomycota, Heterokonta, Cercozoa, Ciliophora, Metazoa, and Streptophyta.

<sup>5</sup>ITS2-F primer targets included: Ascomycota, Basidiomycota, Chytridiomycota, Glomeromycota, and Zygomycota. Non-target reads were one OTU of Viridiplantae.

<sup>6</sup>V4AM non-target reads included: Amoebozoa, Cercozoa, Ascomycota, Basidiomycota and Viridiplantae.

pathogen/saprobe/endophyte, was significantly associated with high yield soils in farm 10 (ALDEx analysis) with a significant positive correlation ( $p < 0.05$ ) with high sand, Fe, and %P saturation. An unknown *Hymenoscyphus* sp.—endophyte/ectomycorrhizal/saprobe seen in low yield farm 1 soil—was significantly ( $p < 0.05$ ) positively correlated with high moisture. Both *Hymenoscyphus menthae* and *Setophoma*

*terrestris* OTUs were associated with high yield sites, but correlated negatively with moisture ( $p < 0.05$ ,  $p < 0.01$ , respectively). *Hymenoscyphus menthae* potentially plays a mycorrhizal/saprobic role in soil, and *S. terrestris* is identified as a potential fungal pathogen/plant pathogen/saprobe. Other non-significant trends can be seen within row clusters, where positive correlations to soil factors on the left side of the heatmap



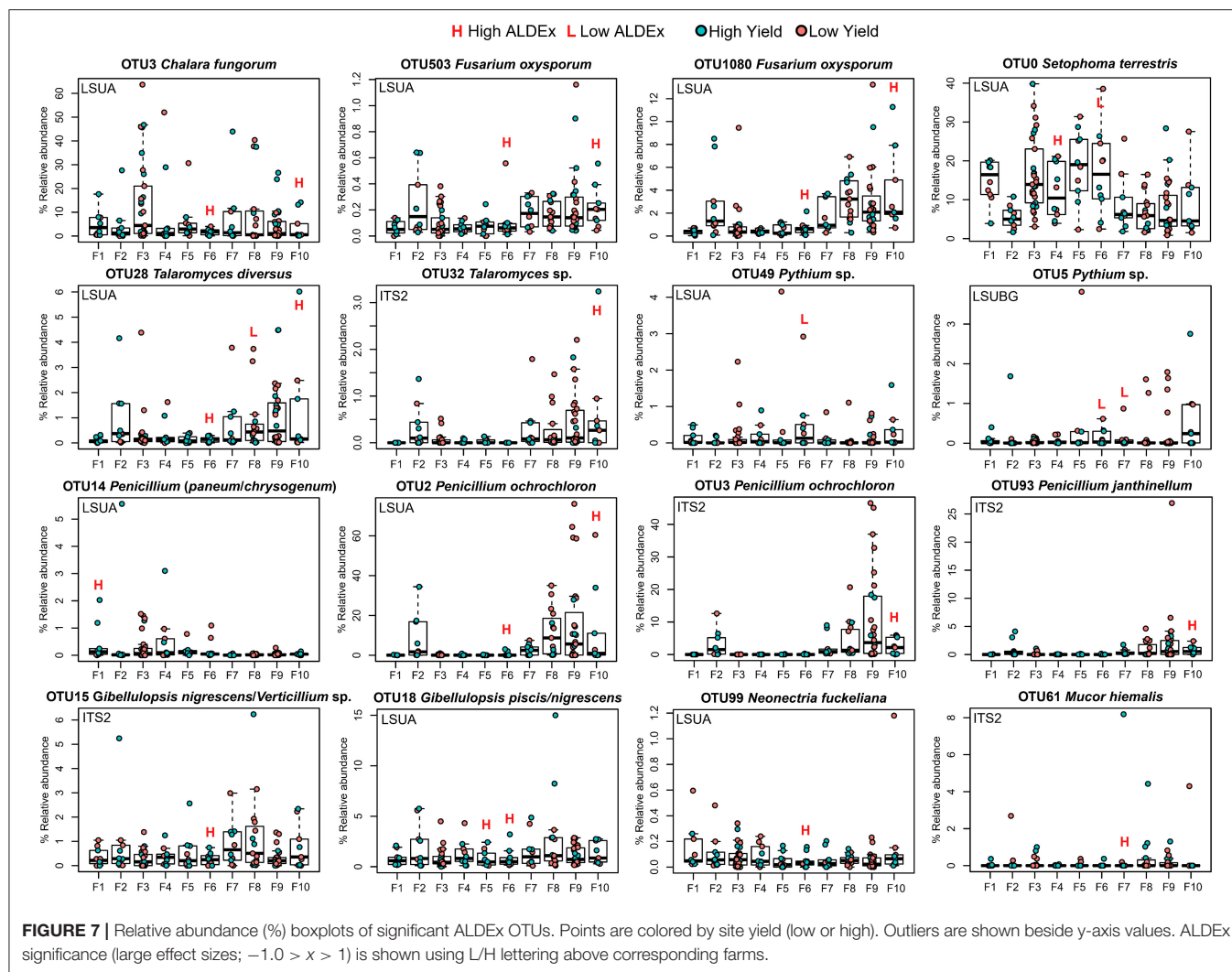
(moisture, OM, sand, Fe, %P saturation, Al and yield) are visible with the topmost OTU cluster, and the opposite trend is seen with taxa in the bottom cluster (a negative correlation with moisture, OM, sand, Fe). OTUs of *Clavicipitaceae*, *Dendryphion*, and one *F. oxysporum* found in high yield soils were positively but not significantly correlated with yield.

A Pearson correlation plot combining yield, soil physical/chemical factors, and select diversity indices (ITS2 Fisher's alpha diversity, LSUA ACE richness estimate, LSUA Phylogenetic Diversity) was used to visualize overall trends in the data (Figure 10). Diversity and evenness indices were trimmed to remove duplicate patterns (i.e., ITS2 and LSUA PD indices correlated identically with other factors, so only one was retained). No single soil physicochemical component or diversity/evenness index highly ( $>0.75$  or  $<-0.75$ ) correlated with yield, but pH had a strong negative correlation ( $-0.5 < x < -0.75$ ), confirming that higher yields were typically seen in soils with pH slightly below neutral. Other partially correlated factors included CEC, moisture, OM and Al. The three fungal

diversity and evenness indices are also partially correlated with Fe and moisture. Neither Shannon's  $H'$  or Simpson's  $D$  were significantly correlated with any other factor (data not shown), confirming the non-parametric nature of the OTU abundances found in these datasets. We intended to develop a structural equation model (SEM) to determine the interaction effects of each variable on the dependent variable (yield). The SEM was weakly correlated, which may be due to underlying complex interactions of numerous soil and other environmental factors that are different in different sites and across farms, as well as the aforementioned batch effect between the two NGS runs.

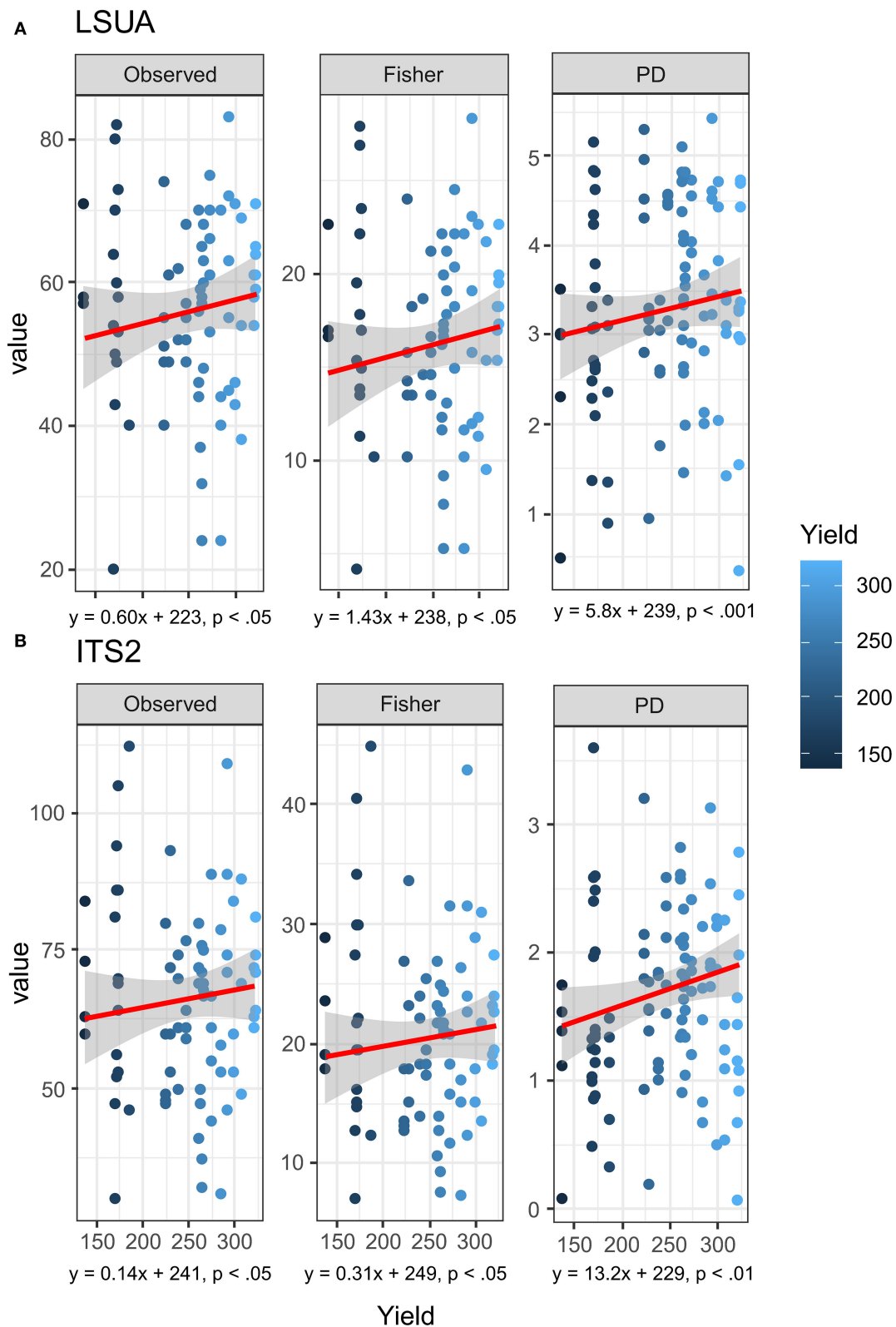
When combining all the trends in soil chemistry, yield, and the corn root mycobiome, we see unique patterns within individual farms (Figure 3, Supplementary Table 1). In farm 1, chemical differences in %Al saturation, pH, %Na, Zn, Fe had no relationship with overall differences in corn mycobiomes (non-significant NMDS separation of high and low yield, data not shown), but some interesting indicator taxa, including *Claroideoglossum* sp., *Neomassariosphaeria typhicola*, *Pythium*



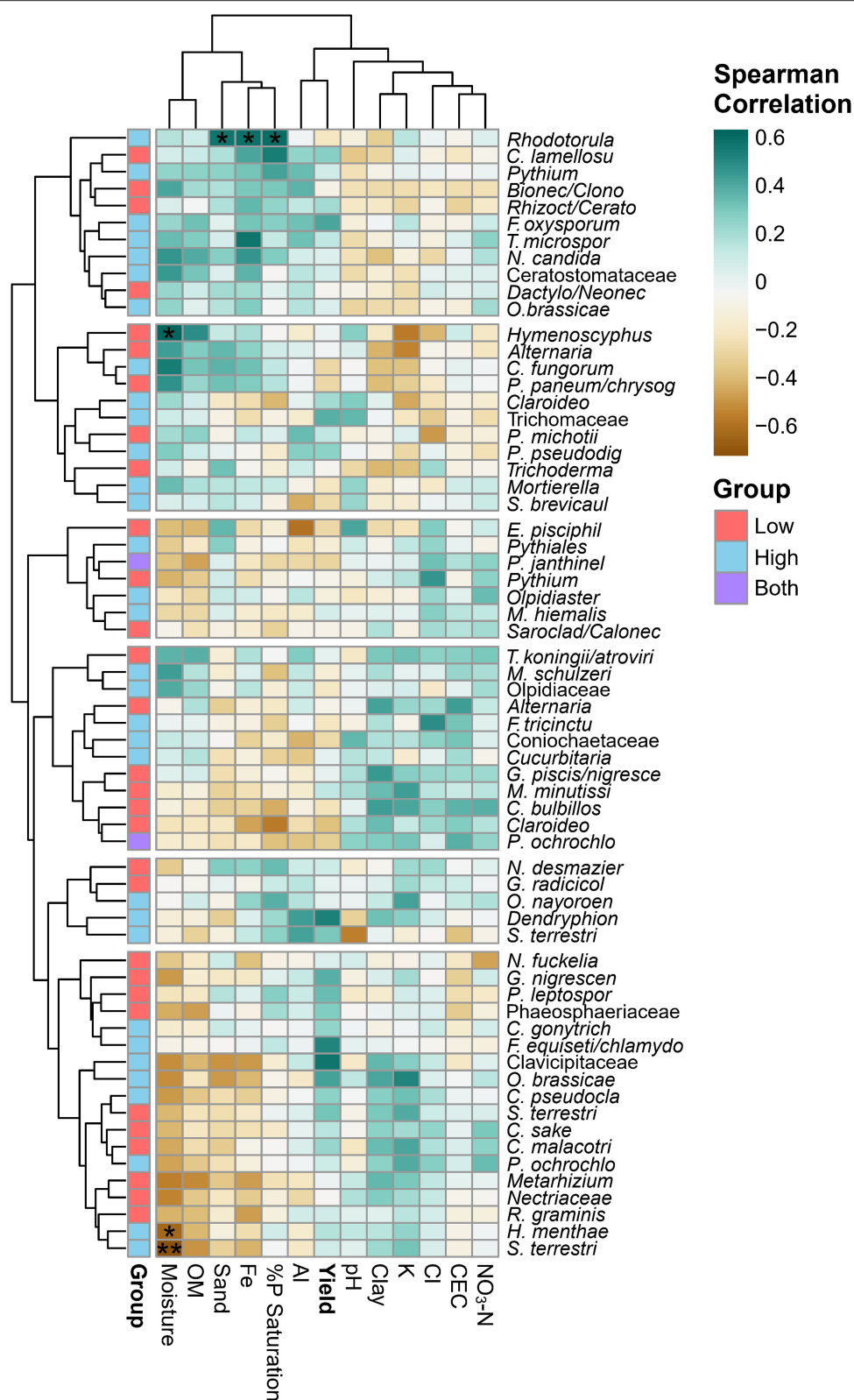


spp., and *Penicillium* sp. were observed. In farm 2, no significant relationship between soil physicochemical parameters, yield, or root mycobiomes were observed, most likely due to the negligible difference in yield between sites. Farms 3, 8, and 9 had few differential taxa between high and low yield soils with no significant separation between the total microbial profile of H/L yielding sites. However, noticeable chemical differences including pH, Mn, Al, clay, and Reactive C were observed in farm 9, with a 6.32 t/ha (94 bu/ac) difference in yield between productivity sites. Farm 8 had 2.69 t/ha (40 bu/ac) difference in yield, with moderate differences in chemical factors including pH, Mn, CEC, Mg, and %P saturation. Farm 3, with a 3.03 t/ha (45 bu/ac) difference showed variation in physicochemical factors such as Mn, CEC, Mg, Silt, and B. In these three cases, we can surmise that the mycobiome had little relation to yield when compared to the influence of soil physicochemical parameters, since there were no clear mycobiome separations in NMDS analyses. In farm 4, similar to other high yielding sites, physicochemical factors specifically pH, Mn, OM, %Na, and Zn, seemed to overwhelmingly drive the 6.12 t/ha (91

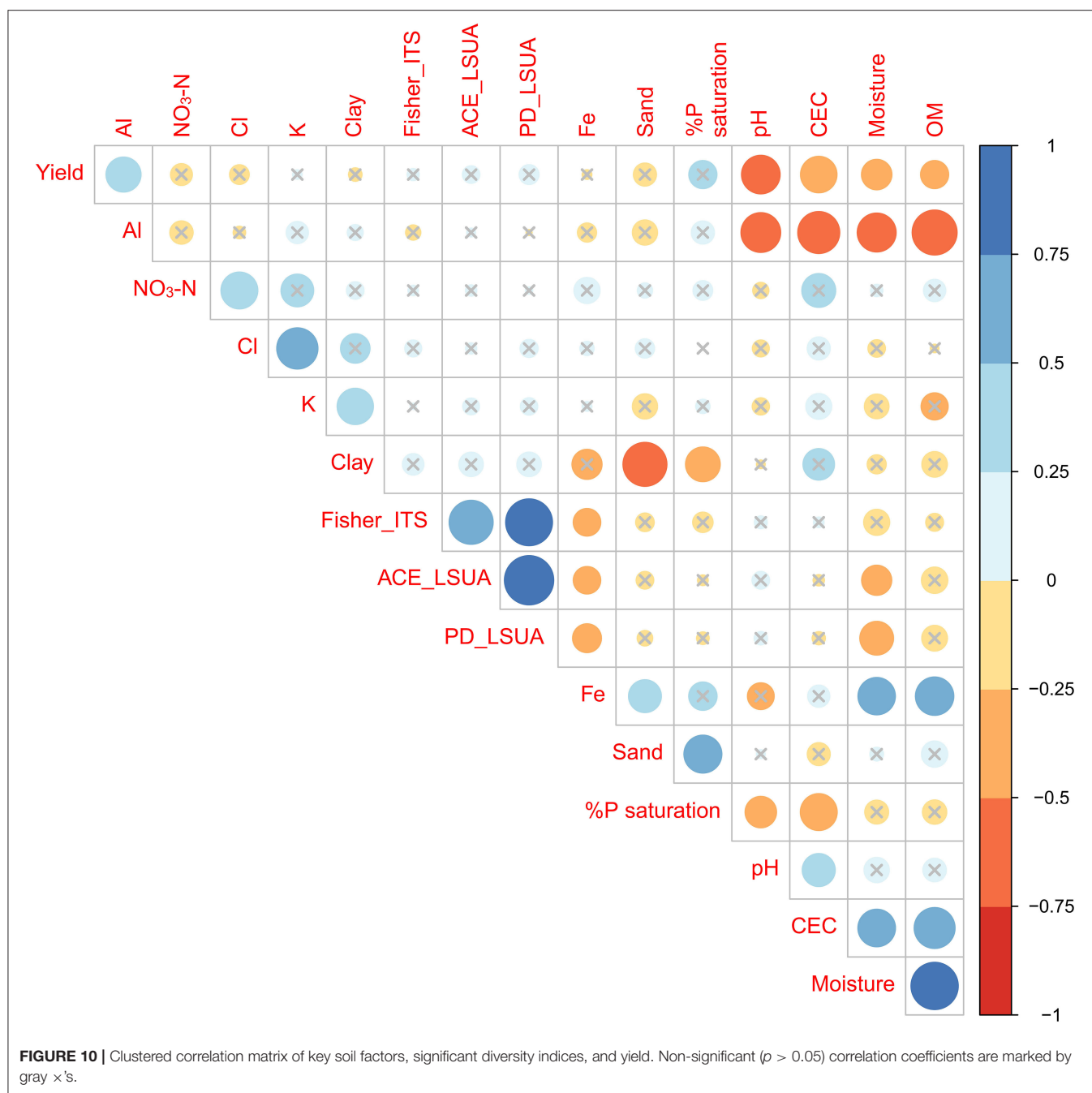
bu/ac) yield difference, with OM and Zn primarily separating sites. This farm also had a significantly high occurrence of *Setophoma terrestris* in both high and low sites, and unknown Pythiales in low sites. Farm 7, with a moderate 3.03 t/ha (45 bu/ac) difference in yield, had different pH, Mn, %Al saturation, Al, and clay in soils, with a unique presence of some taxa including beneficial *Claroideoglossum* spp., pathogenic *Olpidiaster* sp., *Pythium* sp., and *Murispora* sp. Farm 10 had different pH, Mn, %Na, %P saturation, %Al saturation and several distinct fungi, including the pathogens *Conlarium* sp., *Psilachnum pteridii*, beneficial fungi including *Claroideoglossum*, *Exophiala pisciphila*, *Fusarium oxysporum*, *Penicillium* spp. (5 OTUs), and *Rhodotorula mucilaginosa* between low/high yield sites. Farm 6 was unique in having a clear separation of H/L sites in PCA or NMDS ordinations but having physicochemical factors orthogonal to the plane of site separation, with only high Fe significantly associated with high yield sites (data not shown). Despite this, the yield difference was 3.36 t/ha (50 bu/ac), leading us to presume that there was more stochastic physicochemical variability in low yield sites, and heterogeneous



**FIGURE 8** | Kendall–Theil Sen Siegel non-parametric linear regressions on rarefied ( $n = 500$  OTU counts) for **(A)** LSUA and **(B)** ITS2 primers using multiple diversity and evenness indices: OTU richness (Observed), Fisher’s alpha (Fisher) diversity, and Faith’s Phylogenetic Diversity (PD) index.



**FIGURE 9 |** Clustered heatmap of Spearman correlation coefficients between ecologically important indicator taxa (ALDEx) and key soil variables found in this study. Significant Benjamini–Hochberg adjusted  $p$ -values are shown with asterisks: \* $p < 0.05$  and \*\* $p < 0.01$ . Top (soil physicochemistry) and left (ALDEx taxa) dendrograms cluster similar factors using the complete linkage method.



microbial factors potentially affecting to plant health and crop productivity, alongside other unmeasured factors. In farm 6, we identified several fungal species including the potential plant pathogens *Pythium* sp., *Pseudohalonectria* sp., *Myrmecridium* sp., *Setophoma* sp., *Bionectria/Clonostachys* sp., and *Cladorrhinum bulbillosum* in low yield sites. Additionally, there were two *Penicillium* sp., *Conlarium aquaticum/duplumascospora*, *Chalara fungorum*, *Gibellulopsis* sp., and *Trichoderma* sp. that were significantly associated with high yield sites. In contrast to farm 6, farm 10 had a clear separation of productive sites in both PCA and NMDS analyses (data not shown) with some

fungal species such as *Penicillium ochrochloron*, *P. janthinellum*, *Claroideoglossum* spp., *Exophiala pisciphila*, and *Conlarium aquaticum/duplumascospora* present only in high yield sites. Notably, farm 10 also had the largest difference in yield of all sampled sites, 8.61 t/ha (128 bu/ac), and a significant presence of *Penicillium* in high yield sites. However, it is farm 5 where we saw the highest yield differences—21.52 t/ha (320 bu/ac) vs. 16.07 t/ha (239 bu/ac) in high and low yield sites, respectively. This farm had obvious chemical differences such as pH, Mn, %P saturation, B, and Reactive C, with some unique fungal taxa such as *Claroideoglossum* sp., *Exophiala pisciphila*, and



*Oedocephalum naylorense*, one potentially pathogenic and two beneficial fungi. However, with no clear NMDS/PCA separation based on abundance data, it would suggest that the soil chemistry in farm 5 was a driving factor in the higher overall yield and yield differences.

## DISCUSSION

This study focused on associating key fungal species and soil physiochemistry with high or low farm productivity within and across corn fields in southwestern Ontario. Despite the general differences in the 51 soil physicochemical parameters assessed across all of the 10 farms included in this study, factors such as pH, CEC, organic matter, % moisture, % phosphorus saturation, sand, clay, nitrate-nitrogen, potassium, iron, chloride and aluminum linked to site specific productivity differences and the corn root mycobiome. Soil factors, ranked as  $\text{pH} > \text{OM} > \% \text{Na} > \text{Mn} > \text{Al}$ , were primarily involved in site separation in farms with the highest yield differences ( $> 3.36 \text{ t/ha}$  or  $50 \text{ bu/ac}$ ) compared to the fields with minimal productivity differences. The least influential factors, including %MAC,  $\text{NO}_3\text{-N}$ , Cl, and C:N were not correlated with productivity differences. Interestingly, Mn, %Al saturation, Reactive C, B, and Zn—important contributors in individual locations—were not part of the overall yield model, which instead included Sand, pH, % moisture, OM, CEC, %P saturation. Significant positive trends between yield and observed OTU, Fisher's alpha and Phylogenetic Distance were seen, with higher yields having higher richness and diversities in primers amplifying general fungi and Ascomycetes.

It was unsurprising that soil texture (sand and clay) was significant in modeling differences between low and high yield corn in adjacent sites, and across all the farms in the study region. Soil texture classification systems that measure and categorize the percent silt, sand, and clay, allow soil scientists to determine water and nutrient retention/release, both of which control microbial access to soil organic matter (SOM). High clay soils, in particular, limit decomposition by reducing microbial access to SOM or by protecting organic matter from enzyme attack (Baldock and Skjemstad, 2000). Despite management practices that attempt to increase uniformity (tillage, fertilization, digital field mapping), soil heterogeneity still occurs at the field level, and this clearly still has a strong effect on plant productivity, and the incidence of pests and pathogen loads (Dordas, 2008; Patzold et al., 2008; Hbirkou et al., 2011; Veresoglou et al., 2013).

Differences in abiotic and biotic factors in soils as well as stochastic processes are major contributors to productivity differences within sites having the same cropping history, crop genotype, and management practices (Chang et al., 2017). There were 8 different hybrid corn varieties among the 10 sites (Table 1), so it was difficult to correlate yield or mycobiome differences with corn variety. Many researchers claim that plant genotypes are responsible for the composition of root exudates that influence the microbial recruitment in the rhizosphere (Patel et al., 2015), and we can assume that a portion of the overall unexplained variation was also due to genotypic variations between cultivars. Soil characteristics, types of fertilization,

and crop management practices can all strongly influence soil properties, rhizosphere and root microbial abundance, diversity, and resilience (Xu et al., 2009; Antoninka et al., 2015; Tkacz and Poole, 2015), as well as crop and soil productivity in changing environments, climatic conditions, and increased anthropogenic activities (Stockmann et al., 2015; Pii et al., 2016). There was no clear, singular mechanism linking productivity differences to the mycobiome in our study, which might be due to multiple soil and climatic factors including but not limited to soil physicochemical parameters such as moisture content, water holding capacity, particle size differences, difference in the cropping regimes of individual farms, geographic location of the farm, stage and time of sampling, underlying differences in pathogen pressure depending on farm-specific rotation practices, and differences in functional community abundance and diversity driven by years of repeated farming practices. Physicochemical parameters such as pH, %P saturation, CEC, sand, and clay showed a strong relationship with corn productivity in most of the fields sampled in this study. Tedersoo et al. (2014) found the association of edaphic factors such as mean annual precipitation, pH and the concentrations of P and Ca with fungal diversity. Fungal communities may have different response patterns to fertilization and crop rotation than bacteria. In a study by Ai et al. (2018), soil fungi were more affected by crop rotation type (wheat-maize and wheat-soybean), indicating that plant-fungal communities were more tightly linked to one another than bacterial-fungal or bacterial-plant communities in arable soils (Cassman et al., 2016).

Chen et al. (2020) demonstrated that a balanced fertilization regime of P and K increased yield by enhancing the activity of  $\text{PO}_4$  solubilizing microbes, and fertilization using only N and K did not increase yield, indicating that in addition to balancing fertilization, other approaches are required for quickly improving crop yield according to the nature of the soil. Our results corroborate this finding, asserting that the right chemical balance is essential for promoting the establishment of beneficial mycobiomes that are key to enhancing plant productivity. Despite the data suggesting a strong link between crop productivity and selected soil factors, we also see different mycobiomes between healthy and unhealthy sites within farms. There were numerous known antagonistic or beneficial fungi identified in the ITS2 and LSUA datasets, alongside others that did not have any immediately clear ecological roles. In some cases, fungi that are commonly regarded as pathogenic were found in high yield soils in significantly higher abundances than in low yield soils, while the opposite was true in other cases. This could indicate the possibility of non-pathogenic strains of known fungal pathogens (Chulze et al., 2014) or alternative ecological roles that have yet to be revealed.

Corn producing higher yields had higher root-associated fungal phylogenetic diversity and OTU richness than low yield corn in this study (considering general fungi as well as only ascomycetes). However, because the ITS2 primer preferentially amplifies the shorter Ascomycota over the longer Basidiomycota amplicons (see Technical Shortfalls), it is likely that this significant result in both primers (ITS2 and LSUA) is mostly driven by the overrepresentation of the Ascomycota in our datasets. Soil structure and stability has been linked to higher

bacterial (Awasthi et al., 2014; Tardy et al., 2014; Maron et al., 2018) and fungal (Yang et al., 2017) functional and taxonomic diversity. In this case, high root-associated fungal diversity may be linked to corn variety-specific root exudates that are released in order to recruit healthy mycorrhizospheres (Rillig and Mummey, 2006), that in turn support plant growth through pathogen protection, nutrient acquisition, and other mechanisms (Shiomi et al., 2006; Ikram et al., 2020). Unfortunately, there was an inadequate number of corn hybrid replicates across farms to test this interaction. Higher soil and root-associated fungal diversity and richness may also be attributed to management differences including cover cropping or specific crop rotations that promote the colonization of the soil rhizosphere (Lehman et al., 2012; Njeru et al., 2014; Borrell et al., 2017). Lehman et al. (2012) demonstrated that a fall cover cropping mixture (oats-pea) had significantly higher AMF propagules  $\text{g}^{-1}$  than no-crop. Lower diversity (and potentially lower yield) can also be the result of degradative management practices including long-term fertilization (Beauregard et al., 2010; Zhou et al., 2016), or tillage (Schmidt et al., 2019).

Soil chemical and/or mycobiome profiles are fairly distinct in all the sampled locations, except for farm 2 and 4. No obvious differences were seen in soil physicochemical parameters, the corn root mycobiome, or yield in farm 2, which may be due to environmental impact on the quality of NDVI imaging (Johnson et al., 2018). Otherwise, it is possible that sources of plant stress during the imaging stage was subsequently corrected through natural or mediated farming practices, resulting in narrowing yield differences at the time of sampling. Extremely high levels of OM in farm 4 likely masked the influence of other factors on productivity and the mycobiome. As reported by Sul et al. (2013), soil organic matter was the most important factor explaining differences in the microbial community structure in agricultural soils. Despite farm 10 having the largest yield difference between stressed and healthy plants, it was farm 5 that exemplified an ideal harvest situation, where the healthy corn plants had the highest yield of all measured sites (21.52 t/ha; 320 bu/ac), not to mention a substantially higher yield in stressed, low yield plots (16.07 t/ha; 239 bu/ac) than average Ontario values (10.33 t/ha; 153.6 bu/ac). This may be due to synergistic effects between optimal soil health conditions, the mycobiome, crop genetics, management practices, and other unmeasured variables (see below). Farm 5 also has a silt-loam soil type, one that is typically the best suited for agricultural purposes (Haraldsen et al., 2000).

The mycobiome species identified in this study may be affected by differences in crop genetics, cropping regimes, farm management, stochastic soil processes, heterogeneous microbial distributions, on top of any other indiscernible effects of soil physiochemistry and abiotic/biotic factors. Some of these unmeasured variables potentially affecting our study include biotic factors such as nematodes, earthworms, and rhizosphere/bulk soil bacteria, as well as abiotic factors such as water table height, growing season temperature, and monthly precipitation. Plant-parasitic nematodes (e.g., root-knot and root-lesion nematodes) have long since been implicated in corn, soybean, wheat, tomato, and other crop losses in North America (McSorley and Gallaher, 1993; Grabau and Chen, 2016; Simon

et al., 2018), but were not measured or considered in this paper. Bacterial populations are also an essential component of corn-soil-rhizosphere interactions (Song et al., 2007; Li et al., 2018), but their influence on biotic and abiotic factors in our study sites were not within the scope of this paper. Other unmeasured abiotic factors in this study include groundwater, precipitation, and temperature. Water in all ten farm sites may be an important but incompletely measured variable since soil moisture data was taken only at the time of sample collection. In Ontario, there is generally not enough groundwater available to replenish corn agricultural water use and evaporation. Corn requires ~50 cm (20 in.) of water to produce high yields and this can be supplied over the growing season from a combination of stored water in the soil, rainfall or irrigation (OMAFRA, 2017). In 2017, the average total rainfall levels across sites was 34 cm (<https://climate.weather.gc.ca>), which necessitated specific watering by farmers to prevent drought conditions. Management practices such as differences in planting date, planting density, irrigation (timing of), fertilizer applications, weed management techniques (including cover and companion cropping), etc. (Scharf et al., 2002; Bruns and Abbas, 2006; Kucharik, 2006; Van Roekel and Coulter, 2011; Yeganehpour et al., 2015) are difficult to control for in studies comparing yield differences between farms. In addition to this, the development of various hybrid varieties further introduces sources of variation between locations since each variety is developed for different soil physicochemical and environmental systems (Troyer, 2009; OMAFRA, 2017). These and many other components may have increased the proportion of unexplained variation in the mycorrhizosphere in our study.

Farms 6 and 10 had more potentially influential taxa than all other farms in this study. Two OTUs identified as *Fusarium oxysporum* were significantly associated with high yield sites in farms 6 and 10, but also in high relative abundances in farms 2, 7, and 9. *Fusarium equiseti/chlamydosporum* and *F. tricinctum/avenaceum/acuminatum* OTUs were also significantly associated with low yield sites within farms 7 and 10. In agriculture, pathogenic *Fusarium* species, including *F. oxysporum* and *F. equiseti*, among others, are known to primarily cause corn ear and kernel rot (Wall and Mortimore, 1965). However, *F. oxysporum* is also considered a common member of the plant rhizosphere as a saprotroph (Gordon and Martyn, 1997), and all strains can grow and survive for long periods of time on plant matter in the soil and rhizosphere (Garrett, 1970). Pathogenic species of *Fusarium* produce a wide range of mycotoxins (Rep and Kistler, 2010), have a high level of host specificity, and cause root rot, although others have been found in roots without any disease symptoms (Palmer and Kommedahl, 1969; Olivain and Alabouvette, 1997); all of this depending on the plant species or cultivar. *Fusarium oxysporum* was found as one of the predominant species (at 12.1% isolation frequency) from rotted maize ears and kernels in Chongqing (Zhou D. et al., 2018), and 76% of soil isolates and 35% of stem isolates in New Zealand (Harrow et al., 2010), whereas *F. equiseti* was found at 3.4% isolation frequency (Zhou D. et al., 2018). This species is considered ubiquitous in cropping soils and cereal stem bases (Harrow et al., 2010) and was seen in

our study as well. It is therefore unsurprising that *F. oxysporum* was found in both low and high-yield sites in our study. The *F. avenaceum/acuminatum/tricinctum* species complex (OTU469 in low yield farm 10 sites) is one that has been linked to Fusarium head blight. In temperate regions, *Fusarium avenaceum* is similar to *F. tricinctum*, which is often considered to be a relatively weak pathogen of cereals (Harrow et al., 2010), and morphologically similar to *F. acuminatum*, identified as a causative agent in crown and root rot of wheat (Rep and Kistler, 2010). Harrow et al. (2010) found that *F. avenaceum* was most commonly found in stems but in lower abundances in the soil, indicating their ability to survive for short periods of time.

A fungus identified as *Chalara fungorum* was amplified by the Ascomycete-specific primers with high levels of occurrence across many farms; it was significantly associated with high yield sites in farms 6 and 10 and found in high relative abundance in farm 3. The anamorphic genus *Chalara* represents a polyphyletic assemblage of species with similar morphologies (Koukol, 2011), and the true identity of this OTU remains to be determined. The genus contains some aggressive primary plant pathogens responsible for oak-wilt (conidial stage *Chalara quercina*) (Raj and Kendrick, 2006), the causal agent of narrow-leaved ash dieback (*C. fraxinea*) (Kowalski and Holdenrieder, 2009), myrtle beech dieback in Australia (*C. australis*) (Kile and Walker, 1987), and on *Pseudocyphellaria* “speckle belly” lichens (*C. pseudocyphellariae*) (Etayo and Sancho, 2008; Koukol, 2011). However, most *C. fungorum* observations are as saprobic dematiaceous anamorphs on plant litter—coniferous needles, cones, wood or bark, in temperate and tropical regions, with several recurring on particular substrates (Koukol, 2011). It may also be fungicolous as it was originally described on an old fruiting body of a *Hydnellum* (Saccardo, 1886). Its significant association with high yield sites might be due to its role as a saprobe, but it has the possibility of being a beneficial root-associated partner.

*Penicillium ochrochloron*, *P. janthinellum* and *P. paneum/chrysogenum* were all significantly associated with high yield sites in farms 1, 6 and 10. *Penicillium* species are famous for their production of antibiotics and secondary metabolites with interesting biological activity, and so their significant occurrences in high yield sites lead us to categorize their roles as beneficial fungi in this study. Species such as *P. ochrochloron* show activity against Gram positive and negative bacteria, as well as *Candida albicans* (Rančić et al., 2006), and chitinase extracts from this species have shown to affect the growth of larvae of *Helicoverpa armigera*, a common worldwide crop insect pest (Patil and Jadhav, 2015). Additionally, *P. ochrochloron* has been known to solubilize phosphate (Sánchez-de Prager and Cisneros-Rojas, 2017) through the production of citric and malic acid (Coutinho et al., 2012). It is therefore possible that recruitment or proliferation of *Penicillium* in corn roots may have contributed to yield increases in our high yield sites.

Two *Trichoderma* OTUs were significantly associated with high yield sites in farm 6. *Trichoderma* species (*T. hamatum* and *T. koningii*) can induce biofertilization of crops and can increase production up to 300% (Benítez et al., 2004). *Trichoderma* is

also considered a bio-parasite, and has been proposed as a biocontrol agent for phytopathogenic fungi such as *Bipolaris oryzae*, *Fusarium* spp., *Rhizoctonia solani*, *Sclerotium rolfsii*, *Phytophthora cactorum*, *Sclerotinia sclerotiorum*, *B. cinerea*, and *Pythium* spp. (Benítez et al., 2004; Anuar et al., 2019; Arifin and Ilham, 2019; Sanchez et al., 2019). Inoculation of *Trichoderma* sp. in soil fertilized by tricalcium phosphate showed an increase in plant biomass (dry weight and root) of chickpea plants (Kapri and Tewari, 2010).

Despite farming practices that attempt to increase homogeneity, a field's topography, water table, soil erosion patterns, stochastic processes and other factors (Girvan et al., 2003) continue to create pockets of low productivity within average-to-high producing fields. To sustain demanding agricultural practices, inclusive soil health assessments such as physicochemical balances and biological indicators of soil quality are needed (Doran and Zeiss, 2000). Unraveling the association of the mycobiome with its contribution to rhizosphere health and soil quality will identify which fungi are recruited to or naturally proliferate within high or low productive soil environments, and further uncover the roles of fungi in agricultural systems. Our results suggest that the associations between soil edaphic and biotic factors, the mycobiome, and productivity are complex and that no single fungus or set of a few fungi drive yield in positive or negative ways.

## Technical Shortfalls

The general fungal LSU primers did not recover an adequate number of fungal reads to be a reliable choice for plant tissues due to the large proportion of *Zea mays* reads from root DNA (85.7% of all reads). These primers were originally developed for use in soil samples (Asemaninejad et al., 2016), in which they understandably have better performance than with root-associated DNAs. Unfortunately, this leaves the Basidiomycota poorly represented since the LSUA primers have multiple mismatches to members of Basidiomycota, and Illumina reads of ITS2 amplicons will favor the shorter (and thus more abundant) amplicons of Ascomycota rather than Basidiomycota, which are 30–190 bp longer (Bellemain et al., 2010).

Batch effects were also a confounding factor in our ordination analyses and none of the batch removal tools employed through R resulted in any datasets with visible patterns or clusters. This lead us to believe that (a) the sampling effect differences between runs were strong, (b) it may be difficult to truly generalize clustering patterns between low and high yield sites across farms with different corn varieties, geographic locations, farming/amendment practices, and soil types, and (c) future studies with similar designs will require more randomization of samples between runs to help reduce batch effects.

## Future Research

An immediate next step will be expanding our research to further confirm the key and stable fungal taxa associated with high/low productivity sites by involving structural equation models (SEM), which may help understand stochastic and deterministic ecosystem processes (Antoninka et al., 2009; Pascual-García and Bell, 2020). We hope that a better understanding of the



ecological roles of relatively abundant taxa associated with farm-specific productivity will enable the development of biological products that help to improve under-productive sites. Our preliminary list of fungal root-associated taxa in this study is a starting point for this selection. Further exploring mechanisms to balance key soil physicochemical factors will help to establish and sustain a healthy, beneficial mycobiome. Alongside corn, wheat and soybeans are common rotational crops in Ontario that will also benefit from similar in-depth analyses into root mycobiomes and how they may vary across soil types, farming practices, and overall yields. Analyses like this may help to improve soil productivity regardless of crops grown and improve the nutrient use efficiency as well as reduce the overuse of chemical fertilizer. Excessive agrochemicals, such as N fertilizer, tip nutrient balances and are often not completely consumed by plants (Magdoff, 1991). This is not an economically sustainable practice and results in large-scale environmental issues such as terrestrial and aquatic eutrophication, acidification, atmosphere pollution, and the creation of large hypoxic zones in freshwater and marine water bodies (Good and Beatty, 2011). There will continue to be an incentive to replace short-term fertilization for immediate yield gains with long term sustainable practices to keep up with predicted high future demand for cereal production resulting from increases to the global population and subsequent dietary shifts, especially in underdeveloped regions (FAO, 2009). Developed countries have already nearly reached the maximum biological yield potentials of crops using fertilizers, but we cannot sustain these levels of production without serious consequences (Good and Beatty, 2011). By looking into site-specific farming practices that incorporate biological-fertilizers and natural mechanisms for plant growth promotion, we can reduce the need for damaging long-term abiotic amendments. There is a need to explore the dynamics of stress-tolerant microbes in relation to host species in various habitats and geographical locations to reduce sources of crop stress, the need for excessive farm inputs, and crop production heterogeneity.

## CONCLUSION

Understanding the deterministic process involved in shaping the biogeography of microbial communities and its association with plant productivity remains a mystery, but our research has taken a step to provide some insights through large scale field-based research. In this study we identified soil physicochemical factors and corn root associated fungi within and between low and high producing farms in southwestern Ontario. When modeling for yield differences across farms with varying soil chemical factors, pH, moisture, %P saturation, sand, clay CEC, and OM levels helped to predict corn yield. When looking at individual farms, low yielding sites were often correlated with less than ideal levels of one or more of soil physicochemical factors such as pH, Mn, OM, %Na, CEC and Mg. Associated indicator root fungi were distinct in most of the high or low yielding soils within farms but could not be generalized across farms, perhaps because of variation in soil physicochemical parameters.

However, higher yield sites had greater fungal diversity (Fisher's  $\alpha$  and phylogenetic diversity) than lower yield sites. Locations with large differences in yield (>80 bu/ac or 5.38 t/ha) had substantial differences in soil chemistry, structure, and/or fungal indicator taxa. This confirms previous studies that claim an imbalance in soil physicochemistry not only directly affects crop productivity but may also indirectly influence productivity by modifying the abundance and diversity of soil myco- and microbiomes. Different combinations of fungi and soil features, in addition to other edaphic and biotic components, lead to differences in site-specific plant productivity that are unique to each farm location, which further emphasizes the importance of personalized farm prescriptions to improve pockets of under-productive farm locations. A better understanding of the identities and ecological roles of fungi constituting the soil mycobiome will be needed to bring prescriptive management for sustainable agriculture.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ebi.ac.uk/ena, PRJEB40800>.

## AUTHOR CONTRIBUTIONS

SK, GL, and RGT contributed to the conception and design of the study. SK sampled the fields and did all the meta data analysis. SK and SA took part in sample processing at the lab. SK and GS prepared NGS libraries. NW performed bioinformatic analysis of NGS data. SK, NW, RGT, GL, and GP participated in data interpretation. NW and SK wrote the manuscript. SK, NW, RGT, and GL reviewed and edited the manuscript. SK, NW, RGT, and GL contributed to manuscript revision, and all coauthors read and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.617332/full#supplementary-material>



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# Rethinking Crop Nutrition in Times of Modern Microbiology: Innovative Biofertilizer Technologies

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Global population growth poses a threat to food security in an era of increased ecosystem degradation, climate change, soil erosion, and biodiversity loss. In this context, harnessing naturally-occurring processes such as those provided by soil and plant-associated microorganisms presents a promising strategy to reduce dependency on agrochemicals. Biofertilizers are living microbes that enhance plant nutrition by either by mobilizing or increasing nutrient availability in soils. Various microbial taxa including beneficial bacteria and fungi are currently used as biofertilizers, as they successfully colonize the rhizosphere, rhizoplane or root interior. Despite their great potential to improve soil fertility, biofertilizers have yet to replace conventional chemical fertilizers in commercial agriculture. In the last 10 years, multi-omics studies have made a significant step forward in understanding the drivers, roles, processes, and mechanisms in the plant microbiome. However, translating this knowledge on microbiome functions in order to capitalize on plant nutrition in agroecosystems still remains a challenge. Here, we address the key factors limiting successful field applications of biofertilizers and suggest potential solutions based on emerging strategies for product development. Finally, we discuss the importance of biosafety guidelines and propose new avenues of research for biofertilizer development.

**Keywords:** plant growth promotion, microbiome, plant nutrition, bioprospecting, soil health, sustainable agriculture, inoculation, bioformulation

## INTRODUCTION

Soil and plant-associated microbes play a key role in ecosystem functioning by carrying out numerous biogeochemical cycles and organic matter degradation (Paul, 2015). For this reason, biofertilizers (i.e., microbial-based fertilizers) are considered to be crucial components of sustainable agriculture, with long lasting effects on soil fertility (Bargaz et al., 2018; Singh et al., 2019). The term biofertilizer can be defined as formulations comprised of living microbial cells, either a single strain or multiple strains (mixed or consortium), that promote plant growth by increasing nutrient availability and acquisition (Riaz et al., 2020). Nevertheless, the

term itself has evolved over the last 30 years receiving many interpretations (El-Ghamry et al., 2018; Macik et al., 2020). As stated by Macik et al. (2020), the greatest misconception occurs when including microbial inoculants with other beneficial applications (e.g., biopesticides and phytostimulators) as biofertilizers. Likewise, plant growth-promoting bacteria or rhizobacteria (PGPB/PGPR) and biofertilizers should not be considered an interchangeable term, since not all PGPB/PGPR are biofertilizers (Riaz et al., 2020). Nonetheless, it is worth mentioning that biofertilizers can also provide other direct and indirect benefits for plant growth, such as phytostimulation, abiotic stress tolerance and biocontrol (Ferreira et al., 2019; Liu et al., 2020; Shirmohammadi et al., 2020).

The commercial history of biofertilizers dates back to 1895 using “Nitragin” by Nobbe and Hiltner with a laboratory culture of *Rhizobium* sp. (Singh et al., 2019). In the late 1950s, several studies with arbuscular mycorrhizal fungi inoculants reported positive plant growth promotion (PGP) effects through phosphorus (P) uptake (Koide and Mosse, 2004). However, despite their numerous advantages and low cost, the commercialization of biofertilizers is not widespread. The reasons limiting their use are mostly related to inconsistent responses over different soils, crops and environmental conditions, along with practical aspects related to mass production, shelf-life, appropriate recommendations and ease of use for farmers (Debnath et al., 2019).

In the last 10 years, multi-omics technologies enhanced our understanding of the complexity of microbiomes, as they allowed us to better characterize the structure and function of microbial communities (Kaul et al., 2016). These novel approaches are increasingly applied to describe soil microbial communities and their influence on plant nutrient acquisition and other PGP traits (Saad et al., 2020; Tosi et al., 2020a,b). However, they have yet to be successfully applied in the development of novel and improved biofertilizer technologies (Qiu et al., 2019).

In this review, we focus on the direct mechanisms by which microorganisms enhance the availability and acquisition of essential plant nutrients. Subsequently, we assess the current challenges and constraints faced by the implementation of biofertilizers in agriculture, and we discuss emerging strategies for biofertilizer development (e.g., bioprospecting and formulations). Finally, we address the potential risks that biofertilizers pose to human and environmental health, and conclude by highlighting current knowledge gaps and identifying priorities for future research.

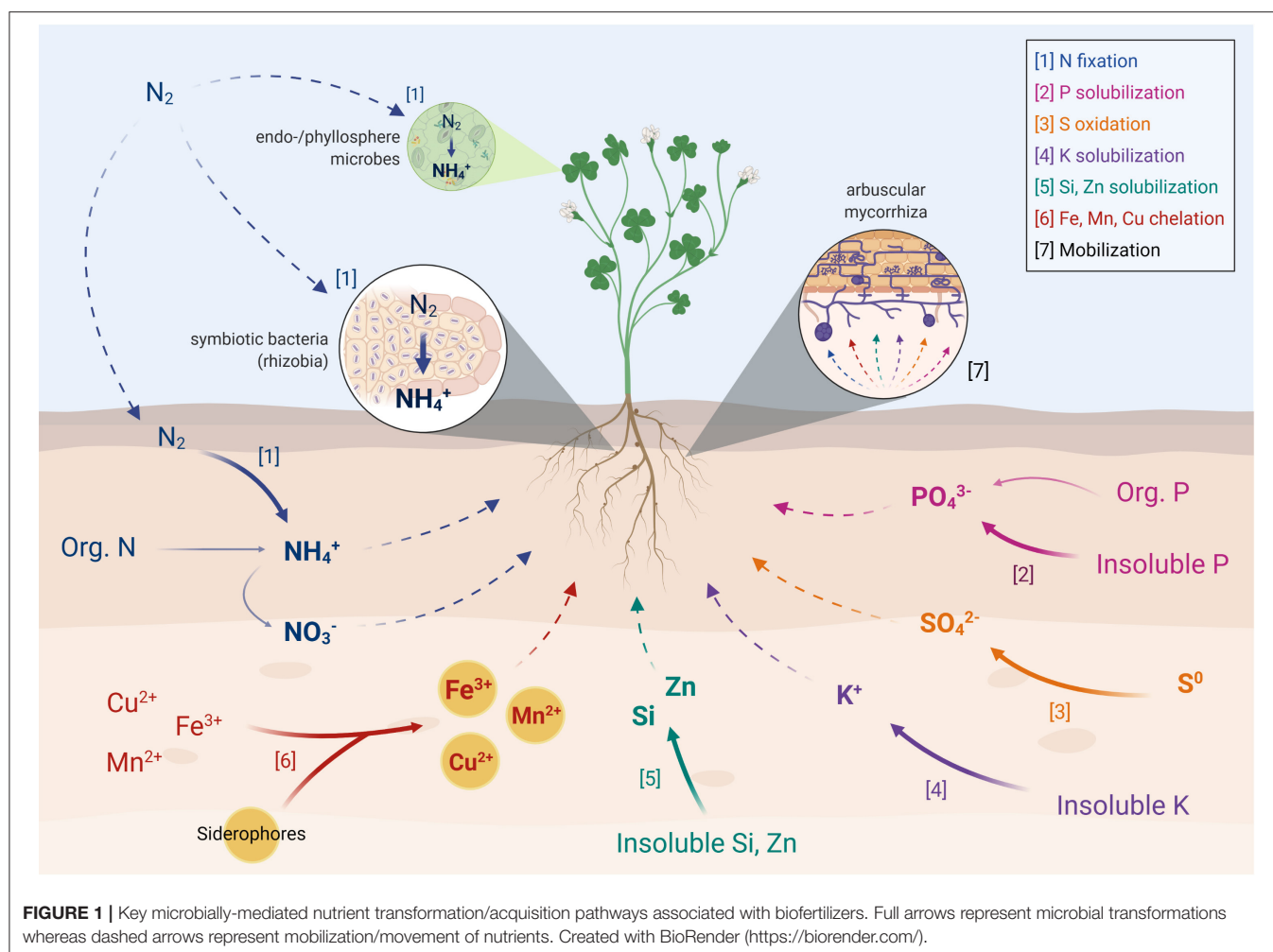
## AN OVERVIEW OF BIOFERTILIZERS: KEY MECHANISMS OF NUTRIENT ACQUISITION

### Nitrogen: N<sub>2</sub>-Fixation

Nitrogen (N) is an essential element for life and it is the fourth most abundant element in all living biomass after hydrogen,

carbon, and oxygen (Howarth, 2009). For example, N is an essential component of chlorophyll, amino acids, nucleic acids, and the energy transfer molecule adenosine triphosphate (ATP) (Werner and Newton, 2005). One important source of N in soils is organic N which requires microbial mineralization to be converted to plant available inorganic N, a combination of ammonification and nitrification (Paul, 2015). However, the major N reservoir is in the atmosphere as N<sub>2</sub>, which is not directly used by plants and only becomes available through Biological N<sub>2</sub>-fixation (BNF) (**Figure 1**). This is an energy-intensive process by which the enzyme nitrogenase converts atmospheric N<sub>2</sub> to ammonia (NH<sub>3</sub>), which is readily available for assimilation by plants and microbes (Dakora et al., 2008). Nitrogenases can be found in a small and diverse group of microorganisms called diazotrophs (N<sub>2</sub>-fixing), which includes symbiotic bacteria, and free-living bacteria and archaea (Moreira-Coello et al., 2019). In agriculture, the most studied symbiotic N<sub>2</sub>-fixing organisms are bacteria known as rhizobia, comprised mostly of the family Rhizobiaceae [i.e., *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Azorhizobium*, *Mesorhizobium*, and *Sinorhizobium* (*Ensifer*)] (Shamseldin et al., 2017). Rhizobia can establish symbiotic relationships with legumes (family Fabaceae) by forming nodules on their roots or stems (Masson-Boivin and Sachs, 2018). These nodules provide an advantage for N<sub>2</sub>-fixation in which nitrogenases are protected in bacteroids from atmospheric O<sub>2</sub>. The oxygen concentration is an important factor determining the amount of N that is fixed, since oxygen is a negative regulator of *nif* gene expression and inhibits nitrogenase activity (Glick, 2015). Several rhizobia, such as *Rhizobium*, *Sinorhizobium* (*Ensifer*), and *Bradyrhizobium*, are commonly used as biofertilizers in agriculture (Carareto Alves et al., 2014). Plants can acquire a significant proportion of their N requirement through associations with the diazotrophs (Dakora et al., 2008). For example, N<sub>2</sub>-fixation could supply ~20–25% of total N requirement in rice, ~30–50% in wheat and up to 70% in sugarcane (Hurek et al., 2002; Gupta and Paterson, 2006; Santi et al., 2013). Yet, the amount of N provided by BNF will vary depending on the plant species and environmental factors, which will ultimately determine a successful colonization (Parnell et al., 2016), as explained below.

In contrast to symbiotic N<sub>2</sub>-fixing bacteria, several heterotrophic free-living diazotrophic microorganisms such as *Azotobacter* sp., *Azospirillum* sp., and cyanobacteria can fix atmospheric N<sub>2</sub> in the rhizosphere and bulk soil. Free-living diazotrophs are particularly important for N acquisition in non-legume crops. For example, increased crop yields were observed in cereals (e.g., wheat, rice, and corn) and a variety of other crops such as sunflower, carrot, oak, sugar beet, sugarcane, tomato, eggplant, pepper, and cotton (Garcha and Maan, 2017). *Azospirillum* species can carry out several PGP functions but are also the most well known free-living diazotrophs, shown to enhance N availability and acquisition in more than 113 plant species (Bashan and De-Bashan, 2010; Pereg et al., 2016; Zeffa et al., 2019).



## Phosphorus: Solubilization and Mineralization

Phosphorus is one of the most important plant nutrients that directly or indirectly affects all biological processes. For example, P is key in all major plant metabolic processes such as photosynthesis, energy transfer, signal transduction, biosynthesis of molecules, and respiration. A considerable amount of P is present in soils, in both inorganic and organic forms, but its availability is one of the main factors limiting plant growth in many ecosystems worldwide (Raghothama, 2015). This is because most soil P is in an occluded or insoluble form, and unavailable for plants, which can uptake P from the soil solution as orthophosphate ions  $\text{H}_2\text{PO}_4^-$  and  $\text{HPO}_4^{2-}$  (Soumare et al., 2020). The concentration of soluble phosphates ( $\text{PO}_4^{3-}$ ) in the soil solution is generally low, around 0.001–0.4 mg P/L in unfertilized soils, which is equivalent to 0.001–0.01% of total-P (Weihrach and Opp, 2018). In addition, it is estimated that ~80% of the P applied via fertilization gets quickly fixed into stable forms in the soil, unavailable for plants (Pradhan et al., 2017).

Soil microbes are capable of converting insoluble soil P into plant available form(s) through various mechanisms of

solubilization and mineralization (Alori et al., 2017) (**Figure 1**). Phosphate-solubilizing microbes (PSM) solubilize inorganic P (e.g., tricalcium phosphate, hydroxyapatite, and rock phosphate) via the production and release of different compounds. One mechanism consists in the excretion of organic acids, hydroxyl ions and  $\text{CO}_2$ , which dissolve the insoluble phosphates directly by lowering the soil pH, then leading to ion exchange of  $\text{PO}_4^{2-}$  by acid ions (Wei et al., 2018). Microbes can also release chelating compounds that capture and mobilize cations from different insoluble phosphates such as  $\text{Ca}^{+2}$ ,  $\text{Al}^{+3}$ , and  $\text{Fe}^{+3}$ , resulting in the release of associated soluble phosphates (Riaz et al., 2020). By increasing P bioavailability, PSM reduce the need for mineral P fertilizer inputs, which in excess can lead to negative environmental impacts such as eutrophication of fresh-water bodies. The most studied P solubilizers belong to the genera *Pseudomonas*, *Bacillus*, *Rhizobium*, *Enterobacter*, *Penicillium*, and *Aspergillus* (De Freitas et al., 1997; Anand et al., 2016).

Another important process by which soil microorganisms can increase P bioavailability is by the mineralization of organic phosphate compounds (e.g., inositol hexaphosphate and phytate) (Alori et al., 2017). This process is mediated by phosphatases (e.g., phosphodiesterases and phosphomonoesterases) and phytases

that help release phosphate from organic P compounds, which can then be taken up by plants (Pradhan et al., 2017).

## Potassium: Solubilization

Potassium (K) is a vital plant macronutrient and a major inorganic cation in the plant cytoplasm, essential for cell constitution and functioning, implicated in photosynthesis, protein synthesis, and many other primary metabolic functions. Potassium is also the second most abundant nutrient in soil after N, and one of the most abundant elements on Earth. However, ~98% of soil K is present in a non-exchangeable form, trapped within crystal structures of the minerals feldspar and mica (e.g., muscovite, biotite). Another 1–2% is adsorbed onto clay particles and organic matter, while only 0.1–0.2% is in the soil solution and directly available for plant uptake (Srivastava et al., 2019). Currently, Canada is the world's largest potash producer with approximately one-third of the world potash reserves located in southern Saskatchewan (Broughton, 2019). However, especially in the Southern Hemisphere, several countries with little or no potash production are highly-dependent on the import of fertilizers and need alternatives to increase soil K availability.

Microorganisms can increase K availability via solubilization, a process that plays a key role in the K cycle by making K available to plants (Sattar et al., 2019; Macik et al., 2020) (Figure 1). Similar to P, the most well-known mechanism of microbial K solubilization involves the synthesis and discharge of organic acids (i.e., tartaric, citric, oxalic, gluconic, lactic, and malic acid) (Sattar et al., 2019). These organic acids lead to the acidification of the surrounding environment and therefore the release (acidolysis) of  $K^+$  from minerals (Sattar et al., 2019). Other important K release mechanisms include chelation, and exchange reactions involving organic acids (Sharma et al., 2016). Several groups of soil bacteria (e.g., *Bacillus*, *Rhizobium*, *Acidithiobacillus*, *Paenibacillus*, *Pseudomonas*, and *Burkholderia*) and fungi (*Aspergillus*, *Cladosporium*, *Macrophomina*, *Sclerotinia*, *Trichoderma*, *Glomus*, and *Penicillium*) can solubilize K minerals (Kour et al., 2020).

## Sulfur: Oxidation

Sulfur (S) is an essential nutrient for plant growth, implicated in the conformation of biomolecules such as proteins, glutathione, chloroplast membrane lipids, coenzymes, and vitamins. Most S in soils (~95%) is in an organic form (C-bonded S or sulfate esters), while inorganic forms are less common (5–10%). The most common form of inorganic S is sulfate ( $SO_4^{2-}$ ), which is readily available for plant uptake and is present either dissolved in the soil solution or adsorbed to soil particles (Scherer, 2009). In the last decades, S deficiency in agricultural soils increased on a worldwide scale, likely as a consequence of the decline in atmospheric deposition of S due to the reductions in  $SO_2$  emissions and the use of low-S fertilizers (Ercoli et al., 2012). Consequently, S fertilizers have received increasing attention, with elemental S ( $S^0$ ) as the most common form of S fertilizer. Elemental S constitutes a highly concentrated form of S but needs

to be oxidized to  $SO_4^{2-}$  in order to become available to plants (Scherer, 2009).

The application of S-oxidizing microbes can help by both optimizing S fertilization and minimizing environmental risks caused by S leaching (Figure 1). Sulfur-oxidizing bacteria can use  $S^0$  as an energy source, releasing plant-available sulfate. Hence, their inoculation together with  $S^0$  fertilizers can speed up its conversion to sulfates, potentially leading to higher crop yields (Pujar et al., 2014). Sulfur-oxidizing biofertilizers have been recommended for grain crops (e.g., oilseed species, oats) and horticultural crops (e.g., onion, cauliflower, ginger, garlic) (Santra et al., 2015). Sulfur oxidation in soil is carried out by a variety of archaea and bacteria such as the genera *Xanthobacter*, *Alcaligenes*, *Bacillus*, *Pseudomonas*, *Streptomyces*, and *Thiobacillus*, as well as fungi including *Fusarium*, *Aspergillus*, and *Penicillium* (Grayston et al., 1986; Germida and Janzen, 1993; Macik et al., 2020).

## Micronutrients: Chelation and Solubilization

Micronutrients such as iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), boron (B), molybdenum (Mo), chlorine (Cl), nickel (Ni), cobalt (Co), and silicon (Si), are essential for plants (Shukla et al., 2018). These are essential for plant development as they are involved in critical enzymatic reactions including photosynthesis, respiration, water oxidation, and oxidative stress protection (Castro et al., 2018). In fact, several studies revealed that micronutrient deficiencies hamper crop production in many areas of the world; especially in alkaline soils with low organic matter content (Rashid and Ryan, 2004).

One of the most studied mechanisms for increasing micronutrient availability is iron sequestration via siderophores (Rroço et al., 2003). Under Fe-limiting conditions, these low molecular weight chelating compounds scavenge  $Fe^{3+}$  (the most common form in soils) from the mineral phases, forming soluble  $Fe^{3+}$  complexes that are accessible for plant uptake (Figure 1). In general, plant species such as barley, rye, and wheat, can produce high concentrations of siderophores and, thus are more resistant to iron deficiency (Ahmed and Holmström, 2014). However, other crops (e.g., maize, sorghum, and rice) with lower siderophore production can benefit from siderophore-producing microorganisms. There are three main classes of fungal siderophores (i.e., rhodotorulic acid, ferrichromes, and fusarinines) and four classes of bacterial siderophores (i.e., phenol-catecholates, hydroxamates, carboxylate, and pyoverdines) (Crowley, 2006). In agricultural plants, siderophore production by *Pseudomonas fluorescens* was shown to play a role in Fe nutrition and PGP in tomato (Nagata, 2017), pea (Lurthy et al., 2020) and sorghum (Abbaszadeh-Dahaji et al., 2020) under Fe limiting conditions. Apart from Fe, siderophores are also known to bind other metals (e.g.,  $Al^{3+}$ ,  $Cd^{2+}$ ,  $Co^{2+}$ ,  $Cu^{2+}$ ,  $Hg^{2+}$ ,  $Mn^{2+}$ ,  $Ni^{2+}$ , and  $Pb^{2+}$ ) (Saha et al., 2013) (Figure 1).

Zinc deficiency is the most important micronutrient problem in crops, causing root necrosis, reduction of biomass and yield, and high plant mortality (Caldelas and Weiss, 2017). Also, it is estimated that more than 84% of total soil Zn occurs as



structurally lattice bound [e.g., zincite (ZnO) and zinc sulfide (ZnS)], while only 1% is in water soluble form and available for plant uptake (Sharma et al., 2013; Prasad et al., 2016). Consequently, Zn is now integrated into chemical fertilizers (i.e., applied along with NPK) for most crops in several countries (Prasad et al., 2016). However, most of this water-soluble Zn (96–99%) is rapidly converted to insoluble forms, and only 1–4% of the total applied Zn can be used by plants (Kushwaha et al., 2020). A solution to this problem is the application of Zn-solubilizing microbes (ZSM) as biofertilizers to increase Zn availability in soils where this micronutrient is present in high concentrations but insoluble forms (Sammauria et al., 2020) (**Figure 1**). Similarly to other solubilizers, these ZSM are capable of solubilizing Zn by acidification, chelation, and chemical transformation (Kushwaha et al., 2020; Macik et al., 2020).

In 2013, Si was classified as a beneficial nutrient by the Association of American Plant Food Control Officials (AAPFCO), due to the increasing knowledge of Si effects on plant growth and protection (Heckman, 2013). In soils, most Si is in recalcitrant silicate minerals (i.e., aluminum silicates and quartz) and only a much smaller fraction is available for plants (Greger et al., 2018). The plant-available Si is in the form of monosilicic acid ( $\text{H}_4\text{SiO}_4$ ), present both in the soil solution and adsorbed phases (Fe and Al oxides/hydroxides) (Tubaña and Heckman, 2015). Silicate-solubilizing bacteria occurring in soils and rhizosphere act by solubilizing silicates (**Figure 1**). The most studied species are from the genera *Burkholderia*, *Aeromonas*, *Rhizobium*, *Enterobacter*, and *Bacillus* (Santi and Goenadi, 2017; Lee et al., 2019).

## Nutrient Mobilization and the Role of Root-Associated Fungi

The most studied case among root-associated fungi are arbuscular mycorrhizal fungi (AMF), obligate symbionts from the phylum Glomeromycota which can form symbiotic relationships with ~80% of land plant species, including agricultural crops (Berruti et al., 2016). Among other benefits, AMF can enhance the uptake of mineral nutrients (i.e., P, N, S, Cu, and Zn) and water by their host plants (Bücking and Kafle, 2015) in exchange for carbon sources (Hodge and Fitter, 2010; Veresoglou et al., 2012). The extraradical AMF mycelium increases the volume of soil explored not only by reaching far beyond the rhizosphere but also by penetrating smaller soil pores (Berruti et al., 2016). This is particularly important for the acquisition of less mobile nutrients such as P and, in fact, AMF are well-known for their ability to enhance P acquisition, especially in P-deficient soils (Kumar et al., 2018) (**Figure 1**). Arbuscular mycorrhizae were also shown to facilitate N uptake, mostly in the form of ammonium ( $\text{NH}_4^+$ ) (Hodge and Fitter, 2010; Veresoglou et al., 2012). Over the last decades, several companies manufactured and commercialized AMF inoculants and the global mycorrhiza-based biofertilizer market is projected to be worth 621.6 million US dollars by 2025. These products are especially being encouraged in countries of the Asia Pacific region such as India and China (ReportLinker, 2020) but, as will

be discussed below, their reliability is still limited (Hart et al., 2018).

Besides AMF, efforts are being made to use other endophytic fungi to improve crop nutrient acquisition and growth (Murphy et al., 2018). Among these, root-colonizing dark septate endophytes (DSE) are a diverse group, mostly belonging to the phylum Ascomycota, which could potentially provide benefits ranging from nutrient acquisition to disease and abiotic stress tolerance (He et al., 2019a; Spagnoletti and Giacometti, 2020). These DSE have been found in over 600 plant species, some of them non-mycorrhizal, and several studies show their potential to enhance N uptake in crops such as rice and tomato (Mandayam and Jumpponen, 2005; Mahmoud and Narisawa, 2013; Vergara et al., 2018). Their effect on P acquisition has been less studied, but evidence suggests they could also assist on the solubilization of Ca, Al, and Fe phosphates (Spagnoletti et al., 2017).

## CURRENT CHALLENGES LIMITING BIOFERTILIZER APPLICATIONS

There are several key steps to be overcome by introduced microbe/s before achieving the desired effects in plant growth and fitness: survival, establishment, colonization, and interaction with the plant (e.g., parasitic/symbiotic behavior, PGP performance). This is particularly concerning in the lab-to-field transition, where it is common for a microbial strain with good performance *in vitro* to perform poorly in greenhouse and/or field trials (Parnell et al., 2016; Hart et al., 2018; Keswani et al., 2019). The inoculation outcome is especially hard to predict because we generally consider and control for a limited number of variables, usually not taking into account their intricate interactions (Moutia et al., 2010; Sasaki et al., 2010; Busby et al., 2017). Here, we summarize the main factors associated with inoculation success by classifying them into plant-related, edaphic/environmental, and inoculant-related (e.g., additives, concentration, viability) (Malusà et al., 2016) (**Table 1**), as well as the practical aspects that currently limit the applicability of biofertilizers in agriculture.

## Edaphic and Environmental Conditions

Edaphic and environmental conditions are two major factors behind the variability and low reproducibility of biofertilizers in field trials (Hoeksema et al., 2010; Da Costa et al., 2014; Schütz et al., 2018). Initial steps of biofertilizer testing are carried out in aseptic controlled conditions, which allow for an unbiased characterization of the microorganism under study. Scaling up to growth chamber or greenhouse trials, and especially to field conditions, increases the number of uncontrolled biotic and abiotic factors that can interfere with inoculation success (Nkebiwe et al., 2016; Bacilio et al., 2017). Among the biotic factors that can affect the outcome of inoculation, the most discussed is the presence of competitors, predators, or other antagonists within the resident microbiome (i.e., indigenous and previously introduced microorganisms) (Biró et al., 2000; Vargas et al., 2000; Ortas, 2003). Abiotic factors, either climatic or edaphic, can also influence the effectiveness of biofertilization

**TABLE 1** | Challenges associated with biofertilizer success and potential solutions relying on novel technologies.

Challenges			Potential solution(s)
Edaphic and environmental	Biotic	Negative interactions with the resident microbiome (e.g., competition, predation, and antagonism).	Personalized biofertilizers for a specific farm (e.g., particular soil, crop and management).
	Abiotic	High variability in soil physicochemical properties (e.g., nutrient levels, pH, organic matter content, moisture, temperature, salinity).	Biofertilizers based on optimal range of performance.
	Agricultural practices	Interaction with other agricultural practices (e.g., organic amendments, fertilizers, pesticides, tillage, crop diversification strategies).	
Plant-related	Plant genotype and physiological status	Different outcomes depending on plant genotype due to different degrees of specificity or indirect selection via plant rhizodeposition and root architecture. Variability in different plant growth stages and overall physiological status.	Isolated compounds or “prebiotics” (e.g., benzoxazinoids, coumarins, triterpenes) to attract or favor microbe/s of interest. Genotype-specific inoculum (e.g., compatible microorganisms, pre-adapted microbiome). Optimized application timing.
Inoculant-related	Genetic and physiological traits	Microbes with poor ecologically relevant traits affecting their establishment, colonization, persistence and tolerance to abiotic stresses (e.g., osmotic and temperature).	Pre-adapted microorganisms (e.g., isolated locally), isolation and screening focused on both PGP and ecological traits. Engineered microbial communities (e.g., SynComs) or whole microbiomes. Mixed inoculants with functional redundancy but a wide range of environmental adaptation. Biofilm-forming microorganisms.
	Formulations	Insufficient physical and chemical protection to maintain cell viability and prevent desiccation/contamination.	Processes based on different methods such as alginate microencapsulation and fluidized bed dryer (FBD).
Practical aspects	Costs	Economic feasibility at a commercial scale (bioprospecting, testing, scaling up, storage, and application).	Resource inputs from public and private sectors encouraged by regulatory agencies and policy makers (e.g., incentives, promotion, and awareness).
	Farmer accessibility	Products with limited versatility, reproducibility, shelf-life, practicality (handling and application), adaptability to different agricultural practices. Insufficient collaboration and communication between researchers, industry, and farmers.	
	Regulations	Lack of standardized and universal testing protocols and evaluation guidelines.	
	Intellectual property	Disregard or negligence to protect intellectual property (patent development) and technology transfer.	

on crop nutrient use efficiency and yield. According to a meta-analysis by Schütz et al. (2018), biofertilizers generally perform better under drier climatic conditions, higher soil P levels (especially for N<sub>2</sub>-fixers) and, exclusively for AMF, lower soil organic matter content and neutral pH. Yet, higher growth and yield responses to biofertilization are usually observed under low nutrient availability (Ozturk et al., 2003; Da Costa et al., 2014), as the plant can fully benefit from the interaction with the introduced microbe. The negative relationship between biofertilization success and soil nutrient content has been widely studied both for AMF colonization with soil P (Kaeppler et al., 2000; Jansa et al., 2009), and rhizobia nodulation with soil N (Glyan'ko et al., 2009; Thilakarathna and Raizada, 2017).

Some edaphic properties can also be highly susceptible to agricultural practices, therefore generating additional variability in inoculation success. So far, evidence has shown interacting

effects of microbial inoculation with chemical fertilizers (Ozturk et al., 2003; Jansa et al., 2009; Da Costa et al., 2014), organic amendments (Manandhar et al., 2017; Ulzen et al., 2019), pesticides (Gaiind et al., 2007; Jin et al., 2013b), and tillage (Miller et al., 1995; Mulas et al., 2015). It is also expected that crop diversification practices (e.g., rotations, cover crops) could modulate biofertilization efficiency by modifying resident microbial communities (Maiti et al., 2011; Buysens et al., 2016).

## Plant-Related Factors

Biofertilization can lead to different outcomes depending on the selected crop species or genotype. Although some studies are beginning to identify genetic markers (e.g., quantitative trait loci or QTLs) associated with this differential response (Kaeppler et al., 2000; Remans et al., 2008), the plant factors behind them are not yet clearly understood. In general terms, it is known that

plants can directly and indirectly alter the rhizosphere habitat through rhizodeposits and changes in the root architecture (Somers et al., 2004; Saleem et al., 2018). Among their many rhizodeposits, plant roots secrete signaling molecules such as secondary metabolites (e.g., flavonoids, hormones, antibiotics), some of which are important in the recognition and interaction with PGP microbes (Bais et al., 2004). The degree of specificity of this signaling process can be high for certain symbioses such as rhizobia-legumes, where the compatibility between microbes and host plants is crucial to establish a successful colonization (Hirsch et al., 2003; Thilakarathna and Raizada, 2017). In the case of arbuscular mycorrhiza, host specificity might not be as critical (Koyama et al., 2017) but colonization and PGP can still be affected by the plant genotype (Yao et al., 2001; Linderman and Davis, 2004; Hoeksema et al., 2010). Studies have explained these different levels of AMF-host plant compatibility through differences in root architecture (Declerck et al., 1995), aerial architecture (Liu et al., 2000), and P utilization and uptake efficiency (Kaeppler et al., 2000; Yao et al., 2001). Significant specificity between microbe and plant genotype was also observed for endophytes (Muñoz-Rojas and Caballero-Mellado, 2003; Yoon et al., 2016; Vujanovic and Germida, 2017) and free-living PGPR such as *Azospirillum* spp. (Sasaki et al., 2010; Vargas et al., 2012), *Pseudomonas* sp. (Digat et al., 1990; Safronova et al., 2006), and *Azotobacter* sp. (Mezei et al., 1997; Anbi et al., 2020). Considering that most plant breeding programs do not address plant-microbe interactions, plant genotype-induced variability in biofertilization outcomes remains a major concern. Furthermore, genotype effects are not isolated, but modulated by plant age and physiological status (Dennis et al., 2010), which will be ultimately determined by surrounding environmental conditions and time of inoculation.

## Inoculant-Related Factors

The selected microbial genotype not only determines its PGP functions, but also its compatibility with the plant host genotype (Linderman and Davis, 2004; Vargas et al., 2012; Ehinger et al., 2014). Furthermore, microbial traits also determine key aspects of inoculation such as survival (before and after application), establishment, colonization, and persistence. Generally, inoculant development focuses on genetic and PGP traits, with little or no attention to ecological traits, which will ultimately determine inoculation success under field conditions (Hart et al., 2018; Kaminsky et al., 2019). For example, strains adapted to specific environmental conditions can be selected by focusing on specific traits such as osmotic tolerance (García et al., 2017) or psychrotolerance (Rawat et al., 2019). Another approach consists in the isolation of native strains, which were shown to improve biofertilization performance (Melchiorre et al., 2011; Ahmed et al., 2013; Maltz and Treseder, 2015). Yet, there seems to be a trade-off between establishment and survival traits and PGP traits, posing a challenge at each of the different stages of inoculant development (Parnell et al., 2016; Kaminsky et al., 2019). This trade-off, together with the high specificity of microbial strains to both environment and host genotype, leads to a crucial question: should we aim for more specific and targeted biofertilizers

or, instead, pursuit broad-spectrum versatile products? (Parnell et al., 2016; Bell et al., 2019; Tosi et al., 2020a). In the latter, the outcome would imply either utilizing versatile microbes or formulating mixed inoculants that expand the ecological adaptation range (i.e., functionally redundant strains that encompass a wider range of environmental adaptation). In spite of the challenges arising from the interaction and possible interference among members of a mixed inoculant, which will differ on each particular scenario (Xavier and Germida, 2003; Remans et al., 2008; Ballesteros-Almanza et al., 2010), they bear great potential for overcoming the issues of environmental adaptability.

Besides the identity of the inoculated microbe, biofertilizer concentration, formulation, and delivery practices will also determine how tolerant and protected inoculated microbes will be from environmental constraints. The inoculant formulation should be able to support microbial growth, while protecting an amount of viable cells high enough for an effective response in the plant (Herrmann and Lesueur, 2013; Bashan et al., 2014). Additional challenges associated with scaling up and commercializing a microbial product include, but are not limited to, a risk of genetic and physiological changes in the strain, viability loss (particularly by desiccation) and contamination (Parnell et al., 2016; Glick, 2020; Greffe and Michiels, 2020). With the expansion in the biofertilizer industry, novel and more sophisticated formulations have been developed, including a variety of solid, slurry, and liquid carriers, as well as a wide array of additives (e.g., nutrients, stimulants, preservatives) to enhance the physical properties of the inoculant (e.g., adhesives, surfactants) (Bashan et al., 2014, 2016; Preininger et al., 2018). These additives allow microbes to withstand the fluctuating and suboptimal conditions during distribution, storage, and application (Parnell et al., 2016) (further discussed in section New Formulations and Delivery Methods). Even though these carriers and additives seem to be a secondary aspect of biofertilizer development, they can be critical for obtaining successful results (Gomez et al., 1997; Herrmann and Lesueur, 2013; Lee et al., 2016).

Similarly, delivery methods (e.g., on seed or into soil), and application timing and frequency can be critical for inoculation success (Parnell et al., 2016). For example, in a rhizobia inoculation trial on *Pisum sativa*, soil applications of a granular inoculant resulted in higher PGP than seed applications of a liquid formulation (Clayton et al., 2004). Foliar and flower applications were also being considered as a safer and more effective delivery strategy for endophytic or phyllosphere microorganisms (Mitter et al., 2013; Preininger et al., 2018). Application timing and frequency should be also taken into consideration in relation to plant growth stages (Bashan, 1986; Fallik et al., 1988; Linderman and Davis, 2004), as well as other agricultural practices such as fertilization (Pii et al., 2019) and transplant (Sohn et al., 2003). The time elapsed between application and establishment is critical, as it will determine the survival of the microbe to environmental constraints before it can provide any benefits to the plant.

## Practical Aspects

We have discussed major technical limitations that can markedly affect biofertilizer efficiency, but there is also a set of practical aspects that cannot be overlooked in biofertilizer development. One of these concerns is the accessibility and convenience of inoculants for both farmers and manufacturers, especially in comparison with chemical fertilizers. Some key factors related to biofertilizer accessibility are: cost/benefit relationship, versatility, robustness and reproducibility, shelf-life and storage requirements, ease of use (handling and application), adaptability to agricultural practices and machinery, and biosafety (Bashan et al., 2014; Parnell et al., 2016; Bell et al., 2019). Novel products should be accompanied by a better outreach in order to inform farmers on the benefits of biofertilizers, particularly in the longer term, and to facilitate and promote their application (Parnell et al., 2016; Martínez-Hidalgo et al., 2018). A less discussed issue are intellectual property rights and patent development, which can be a valuable tool to transfer technology between the academic and industrial sector, but could also put at risk the free exchange of ideas and materials among researchers (Glick, 2020). Product commercialization also requires proper regulations (Sessitsch et al., 2019) with standardized and universal testing protocols and risk evaluation guidelines (Vílchez et al., 2016; Timmusk et al., 2017). Finally, the research and development sector are still in need of standardized protocols to evaluate inoculation success (Hart et al., 2018; Martínez-Hidalgo et al., 2018; Kaminsky et al., 2019) and to monitor microbes once applied in the field (Schütz et al., 2018; Compant et al., 2019).

## NEW APPROACHES IN BIOFERTILIZER DEVELOPMENT

In recent years, product development strategies have shifted from single-strain to microbial consortia inoculation. These strategies are based on a greater chance of at least one strain escaping competitive exclusion, and thus ensuring inoculant survival and function (Rivett et al., 2018; Tosi et al., 2020a). Microbial consortia can consist of two or more strains that are either closely (Kyei-Boahen et al., 2005; Jin et al., 2013a) or distantly (Ramírez-López et al., 2019; El Maaloum et al., 2020) related that provide an overall additive or synergistic biofertilization effect. One of the most common applications is the co-inoculation of rhizobia and AMF on legumes, as a number of studies report a synergistic effect on plant growth promotion (Xavier and Germida, 2003; Ashrafi et al., 2014; Kamaei et al., 2019). Yet, examples in the literature also show negative effects of AMF on nodule development or non-significant effects on crop yield (Antunes et al., 2006; Menéndez and Paço, 2020). Despite the promising beneficial effects of developing biofertilizers consisting of microbial consortia, it is unknown how these inoculants would establish across a range of agricultural field settings (Finkel et al., 2017). Moreover, even if inoculated microbes colonize their new environment initially, their persistence over time is not guaranteed. Here, we discuss new approaches to develop suitable bioinoculants at commercial scale from screening potential candidate microorganisms, designing the inoculant and optimizing formulations.

## New Culture-Dependent Methods

Since Antony Van Leeuwenhoek's discovery of microorganisms in the 1670s, isolation and cultivation of microbes are the major pillars of microbiology (Oren and Garrity, 2014). Since then, the best practices for culturing new organisms have been developed and published in guides such as the Bergey's Manual of Systematic Bacteriology (Boone et al., 2001). However, in the last 20 years, the use of diffusion chambers (Kaerberlein, 2002; Bollmann et al., 2007) and isolation chips (Ichip) (Nichols et al., 2010), that mimic natural environments, increased the number of cultured colonies and marked a rebirth of culture dependent techniques. Most recently, studies by Lagier et al. (2015, 2016) used "culturomics" to cultivate previously uncultured members of the human gut microbiota. Culturomics uses multiple culture conditions, MALDI-TOF mass spectrometry and 16S rRNA sequencing for the identification of bacterial species (Lagier et al., 2018). The main objective of this technique is to suppress the culture of fast-growing and highly abundant species and to promote the growth of fastidious and/or less abundant microorganisms (Lagier et al., 2015).

Despite its success on the human microbiota, multiple combinations in culturomics (i.e., various growth media, culturing conditions, atmospheres, and times of incubation) have yet to be extended and developed for the soil and plant-associated microbiome. In order to minimize these challenges, Sarhan et al. (2019) suggested a "plant-tailored culturomic technique" that combines culturomics with plant-based media. According to these authors, most studies continue to use general media containing nutrients of animal origin (e.g., nutrient agar, R2A, and LB) to isolate plant associated microbes, whereas plant materials or dehydrated juices powders should be used instead. In fact, P-solubilizing *Bacillus circulans* and N<sub>2</sub>-fixing *Azospirillum brasilense* have been successfully grown on plant-only-based culture media (Youssef et al., 2016; Mourad et al., 2018). In addition, online platforms such as KOMODO (Known Media Database, <http://komodo.modelseed.org>) that includes >18,000 strain-media combinations and >3,300 media variants/compound concentrations can be used as a guide for developing suitable lab media for growing microorganisms (Oberhardt et al., 2015). Therefore, new culturing methods to discover novel isolates with biotechnological applications are key for biofertilizer development. Unfortunately, newly culturable microorganisms (e.g., slow growers) may still be less reliable and cost-effective for mass production with our current technology.

## How to Artificially Select Microbiomes?

There are two main approaches to artificially select microbiomes: "top-down," which modifies an existing microbiome, and "bottom-up," which starts from individual microorganisms to build artificial or engineered microbiomes (e.g., synthetic communities or SynComs). In the "top-down" approach, selected environmental variables (e.g., pH, temperature, redox potential) are used to manipulate an existing microbiome, through ecological selection, to perform desired functions (Lawson et al., 2019). Although this approach is widely used for bioremediation (Atashgahi et al., 2018) and wastewater treatment (Demarche et al., 2012), it has the disadvantage of working with a complex community. In contrast, "bottom-up" approaches offer



the advantage of simplifying these interactions by building artificial communities from pre-selected individual organisms (Raaijmakers, 2015).

Given the high complexity of molecular-scale microbiome processes, most of the challenges in designing microbial inoculants is to identify key beneficial microorganisms (e.g., *Azotobacter*, *Rhizobium*, *Pseudomonas* spp.) that are viable and with a greater chance of environmental colonization, resulting in reliable functional outcomes. One approach is to target keystone taxa (i.e., microbial taxa that are highly connected or highly influential on the community) in a pre-existent or artificially built microbiome (Banerjee et al., 2018). These taxa provide an appealing target for microbial screening, followed by isolation and whole genome sequencing to identify their functional capabilities (Kong et al., 2018). Consequently, their role in regulating the growth and function of other members of the microbiome can be exploited to enhance specific desired functions.

An important tool for identifying key taxa and developing ecosystem-wide dynamic models is through network analyses. Based on high-throughput sequencing data, these networks provide an overview of microbial assemblages and microbe-microbe interactions. With network centrality metrics, they can allow to further detect microbial taxa that hold key topological positions within the network (Toju et al., 2018b). Together, detecting ecologically significant microbial interactions, with proper experimentation, will provide powerful methods in developing new biofertilizers.

## Creating Synthetic Communities

Recent culture independent techniques and new culture collections have paved the way for developing artificially constructed communities, also known as “artificial microbial consortia” (AMC) or “synthetic communities” (SynComs). Here, core microbiomes are used to recreate the structure and function of the microbiome. A great advantage of SynComs studies is that it allows for a detailed evaluation under controlled conditions, in which members can be added, eliminated, or substituted as needed (Vorholt et al., 2017). These studies can also help to elucidate different aspects of the spatial structure, microbial social interactions and how phenotypes interact and compete for space (Rodríguez Amor and Dal Bello, 2019). Yet, by definition, SynComs attempt to emulate the natural microbiome with less complexity, retaining only the indispensable microbial taxa, which could pose its own limitations (Vorholt et al., 2017; Satyanarayana et al., 2019). For example, SynComs with lower complexity might bypass important associations or inter-relationships, which might be critical at the functional level and, therefore, unsuitable for field applications. Highly complex SynComs, on the other hand, have their own designing limitations but a better chance of keeping associations intact (Satyanarayana et al., 2019). Despite its limitations, these reduced-complexity systems are particularly useful when a metabolic pathway is either too energy intensive or too complex to be accomplished by a single or few taxa.

In plant sciences, SynComs were first introduced in studies using *Arabidopsis thaliana* under gnotobiotic conditions (Bai

et al., 2015; Lebeis et al., 2015). In studies by Castrillo et al. (2017), SynComs were used to investigate links between *Arabidopsis* phosphate starvation response, immune system function, and root microbiome assembly. In agricultural crops, Armanhi et al. (2018) designed a SynCom comprised of naturally occurring bacterial groups in the sugarcane microbiome and tested using maize as a model plant. These authors found that inoculated plants had an assemblage pattern similar to those found in sugarcane, which demonstrates a successful colonization of the synthetic community. However, Armanhi et al. (2018) conducted their SynCom assembly by choosing highly abundant bacterial groups, and we propose a selection based on microbe-microbe interactions, functional traits (e.g., nutrient solubilization/mineralization) and (or) colonization abilities (e.g., production of antimicrobial compounds). Moreover, biofertilizers can be developed with SynComs designed with high functional redundancy, in order to increase environmental adaptability and overcome some of the challenges of products currently available in the market.

## Personalized Microbial Inoculants and Plant Prebiotics

Inspired by the concept of personalized diagnosis in medicine, Schlaeppli and Bulgarelli (2015) proposed a similar strategy in agricultural systems. This strategy consists on customizing tools such as microbial inoculants into farming practices. Similar to fertilizer consultants that advise farmers on application strategies, Bell et al. (2019) proposed a customizable field-scale microbial inoculant that, with appropriate implementation, could have long-lasting effects. Considering that soil conditions might change dramatically over short distances (Peukert et al., 2016), product development strategies of “one formulation applied for all fields” seems unrealistic. One strategy is the on-farm production of mycorrhizae-based inoculants, in which studies have shown their effects on potato (Douds et al., 2007; Goetten et al., 2016) and eggplant (Douds et al., 2017) growth and nutrition. These locally produced inoculants often have low costs and are applied shortly after production, without the need of shipping and storage (Douds et al., 2005). Yet, it is important to consider how these products will be feasible or cost-effective on a global scale. A good starting point could be establishing an optimal range for biofertilizer performance, in which inoculants would be introduced to conditions best resembling the soils they were isolated from. Here, different formulations can be designed for particular soils and(or) plant-root systems with the incorporation of certain aspects of precision farming, thus identifying areas in a particular field that might be more suitable to one formulation or another.

Another strategy is to use root exudates to stimulate the beneficial plant-associated microbiota. These exudates consist predominately of sugars and organic acids, but also flavonoids, amino acids, fatty acids, hormones, antimicrobial compounds, and vitamins (Bulgarelli et al., 2013). They can serve as growth substrates or signals for suitable microbes that strongly influences rhizosphere microbiome composition and dynamics (Philippot et al., 2013; Mitter et al., 2017; Sasse et al., 2018).

For example, benzoxazinoids (BXs) are major secondary defense metabolites in the Poaceae family (e.g., maize, wheat, and barley) that are typically produced during relatively early plant growth stages (Cotton et al., 2019). Benzoxazinoids and their breakdown products are known to be biocidal to some soil-borne bacteria and fungi and act as important regulators of belowground plant–microbe interactions (Schütz et al., 2019). Despite their allelochemical properties, studies by Neal et al. (2012) revealed that BXs may act as recruitment signals for *Pseudomonas putida* KT2440 in maize plants. In addition, *P. putida* has been previously studied for their ability to solubilize phosphate and thus promote growth of leguminous (Rosas et al., 2006) and maize (Sarikhani et al., 2020) plants. Hence, BXs could be exploited to recruit beneficial microbes such as P-solubilizing *P. putida* in field conditions.

Coumarins are a family of plant-derived secondary metabolites exuded by plants that have been extensively studied for their role in induced systemic resistance (ISR) (Stringlis et al., 2018; Pascale et al., 2020). However, Tsai and Schmidt (2017) reported their involvement as Fe-mobilizing compounds in response to Fe deficiency of dicotyledonous plants. Moreover, Voges et al. (2019) demonstrated the role of Fe-mobilizing coumarins in structuring the *A. thaliana* root bacterial community by inhibiting the growth of *Pseudomonas* spp. via a redox-mediated mechanism. Other molecules, such as triterpenes, can also promote the enrichment of Bacteroidetes and the depletion of Deltaproteobacteria in *A. thaliana* (Huang et al., 2019). Similarly, Koprivova et al. (2019) reported that a specific concentrations of plant derived camalexin concentration is necessary for proper interaction with a plant growth-promoting *Pseudomonas* sp. strain.

Multiple lines of evidence show that root exudates could be used as compounds to stimulate the growth of beneficial microbiota, rather than introducing microbes by inoculation. A similar approach was proposed by Arif et al. (2020), in which the authors suggested that particular soil amendments could act as “prebiotics” to promote microbial functions. Qiu et al. (2019) also suggested that synthesized compounds can be added to crops to attract or favor particular microbes. Yet, we propose that these “plant prebiotics” could be used in combination with microbial inoculants to enhance biofertilizer efficiency. By acting as signaling molecules, these compounds could potentially attract introduced microbes to the rhizosphere, thus giving them an advantage over other microorganisms for early colonization.

## Biofilmed Biofertilizers

Biofilms consist of associated microorganisms, either from a single or multiple species, adhering to the biotic or abiotic surfaces in a self-produced matrix of extracellular polymeric substances (EPS) (Rana et al., 2020). This matrix provides the structure and protection by which microbes have the ability to chemically link with each other by quorum sensing (QS) and work as one unit (Li and Tian, 2012; Vlamakis et al., 2013). In soils, microbial communities such as bacteria and fungi can form biofilms on abiotic surfaces such as ore (minerals), water-air interfaces, and dead organisms (Rekadwad and Khobragade, 2017). Moreover, biofilm formation in the

rhizosphere is an important trait that prevents microorganisms from being detached from plant roots by various natural processes (Velmourougane et al., 2017).

In recent years, biofilmed biofertilizers (BFBFs) (i.e., biofertilizers containing microbial communities capable of forming biofilms) have emerged as a new inoculant strategy to improve biofertilizer efficiency and sustain soil fertility (Parween et al., 2017). The idea behind BFBFs is that biofilm formation will create a more suitable environment for biofertilizers to compete with resident organisms and to cope with the heterogeneity of biotic and abiotic factors in soil (Ünal Turhan et al., 2019). For example, several studies have shown that biofilmed biofertilizers augmented P-solubilization (Swarnalakshmi et al., 2013), N<sub>2</sub> fixation (Wang et al., 2017), siderophore production (Ricci et al., 2019), and Zn solubilization (Triveni and Jhansi, 2017). In addition, studies by Kopycińska et al. (2018) highlighted the role of biofilm formation, by exopolysaccharides (EPS) production, in *Rhizobium leguminosarum* during Zn stress. These authors found that EPS-deficient *R. leguminosarum* mutants were more sensitive to Zn exposure, whereas cell viability and root attachment were significantly higher in EPS producing strains.

Multi-species biofilms were also found to be more resilient in comparison to single-species biofilms (Velmourougane et al., 2017). In fact, natural rhizobacterial biofilms are often in mixed communities with interspecies interactions. This assembly is usually more advantageous than single planktonic cells, with optimal and maximal use of nutrients and resources (Nayak et al., 2020). For example, fungal–bacterial biofilms have been shown to enhance nutrient uptake and environmental stress tolerance compared to mono- or mixed-cultures of no biofilm-forming microorganisms (Hassani et al., 2018). Taktek et al. (2017) studied two hyphobacteria (*Rhizobium miluonense* and *Burkholderia anthina*) and two mycorrhizobacteria (*Rahnella* sp. and *Burkholderia phenazinium*) that strongly attaches to the surface of the arbuscular mycorrhizal fungus *Rhizoglyphus irregulare*. These authors demonstrated that *B. anthina* can strongly adhere to abiotic and biotic surfaces and allow a higher phosphate solubilization than other isolates tested. Beneficial inter-kingdom biofilm formation were also reported in *Bacillus* sp. with *Gigaspora margarita* (Cruz and Ishii, 2012) and *Pseudomonas fluorescens* with *Laccaria bicolor* (Noirot-Gros et al., 2018).

Biofilmed biofertilizers have potential applications to improve ecosystem functioning and sustainability, which includes enhancing soil fertility and protecting the host plant under adverse conditions. However, biofilms are known to be problematic in many industrial settings as they often clog pipes and tubing (Vlamakis et al., 2013). Consequently, additional studies are needed to test BFBFs efficiency at a field scale and to determine optimal processes for a large-scale production and reliable results.

## New Formulations and Delivery Methods

Following the selection of microorganisms and their functions, a suitable formulation is required to ensure microbial cell viability during storage and application. In fact, most governments regulate quality standards mandating a minimum number of

viable cells and a threshold for any potential contaminants (e.g., chemical or microbial) (Herrmann et al., 2015; García de Salamone et al., 2019). Hence, different bioformulations have been developed and are broadly divided into those using solid materials as carriers or liquid formulations.

The most commonly used solid carriers are peat, rock phosphate, charcoal, lignite, vermiculite, clay, diatomaceous earth, talc, cellulose, and polymers such as xanthan gum (Mishra and Arora, 2016). Liquid formulations, also known as flowable or aqueous suspensions, consist of microbial suspensions in water, oils, or emulsions (Mondal and Dalai, 2017). In addition, bioformulations may contain additives such as methyl cellulose, starch and silica gel to improve their physical, chemical, and nutritional properties (Macik et al., 2020). The main disadvantages of liquid bioformulations are that the metabolic activity of beneficial microbes decrease rapidly after manufacturing and they often have higher contamination risks (Kaminsky et al., 2019; Macik et al., 2020; Vassilev et al., 2020). On the other hand, solid formulations (e.g., powders and granules) are challenging for non-sporulating bacteria, as desiccation disrupts cell membranes, causing cell death and overall loss of viability during rehydration (Berninger et al., 2018). This can lead to major setbacks for product commercialization. Two strategies to overcome these limitations are using microbial encapsulation with polymeric hydrogels and drying methods using a fluid bed dryer (FBD).

Polymeric hydrogels consist of crosslinked polymers chains with high affinity for water that are used for a variety of technological applications (Kobayashi, 2018). These hydrogels provide: (i) an aqueous environment that helps maintain the biological function of the encapsulated material and (ii) a diffusion barrier that allows the passage of molecules with a given size threshold (Pérez-Luna and González-Reynoso, 2018). Several naturally occurring (e.g., polysaccharides) and synthetic (e.g., polyacrylamide, polyurethane) polymers have been widely used for microbial encapsulation; yet, alginate has been particularly attractive specially in biomedical applications due to its structural similarity to extracellular matrices of living tissues (Gasperini et al., 2014). Alginate is a polysaccharide derived from different brown algae (e.g., *Phaeophyceae*) and bacteria (e.g., *Azotobacter* and *Pseudomonas*) (Lee and Mooney, 2012), and its main advantages are high biocompatibility in supporting cell survival, low toxicity and ease of gelation (Gasperini et al., 2014).

In biofertilizer applications, microbial cell entrapment with alginate allows a gradual and controlled release of microbial inoculants in the soil (Sahu and Brahmaaprakash, 2016). The sticky nature of alginate may also help microbial cells to easily adhere to seeds and resist to environmental stresses (Nayak and Mishra, 2020). Lopes et al. (2020) used alginate beads for encapsulating plant growth-promoting *Trichoderma* spp., and found higher survival rates in freeze-dried encapsulated cells at different storage temperatures. In addition, studies using microbial cell entrapment with alginate have shown higher PGP rates on cotton (He et al., 2016), maize (Pitaktamrong et al., 2018), and hybrid cabbage (Stella et al., 2019) compared to

non-entrapped cells. However, a few drawbacks still limit the use of these formulations at a large-scale in agricultural systems. For example, most natural polymers are heat sensitive and with lower mechanical strength compared to synthetic polymers (Zhu, 2007). In addition, alginate can be relatively costly and the porosity of alginate particles could be a limiting factor for the biofertilizer industry (Reis et al., 2006; Sahu and Brahmaaprakash, 2016).

A new approach for developing formulation methods is by using fluid bed or fluidized bed dryer (FBD) to increase inoculant survival rate and reduce contamination. Fluid bed dryer has been extensively used by the pharmaceutical and food industry to reduce the moisture of powders and granules (Dewettinck and Huyghebaert, 1999). In this process, particles to be coated are maintained suspended against gravity in an upward flowing air stream causing them to behave as a fluid (Sahu and Brahmaaprakash, 2016). Then, the coating material is sprayed through a nozzle onto particles and electrical heaters are employed for drying the material (Schoebitz et al., 2013). One of the main advantages of this process is that it operates in a temperature of ~37 to 40°C, which is milder than spray-drying and more suitable for mesophilic organisms (Sahu et al., 2018). In fact, Gangaraddi and Brahmaaprakash (2018b) reported longer shelf-life of *Rhizobium* spp. inoculants in a FBD formulation, in which cell viability was maintained for up to 120 days. Higher cell viability using FBD have also been shown for biocontrol (Larena et al., 2003; Sabuquillo et al., 2006; Gotor-Vila et al., 2017) and for plant growth-promoting (Gangaraddi and Brahmaaprakash, 2018a) inoculants. In spite of these results, the use of FBD technology in microbial inoculant formulations is not common. Future studies are still needed on optimizing temperature cycles of FBD for inoculant formulations and to assess survival rates after field applications. Yet, both methods using alginate or FBD have promising effects in reducing formulation inconsistency by preserving microbial cell density during storage. Furthermore, these techniques may open new possibilities to extend shelf-life of biofertilizers containing non-spore forming Gram-negative bacteria.

## BIOSAFETY OF BIOFERTILIZERS

As discussed in previous sections, biofertilizers have great potential to replace chemical inputs in agricultural systems but still face several challenges in terms of technical reliability and accessibility to farmers. Yet, an important consideration is their potential impact on both human and ecosystem health. Due to the extensive nature of these risks, we believe that all inoculant research and development should be assessed through a “One Health” approach that regards plant, animal, human, and ecosystem health (van Bruggen et al., 2019). Currently, our understanding of both the introduced microorganism/s and the complex interactions that occur in the plant-soil interface is still limited to fully diagnose the risk posed by a specific product. However, research efforts have been made to fill knowledge gaps and to elaborate guidelines for assessing risks associated with different inoculants (Vílchez et al., 2016). Understanding the



mechanisms behind successful PGP is as important as properly assessing the risks associated with the microorganisms that will be introduced in agricultural systems in a large scale.

## Human Health Risks

The plant-soil interface hosts a vast number of genetically and functionally diverse microorganisms, some of which interact with the plant in different ways (beneficial, neutral, and pathogenic). Even though these interactions can lead to completely different outcomes in terms of plant fitness, they are known to share many features, to the extent that some symbiotic microbes could switch to a parasitic behavior with the same host (Kogel et al., 2006). In the last decades, various root-associated bacteria, some of them studied for their PGP traits, were found to be opportunistic plant and human pathogens (Berg et al., 2013). Potential human pathogenic bacteria can be found among several genera, including *Burkholderia*, *Pseudomonas*, *Rhodococcus*, *Serratia*, *Acinetobacter*, *Stenotrophomonas*, *Enterobacter*, *Ochrobactrum*, *Klebsiella*, *Ralstonia*, and *Bacillus* (Martínez-Hidalgo et al., 2018; Keswani et al., 2019). A well-studied case is that of the *Burkholderia cepacia* complex, a group of phenotypically associated bacterial species which have known PGP traits, including N<sub>2</sub>-fixation, but can also be opportunistic human pathogens (Chiarini et al., 2006; Eberl and Vandamme, 2016). Another intensively debated bacterial genus is *Pseudomonas*, which encompasses several PGP species (e.g., *P. fluorescens*, *P. putida*, *P. putrefaciens*, *P. stutzeri*, and *P. pseudoalcaligenes*) but also the pathogenic species *P. aeruginosa*, an opportunistic pathogen causing respiratory tract infections in humans (Mendes et al., 2013).

Currently, the majority of commercial biofertilizers consist of formulations of N<sub>2</sub>-fixing organisms (e.g., *Actinorhizobium* spp., *Azospirillum* spp., *Azotobacter* spp., and *Rhizobium* spp.), which have a low health risk and a history of safe application (Martínez-Hidalgo et al., 2018; Sessitsch et al., 2019). Yet, other bacterial species or strains which could provide useful functions for agriculture could also pose a biosafety threat. For instance, Actinobacteria are thought to have low pathogenic risk, but some species such as *Streptomyces somaliensis* can cause disease in humans (Martínez-Hidalgo et al., 2018; Olanrewaju and Babalola, 2019). In other cases, the presence of pathogenic species or strains within a taxonomic group can lead to drastic measures that would not be necessary with the proper testing methods and risk evaluation protocols. The *B. cepacia* complex was restricted for field application in the United States (Martínez-Hidalgo et al., 2018), even though its pathogenicity and ecology were found to be variable among species (Chiarini et al., 2006). The evaluation of pathogenicity and risk associated with a particular species or strain is unclear. More research is needed to identify genotypic and phenotypic differences between a plant or soil opportunistic pathogens and their clinical counterparts (Mendes et al., 2013; Martínez-Hidalgo et al., 2018). Genetic analyses could provide information about presence or absence of known virulence factors such as quorum sensing, motility, siderophore production, and lipopolysaccharide biosynthesis (Guttmann and Ellar, 2000; Eberl and Vandamme, 2016). In this sense, a powerful tool is whole genome sequencing to compare

potentially pathogenic and mutualistic members of genus or species (Martínez-Hidalgo et al., 2018; Keswani et al., 2019). Another crucial step is understanding the role of horizontal gene transfer in pathogenicity (Dobrindt et al., 2004), particularly among members of the same species or strain. This information will allow to develop proper screening tests which could be followed by toxicity and pathogenicity testing on model hosts such as plants or nematodes (Martínez-Hidalgo et al., 2018). However, in the case of host-specific pathogens, such as some *Burkholderia* spp., model hosts might be insufficient to discard potential risks for humans (Eberl and Vandamme, 2016).

For all the above mentioned, it is clear that proper biosafety screening of any PGPB strain should become a standard practice in biofertilizer production, ensuring the safety of the product before exposing personnel, consumers, and natural resources (Berg et al., 2013; Keswani et al., 2019). More research is needed on potential allergens (Keswani et al., 2019) and fungal biofertilizers, including AMF, which might not be directly harmful for humans but could carry undesired microbial species in their formulation (Agnolucci et al., 2019). Moreover, it is also important to understand the environmental conditions that could promote the proliferation of opportunistic pathogens. For example, while some characteristics of the rhizosphere environment could be favoring opportunistic pathogenic bacteria (e.g., high nutrient availability, UV protection), others, such as high microbial diversity, could limit their survival (Matos et al., 2005; Mendes et al., 2013). Finally, there is a need for improved consistency when evaluating risk and establishing regulatory frameworks such as the Risk Groups (Europe) and Biosafety Level or BSL (United States) (Vílchez et al., 2016). Vílchez et al. (2016) developed an evaluation system, the “Environmental and Human Safety Index (EHSI)” based on a panel of tests which could be used to evaluate the safety of PGP bacteria in an objective and reliable manner, encompassing not only human but also environmental health. More effort must be invested in similar projects, with a special emphasis on international and interdisciplinary exchange and cooperation.

## Environmental Health Risks: Effects on the Resident Biota

Ideally, a biofertilizer should cause a minimum and/or controlled impact in terms of dispersion, persistence, alteration of microbial function and biogeochemical cycling, and alteration of macroflora and macrofauna (Bashan et al., 2014). A major concern is the effect of the introduced microorganism/s on the resident microbiome, which can occur from direct ecological interactions, either synergistic or antagonistic (e.g., competition, inhibition), as well as horizontal gene transfer (Glick, 2020; Mawarda et al., 2020). Introduced microorganisms can also modify resident microbial communities indirectly, by modulating plant physiology and morphology. For example, some PGP are known to modify root architecture and exudation (Vacheron et al., 2013), which can alter rhizosphere communities (Jones, 1998; Saleem et al., 2018). In mixed plant communities, indirect effects on the resident microbiome could also be expected if the introduced microorganism induces changes in



plant diversity and composition, as was observed with some AMF inoculants (Hart et al., 2018; Keswani et al., 2019).

Inoculation of rhizobia, widely utilized in biofertilizers, have shown significant impacts on soil and plant-associated microbial communities, as found in soybean (Zhong et al., 2019) and alfalfa (Schwieger and Tebbe, 2000) crops. These effects were not limited to the rhizosphere; field inoculation of *Phaseolus vulgaris* with two indigenous rhizobia strains (separately or combined) modified the structure and increased the phylotype richness of bacterial communities in the bulk soil (Trabelsi et al., 2011). Changes in microbial structure can result from both positive and negative interactions of rhizobia with the rhizosphere microbiome. *Azospirillum*, a bacterial genus characterized by free-living organisms with N<sub>2</sub>-fixing capabilities among other PGP traits, has shown variable effects on the resident microbiome (Trabelsi and Mhamdi, 2013). Inoculation of maize with *A. lipoferum* induced a shift in the composition of rhizosphere bacterial communities which lasted for at least one month (Baudoin et al., 2009). Yet, variable results were observed when inoculating the same or other crops with *A. brasilense* (Herschkovitz et al., 2005; Lerner et al., 2006; Correa et al., 2007), even though this bacterium can induce physiological and morphological changes in the root system. According to Trabelsi and Mhamdi (2013), effects of *Azospirillum* inoculation on rhizosphere microorganisms is likely driven by N dynamics, although evidence suggests that a wide array of factors are involved. While rhizobia and *Azospirillum* have been more widely studied regarding their effects on the resident microbiome, research on other taxa such as *Pseudomonas* sp. remains limited, even though they could potentially modify both bacterial and fungal communities (Andreote et al., 2009; Gao et al., 2012).

Among fungal biofertilizers, those based on AMF are the most widespread and commercially available, even though ecological risks of field application are not being properly assessed (Hart et al., 2018). Inoculation with foreign AMF can affect native AMF communities, for example displacing them and reducing their diversity (Koch et al., 2011) or modifying their composition (Jin et al., 2013a), although a response in the native AMF community is not always observed (Antunes et al., 2009). In the study by Jin et al. (2013b), a diverse AMF inoculum had less beneficial effects on plant fitness but had a lower impact on the composition of subsequent AMF communities. Besides native mycorrhizal fungi, introduced AMF can disturb other microbial communities in soil, particularly those surrounding their extraradical mycelium, (i.e., the mycorrhizosphere) (Trabelsi and Mhamdi, 2013). So far, the effect of AMF on soil microbial communities seems to be variable and modulated by several factors (Marschner and Timonen, 2005; Cavagnaro et al., 2006; Monokrousos et al., 2020). Remarkably, bacteria inhabiting the mycorrhizosphere, some of which may already be present in commercial inoculants, have shown PGP activity and are thought to act synergistically with AMF (Agnolucci et al., 2019). Lastly, non-AMF fungal inoculants have been less studied but there is some evidence for endophytic fungi (Casas et al., 2011; Rojas et al., 2016) and *Trichoderma* spp. (Jangir et al., 2019) to induce changes in local microbial communities.

The impact that introduced microbes may have on the resident microbial communities will depend to some extent on their abundance, survival, and permanence in the system (Ambrosini et al., 2016). This means that the same characteristics that will help guarantee a successful plant growth promotion, are also the ones increasing the risk for invasion. Yet, not enough studies focus on the effect of mass and repetitive inoculation, or effects in the long term or in subsequent crops (Trabelsi and Mhamdi, 2013; Mawarda et al., 2020). According to Hart et al. (2018), long-term effects are also important for AMF inoculants, although their complex genetic organization will definitely challenge the assessment of their establishment and persistence. These effects are particularly concerning if we consider that inoculation effects could remain even after the introduced microbial population decreases (Mawarda et al., 2020). One of the mechanisms behind these “legacy” effects is through plant-soil feedbacks, something more likely to occur when the introduced microorganism is symbiotic or has a high affinity for a specific plant (e.g., a non-target plant present in the agroecosystem) (Ambrosini et al., 2016; Keswani et al., 2019).

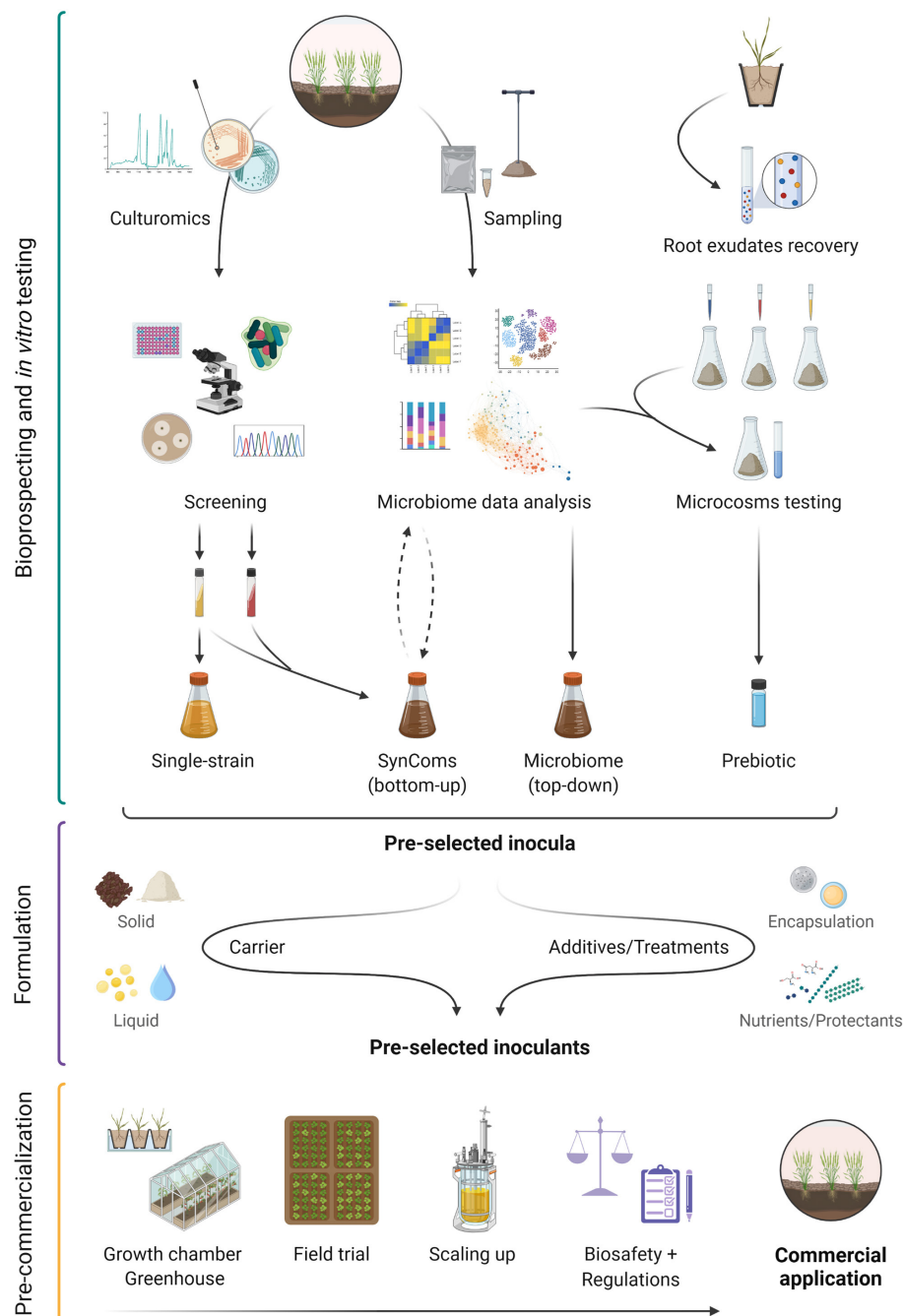
The variable effects observed on the resident microbiome when introducing a microorganisms via inoculation suggest the outcome is driven by several factors, including the host plant (Marschner and Timonen, 2005). For example, Correa et al. (2007) found that the plant genotype modulated the genetic and physiological response of phyllosphere and rhizoplane bacterial communities to *A. brasilense* seed inoculation. In another study, the response of rhizosphere bacterial communities to *A. brasilense* changed with the crop growth stage, being more affected at jointing than grain-filling stage (Di Salvo et al., 2018). On the other hand, the resident microbiome might present different susceptibility and buffering capacity levels depending on its diversity (Eisenhauer et al., 2013; Trabelsi and Mhamdi, 2013) and the presence of specific antagonists or synergistic taxa (Mar Vázquez et al., 2000; Asiloglu et al., 2020). Similarly, environmental conditions may play a key role regulating the impact of introduced microorganisms, as observed for soil moisture (He et al., 2019b; Monokrousos et al., 2020) and light intensity (Marschner and Timonen, 2005).

Overall, most studies analyzing the impact of biofertilizers seem to focus on structural aspects and not enough on functionality (Trabelsi and Mhamdi, 2013). Understanding the functional implications of those changes is crucial, since they will directly impact ecosystem functioning and health. Multi-omics and data analysis are key tools to begin to understand the complex dynamics taking place and to predict microbial response under natural conditions (Ambrosini et al., 2016; Timmusk et al., 2017). Another question that remains is how persistent the environmental impact of the introduced microbes is (i.e., long-term and legacy effects). Moreover, further research is needed in the specific case of genetically modified microorganisms, including effects of metabolic load (Glick, 2020), and risks of horizontal gene transfer and dispersion (Hirsch, 2004; Bellanger et al., 2014). Diverse inoculants could be a safer alternative in terms of environmental impact, as suggested by studies on AMF or *Bacillus* spp.

inoculation (Jin et al., 2013a; Gadhave et al., 2018). Still, further research is needed to fully understand the impact of different types of mixed inoculants (either based on same species, different species or different strains) on the resident microbiome.

## CONCLUSIONS AND FUTURE PERSPECTIVES

The global biofertilizer market has an estimated value of 2.3 billion US dollars and it is projected to increase to 3.9 billion in



**FIGURE 2 |** Overview of biofertilizer development workflow from potential candidate microorganisms to commercial applications with four novel approaches: single-strain inoculant obtained using emerging culture-based methods (e.g., culturomics), synthetic microbial communities (SynComs) obtained from a bottom-up approach, whole microbiomes recovered from natural or engineered ecosystems (top-down approach), and prebiotics obtained from root exudates. Following the initial stages of bioprospecting and *in vitro* testing, selected inocula and/or prebiotics require a proper formulation to ensure shelf life and protection. Finally, product pre-commercialization steps include *in planta* trials under controlled (growth chamber/greenhouse) and uncontrolled (field) conditions, production scale-up to a commercial scale, proper biosafety screening tests (e.g., toxicity and pathogenicity), and compliance with existing regulations. Created with BioRender (<https://biorender.com/>).

2025 (Marketandmarkets, 2020). Despite their great potential and long-term effects, biofertilizer products still face major challenges limiting their use in agricultural settings. These are often associated with limited shelf-life and the survival of inoculated strains in vastly different environments.

In this review, we highlighted the application of multi-omics approaches and emerging technologies in biofertilizer development (Figure 2). As discussed by Saad et al. (2020) and Toju et al. (2018a), keystone taxa provide an appealing target to optimize the outcomes of biofertilization due their role in regulating the growth/function of other members in the plant microbiome. We propose that these taxa and other members of the core microbiome could be further explored to manipulate microbiomes or design synthetic microbial communities (e.g., SynComs). At the same time, emerging culture-based methods (e.g., “culturomics”) can be used to discover novel isolates with biofertilizer applications. As an alternative, or in combination with, we suggest the use of “plant prebiotics,” that act as signaling molecules to attract beneficial microbes, thus enhancing biofertilizer efficiency. These studies can be further integrated into a global database systematizing different outcomes, environmental conditions, targeted plant genotypes, soil types, and growing seasons.

The success of biofertilizers, however, not only depends on selecting specific microorganisms or functions, but also on developing new formulations to ensure the survival of inoculated strain(s). Here, we reviewed different methods in which bioformulations could be improved by using biofilm-producing strains, microencapsulation with alginate, and processes based on fluidized bed dryer (FBD). Ideally, new technologies should target carriers and additives that are cost-effective and easy to use but, most importantly, able to support a higher number of viable cells during storage and application.

Simultaneously, the biosafety of inoculated microbes should be assessed through a “One Health” approach. This step includes proper screening tests (e.g., toxicity and pathogenicity testing)

to ensure their safety before exposing personnel, consumers and natural resources. Moreover, continued studies on ecological interactions and how plants shape their microbiome in agricultural systems are still essential. This is particularly critical in the context of climate change, where key biogeochemical processes carried out by soil microorganisms may be affected.

Due to the complexity and genetic diversity within the soil and plant microbiome, it is unlikely that one formulation will be effective for all fields. Yet, it is unfeasible and unrealistic to design specific biofertilizers for each particular field. For this reason, in agreement with Bell et al. (2019), we suggest that continuous product design, refinement, and validation should be oriented toward optimal and sub-optimal environmental ranges for microbial products (i.e., crop, climate, soil type, and agricultural practices). Finally, significant resource inputs from both public and private sectors are needed to fill critical knowledge gaps in the field. This effort must be accompanied by the encouragement of regulatory agencies and policy makers supporting sustainable practices and biofertilizers.

## AUTHOR CONTRIBUTIONS

EKM, MT, and DO collected literature and wrote the manuscript. EKM edited the manuscript and MT designed the figures. JJG and KED provided critical feedback. All authors conceived and planned the scope, and approved the final version manuscript.

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# Profiling of Metabolites of *Bacillus* spp. and Their Application in Sustainable Plant Growth Promotion and Biocontrol

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*Bacillus* spp. are well-characterized as efficient bioinoculants for sustainable plant growth promotion and biocontrol of phytopathogens. Members of this spp. exhibit the multifaceted beneficial traits that are involved in plant nutrition and antimicrobial activities against phytopathogens. Keeping in view their diverse potential, this study targeted the detailed characterization of three root-colonizing *Bacillus* strains namely *B. amyloliquefaciens*, *B. subtilis*, and *B. tequilensis*, characterized based on 16S rRNA sequencing homology. The strains exhibited better plant growth promotion and potent broad-spectrum antifungal activities and exerted 43–86% *in-vitro* inhibition of growth of eight fungal pathogens. All strains produced indole acetic acid (IAA) in the range of 0.067–0.147  $\mu$ M and were positive for the production of extracellular enzymes such as cellulase, lipase, and protease. Ultra-performance Liquid Chromatography-Electrospray Ionization-Mass Spectrometry (UPLC-ESI-MS/MS) analysis revealed the production of antifungal metabolites (AFMs) such as surfactins, iturins, fengycins, macrolactins, bacillomycin-D, and catechol-based siderophore bacillibactin which were further confirmed by amplifying the genes involved in the biosynthesis of these antimicrobial lipopeptides. When compared for the amounts of different cyclic-peptides produced by three *Bacillus* strains, *B. amyloliquefaciens* SB-1 showed the most noticeable amounts of all the antifungal compounds. Plant experiment results revealed that inoculation with phytohormone producing *Bacillus* spp. strains demonstrated substantial growth improvement of wheat biomass, number of spikes, and dry weight of shoots and roots. Results of this study indicate the biocontrol and biofertilizer potential of *Bacillus* spp. for sustainable plant nutrient management, growth promotion, and effective biocontrol of crop plants, particularly cultivated in the South Asian region.

**Keywords:** biopriming, *Bacillus amyloliquefaciens*, cyclic-lipopeptides, mass spectrometry, siderophores, biofertilizer



## INTRODUCTION

Increasing environmental concerns and food safety issues gave momentum to the use of bio-fertilizers and biological control agents in agriculture sector world-wide. Moreover, to combat the threatening challenges of non-degradable and recalcitrant agrochemicals, and increased pesticide resistance, plant growth promoting bacteria (PGPB), and their bioactive metabolites got global attention (Bhardwaj et al., 2014). PGPB exert multifaceted benefits to the plants and enrich soil nutrients by means of phosphate/potassium/zinc mineralization, release of plant growth promoting regulators and hormones, and nitrogen fixation (Hayat et al., 2010; Saharan and Nehra, 2011). Furthermore, their contribution in plant defense through the production of metabolites make them significant and key players in sustainable agriculture (Sinha et al., 2014).

Currently, main plant growth promoting rhizobacteria (PGPR) include *Azotobacter*, *Azospirillum*, and *Rhizobium* [N<sub>2</sub>-fixers], *Gluconacetobacter*, *Enterobacter*, *Azoarcus*, *Klebsiella*, *Burkholderia*, *Pantoea*, *Stenotrophomonas* spp. [mineral mobilizers and plant-hormone regulators], *Bacillus* and *Pseudomonas* [PGP and biocontrol agents]. Among these predominant and the most widely studied are *Bacillus* and *Pseudomonas* spp. for their characteristic roles in nutrient acquisition and assimilation, secretion and modulation of hormones, plant beneficial secondary metabolites, antifungal metabolites (AFMs), antibiotics, and various extracellular signaling compounds (Ahemad and Kibret, 2014; Agler et al., 2016). *Bacillus* spp. have been in limelight for their long shelf-life in bio-formulations, effective colonization of plant tissues, and broad-spectrum antifungal abilities (Souza et al., 2014). Almost 4–5% genome of *Bacillus* spp. is dedicated for the production of structurally diverse antimicrobial compounds that have demonstrated varying antagonism against fungal and bacterial phytopathogens. Most significant among these antimicrobials are cyclic-lipopeptides (CLPs) constituting iturins, fengycins, and surfactins that are pivotal in root colonization by *Bacillus* spp. (Aira et al., 2010; Carvalhais et al., 2013).

Many research studies have investigated the biofertilizer and biocontrol potential of *Bacillus* spp., however, less is known about the complete metabolomic profile, account of the plant growth-promoting traits, secondary metabolites and their variants produced by a single strain. For instance, endophytic *B. subtilis* strain ALB629 was evaluated for its plant growth-promoting traits but its antagonistic secondary metabolites were not identified (Falcão et al., 2014). Likewise, PGPB *Bacillus* strains RM-2 and BPR7 were evaluated for *in-vitro* antagonistic activities, nonetheless, they were not subjected to detailed characterization of their secondary metabolites (Kumar et al., 2012; Minaxi et al., 2012; Shobana et al., 2020).

The following study aimed to identify antifungal *Bacillus* spp., characterization and relative quantification of their strain-specific antagonistic metabolites, and analysis of their biofertilizer potential. Based on the biocontrol potential, three *Bacillus* spp. isolates, i.e., SB-1, A-2, A-3, were hypothesized to be active against phytopathogens of important economic crops and subjected to detailed characterization. Antagonistic metabolites of these isolates were comprehensively documented using

polyphasic approach with the identification of putative genes involved in the production of these metabolites. Furthermore, bacterial isolates were screened for their plant growth-promoting (PGP) traits and their effects as bioinoculants for wheat were evaluated *in-vivo* to be used as multifaceted biofertilizer and biopesticides for various crop plants.

## MATERIALS AND METHODS

### Isolation and Screening of Bacterial Isolates With Antagonistic Activity

Bacteria were isolated from sugarcane, rice (Lahore, Pakistan, 31.5204° N, 74.3587° E), and corn (Kasur, Pakistan, 31.1179° N, 74.4408° E), using standard serial dilution plating (Somasegaran and Hoben, 1985, **Supplementary Tables 1–3**). Pure bacterial cultures were obtained by streaking single colonies on Luria-Bertani (LB) agar plates incubated at 37°C for 48 h. Antagonistic activity of bacterial isolates was checked against fungal phytopathogens (diseases, affected crops, host plants are described in **Supplementary Table 4**) including *Aspergillus niger* (NCBI accession no. MN786323), *A. flavus*, *Fusarium oxysporum* (NCBI accession no. MN636869), *F. moniliforme* (NCBI accession no. MN636870), *F. solani*, *Colletotrichum falcatum*, *Curvularia* sp. (NCBI accession no. MN636871), and *Rhizopus* sp. (NCBI accession no. MN636450), using plate bioassays. Fungal cultures were obtained from the culture collection of Mycology Laboratory, Forman Christian College (A Chartered University) Lahore, Pakistan and maintained on potato-dextrose agar (PDA) plates at 26–28°C.

### In-vitro Inhibition of Mycelial Growth of Pathogens by Bacillus spp.

Dual culture assay described by Sakthivel and Gnanama-nickam (1986) was used to determine the antagonistic activity of bacterial isolates against fungal phytopathogens. In a complementary antagonistic experiment, agar well-diffusion assay was performed (Magaldi et al., 2004). Results were expressed as percentage inhibition zones by antagonistic bacteria to suppress the growth of fungal mycelia. Each experiment was performed twice in triplicates and percentage inhibition was calculated as

$$I = (C - T/C) \times 100$$

Where

I = % inhibition, C = fungal diameter in control plate, T = fungal diameter in test plate.

### Biochemical and Molecular Characterization of Bacillus Isolates

Based on antifungal activity, three bacterial isolates [SB-1 (sugarcane), A-2 (rice), and A-3 (corn)] were selected for detailed studies. These isolates were biochemically characterized using QTS-24 bacterial identification kits (DESTO Laboratories, Karachi, Pakistan) using manufacturer's protocol. Bacterial DNA was isolated using GeneJET genomic DNA purification kit (Thermo Fisher Scientific, USA, catalog number: K0721). 16S rRNA gene was amplified from genomic DNA of isolates SB-1, A2, and A3. Primers and PCR conditions are described in

**TABLE 1** | PCR conditions used for amplification of genes from *Bacillus* spp. Isolates.

Gene/Antibiotic	Target genes	Primers sequences	PCR Profile (35 cycles each)	Amplicon Size (bp)	References
16S rRNA	<i>FGPS 1509-153</i> <i>FGPS 4-281</i>	5'-AAGGAGGTGATCCAGCCGCA-3' 5'-AGAGTTTGATCCTGGCTCAG-3'	Denaturing: 95°C 2 min Annealing: 55°C 60 s Extension: 72°C 90 s Final Extension: 72°C 10 min	1,500	(Normand, 1995)
Surfactin	<i>sfp-f</i> <i>sfp-r</i>	5'-ATGAAGATTTACGGAATTTA-3' 5'-TTATAAAGCTCTTCGTACG-3'	Denaturing: 94°C 1 min. Annealing: 55°C 60 s Extension: 72°C 60 s Final Extension: 72°C 10 min	675	(Kefi et al., 2015)
Iturin A	<i>ituD-f</i> <i>ituD-r</i>	5'-ATGAACAATCTTGCCCTTTTA-3' 5'-TTATTTTAAATCCGCAATT-3'	Denaturing: 94°C 3 min Annealing: 55°C 60 s Extension: 72°C 90 sec. Final Extension: 72°C 10 min.	1,200	
Fengycin	<i>fenD-f</i> <i>fenD-r</i>	5'-CCTGCAGAAGGAGGAGGACTGAAG-3' 5'-TGCTCATCGTCTTCCGTTTC-3'	Denaturing: 94°C 3 min Annealing: 58°C 60 s Extension: 72°C 60 s Final Extension: 72°C 10 min	300	(Kim et al., 2010)
Bacillomycin D	<i>bmy-f</i> <i>bmy-r</i>	5'-TGAAACAAAGGCATATGCTC-3' 5'-AAAAATGCATCTGCCGTTCC-3'	Denaturing: 94°C 3 min Annealing: 55°C 60 s Extension: 72°C 60 s Final Extension: 72°C 10 min	375	

**Table 1.** A reaction mixture of 50  $\mu$ L was prepared by using Taq buffer 5  $\mu$ L (10X),  $MgCl_2$  2  $\mu$ L (25 mM), Taq polymerase (5 U) 2  $\mu$ L, dNTPs 2  $\mu$ L (2.5 mM), each of forward and reverse primer (20 pmol) 1  $\mu$ L,  $dH_2O$  35  $\mu$ L, and the template DNA 2  $\mu$ L ( $>50$  ng/ $\mu$ L). All PCR reagents were purchased from Fermentas (Thermo Fisher Scientific, USA). PCR products were purified using GeneJET PCR purification kit (Thermo Fisher Scientific, USA, catalog number: K0701) and sequenced with both, forward and reverse primers, by Eurofins Genomics, USA. Amplified sequences were compared with NCBI GenBank sequence database, using BLAST search tool and phylogenetic analysis was performed using MEGA5 platform (Tamura et al., 2011). Nucleotide sequences of isolates SB-1, A2, and A3 were aligned with Clustal X 2.1 program. Bootstrap confidence analysis was performed on 1,000 replicates to determine the reliability of the distance tree topologies obtained. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option).

## Identification and Pseudo-Relative Quantification of Secondary Metabolites by Ultra Performance Liquid Chromatography- Tandem Mass Spectrometry (UPLC-MS/MS) Extraction of Secondary Metabolites

To identify secondary metabolites, bacterial isolates were individually inoculated to 100 mL LB broth and incubated at 37°C for 48 h in shaking incubator at 180 rpm. Bacterial cultures

were pelleted by centrifugation ( $3,900 \times g$  and 4°C for 20 min) and the pH of the supernatants was adjusted to 2.0 with 6N HCl. Secondary metabolites were precipitated by incubating the supernatants overnight at 4°C. The precipitates were recovered by centrifugation ( $3,900 \times g$  and 4°C for 20 min) and dissolved in 5 mL of methanol:  $H_2O$  (2:1, v/v). The extracts were re-centrifuged ( $3,900 \times g$  and 4°C for 20 min), supernatants were dried under vacuum and, re-suspended in 2 mL of methanol, and 10  $\mu$ L were injected for each LC-MS analysis.

## LC-MS/MS and Data Analyses

Sample solutions were injected onto an Eclipse Plus C18 RRHD column (2.1  $\times$  100 mm, 1.8  $\mu$ m; Agilent, Santa Clara, CA, USA) and separated by reversed-phase liquid chromatography using a Waters Acquity UPLC system (Milford, MA, USA) at a flow rate of 0.4 mL/min. The UPLC system was coupled to an Orbitrap Fusion Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an EASY-Max NG electrospray ion source. The mobile phases were water/0.01% formic acid (A) and 100% acetonitrile/0.01% formic acid (B). Secondary metabolites were separated on the column with a 20 min binary solvent elution gradient (0 min, 5% B; 0–15 min, 5–100% B; 15–17 min, 100% B) followed by a 3-min column equilibration at 5% B between injections. The MS parameters were as follows: positive-ion electrospray spray voltage, 3.9 kV; negative-ion electrospray spray voltage, 2.5 kV; capillary temperature, 325°C; S-lens Level, 60%; sheath gas, 50; sweep gas, 1; and auxiliary gas 10. The MS survey scan was carried out over the mass range  $m/z$  100–2,000, with the data recorded in the centroid mode and at a mass resolution of 120 K FWHM ( $m/z$  200), AGC target 4E5, and one microscan with a maximum inject time of 50 ms in the

quadruple isolation mode. A lock mass at  $m/z$  391.28426 from di(2-ethylhexyl) phthalate (a ubiquitous plasticizer) was used for real-time internal mass calibration during the LC-MS runs with the Fourier Transform (FT) MS detection. In the LC-MS/MS runs, the top five most intense ions, with their charge state of 1 and ion counts of  $>5,000$  in each survey scan were selected for MS/MS by collision-induced dissociation (CID) in the linear ion trap or by “higher-energy” collisional dissociation (HCD) in the collision cell in the upfront of the C-trap. Other parameters included: activation isolation window, 2 Da; AGC target for MS/MS, 5E4; maximum inject time, 30 ms; activation time, 10 ms; activation Q, 0.25; and normalized collision energy at 35% for the CID operations. Activation isolation window, 2 Da; AGC target for MS/MS, 5E4; maximum inject time, 30 ms; activation time, 10 ms; activation Q, 0.25; and normalized collision energy at 25% were used for the HCD operations. Raw data files were recorded and processed using the XCalibur 4.1.31.9 (Thermo Scientific) software suite. For relative quantification of secondary metabolites produced by *Bacillus* strains, each sample solution was injected in triplicate and the peak areas were normalized. Bar graphs were plotted with error bars to show the analytical standard deviations.

## IDENTIFICATION OF GENES ENCODING SECONDARY METABOLITES IN BACILLUS STRAINS

Detection of the genes encoding surfactin, iturin, bacillomycin, and fengycin was done using gene-specific primers. Primers used in this study and PCR conditions are shown in **Table 1**. An individual reaction mixture of 50  $\mu$ L was prepared for all the reactions as described above. PCR products were analyzed on 1% agarose gel. The respective bands were excised and purified using a gel extraction kit. PCR products were sequenced (Eurofins) and analyzed using the NCBI BLAST (blastn) and alignment tools.

## IDENTIFICATION AND QUANTIFICATION OF INDOLE-3-ACETIC ACID (IAA)

### Preparation of Samples

Bacterial cultures were grown in 10 mL of LB medium at 180 rpm and 37°C temperature, supplemented with L-tryptophan (100 mg/L). After 7 days of growth, bacterial cells were centrifuged at  $3,900 \times g$  for 30 min (Allegra TM X-22R Centrifuge, Beckman Coulter, California, USA) and the pH of the supernatants was adjusted to pH 2.0 with 6 N HCl. The acidified supernatants were extracted twice with an equal volume (10 mL) of ethyl acetate. After centrifugation at  $3,900 \times g$  for 30 min, the upper organic layers were collected, pooled, and evaporated to dryness.

### Preparation of Indole-3-Acetic Acid Standard Curve and LC-MS Quantification

The reference standard of indole-3-acetic acid (IAA,  $C_{10}H_9NO_2$ , monoisotopic neutral mass = 175.0633, Sigma-Aldrich, USA) was dissolved in methanol:chloroform:H<sub>2</sub>O [50:25:25, v/v] at 0.25 mM to form stock standard solution (**Supplementary Table 5**). Ten- $\mu$ L aliquots of each standard and

sample were injected into the column for the LC-FTMS (Fourier Transform Mass Spectrometry) runs, as described above. The concentrations in the samples were determined from the peak areas of the extracted ion chromatograms of IAA detected at  $m/z$  176.0706  $[M+H]^+$  by interpolation of the linear-regression calibration curve for this compound (**Supplementary Figure 1**).

## Detection of Plant Growth Promoting Traits of *Bacillus* spp.

Lipase production was checked on 1% Tween-20 LB agar plates according to the method of Sierra (1957). Pikovskaya agar medium was used for the detection of phosphatase enzyme (Pikovskaya, 1948). Protease production was checked using skim milk agar plates (Alnahdi, 2012). Extracellular cellulase production was checked on 1% (Carboxymethyl Cellulose) CMC-LB agar plates (Kasana et al., 2008). *In-vitro* zinc solubilization was assessed using the method of Sharma et al. (2012). Siderophore production was detected as per O-CAS method (Louden et al., 2011). Qualitative determination of hydrocyanic acid (HCN) production carried out as per alkaline-picrate method (Millar and Higgins, 1970).

## Plant Experiment

*In-vivo* effect of *Bacillus* spp. as bioinoculants was evaluated using pot experiments in climate control room. Soil used for this experiment was taken (0–50 cm) from wheat field, demonstrated the following physico-chemical properties: temperature (°C) = 21.7, pH = 7.65, moisture (%) = 23, texture = silty loam, electrical conductivity ( $EC_{1:1}$  dS/m) = 1.15, organic matter (OM g/kg) = 29.2, and potential acidity ( $H^+ Al$  mg/kg) = 47.3. Soil was sieved and sterilized in drying oven at 90°C overnight, to eliminate native microbial communities and analyzed post-sterilization to determine any microbial population. Pots were filled with 300 g soil (per pot) and 25 mL of half-strength Hoagland's solution per pot was added (Hoagland and Arnon, 1970). For seed sterilization, 100 seeds of wheat variety, i.e., Faisalabad-2008 were soaked in 100 mL of 0.1 N bleach (sodium hypochlorite; NaClO) solution for 15 min, and given four successive washes of 10 min each with sterile dH<sub>2</sub>O (Chen et al., 2018). Seeds were transferred to 1% water-agar plates and incubated at 22–24°C. Three days old germinated seedlings were transferred in pots (1 seed/pot) and inoculated with 1 mL of individual bacterial culture containing  $1 \times 10^7$  cells/mL in 10 replicates for each treatment. Plants were kept in a climate control room at relative humidity of 60% with a 12 h photoperiod (200  $\mu$ M·m<sup>-2</sup>·s<sup>-1</sup> at pot heights with fluorescent lights, 15/20°C). The experiment was set up in a completely randomized block design (CRBD). The temperature in climate room was maintained at  $20 \pm 2^\circ\text{C}$ ; with light source of  $6,000 \pm 500$  FLUX and light period of  $10 \pm 1$  h. To provide moisture, plants were watered at alternate days using autoclaved distilled water and second dose of half strength Hoagland's solution was applied after 15 days. Plants were harvested after 50 days, roots were thoroughly washed and the root and shoot lengths of individual plants of each treatment were noted. Roots and shoots were detached to determine fresh weights of roots and shoots following drying in an oven for 72 h at 70°C to record dry weights of shoots and roots.



## Statistical Analysis

All the experiments were performed in triplicate and the average of each data was taken. Data was statistically analyzed using the Statistical Package for the Social Sciences (SPSS) software (IBM Statistics 23.0) at  $\alpha = 0.05$ .

## RESULTS

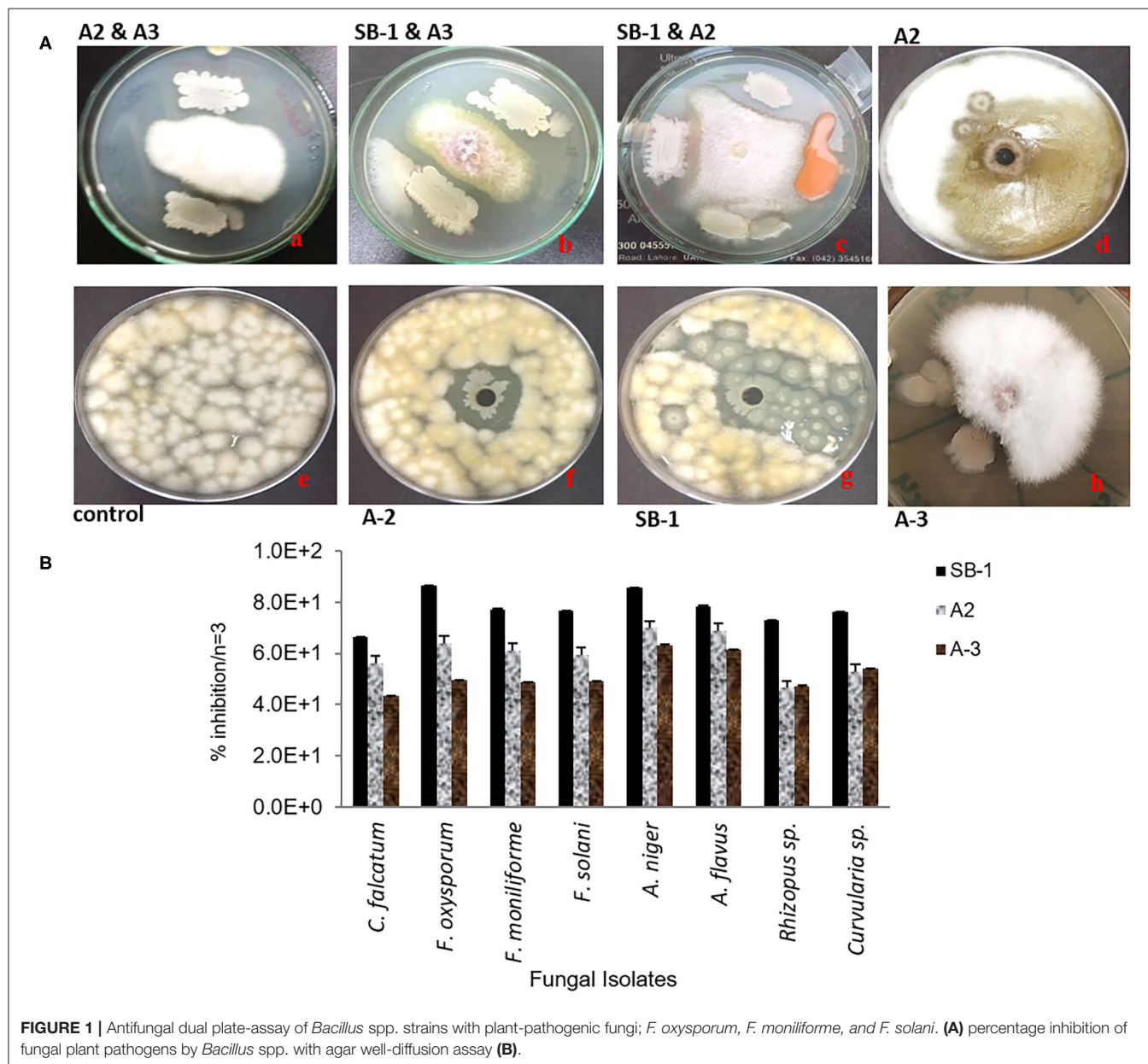
### In-vitro Inhibition of Mycelial Growth

Three isolates; SB-1 (sugarcane stem), A-2 (rice rhizosphere), and A-3 (corn rhizosphere) exhibited broad-spectrum *in-vitro* antifungal activities against all the pathogens tested (Figure 1A). SB-1 showed ~86% inhibition of *F. oxysporum*, whereas, *Bacillus* strains A-2 and A-3 exhibited ~64 and ~50% inhibition of *F. oxysporum*, respectively. Figure 1B highlights the variable

growth inhibition of fungal pathogens by the bacterial isolates. Strain SB-1 also demonstrated >70% inhibition of mycelial growth of *F. moniliforme*, *F. solani*, *A. flavus*, *A. Niger*, and *Curvularia* sp. Whereas, 60~65% suppression of these pathogens was shown by strains A-2 and A-3. Minimum inhibition (~43%) was noted for the sugarcane red-rot pathogen *C. falcatum* by the strain A-3, however, SB-1 exhibited ~66% suppression of this pathogen. *Rhizopus* sp. was moderately inhibited by all three strains tested (52~65%).

### Biochemical and Molecular Characterization of *Bacillus* Isolates

Bacterial isolates were biochemically characterized using QTS-24 bacterial identification kits (Supplementary Table 6). For 16S rRNA gene, sequence data were searched through



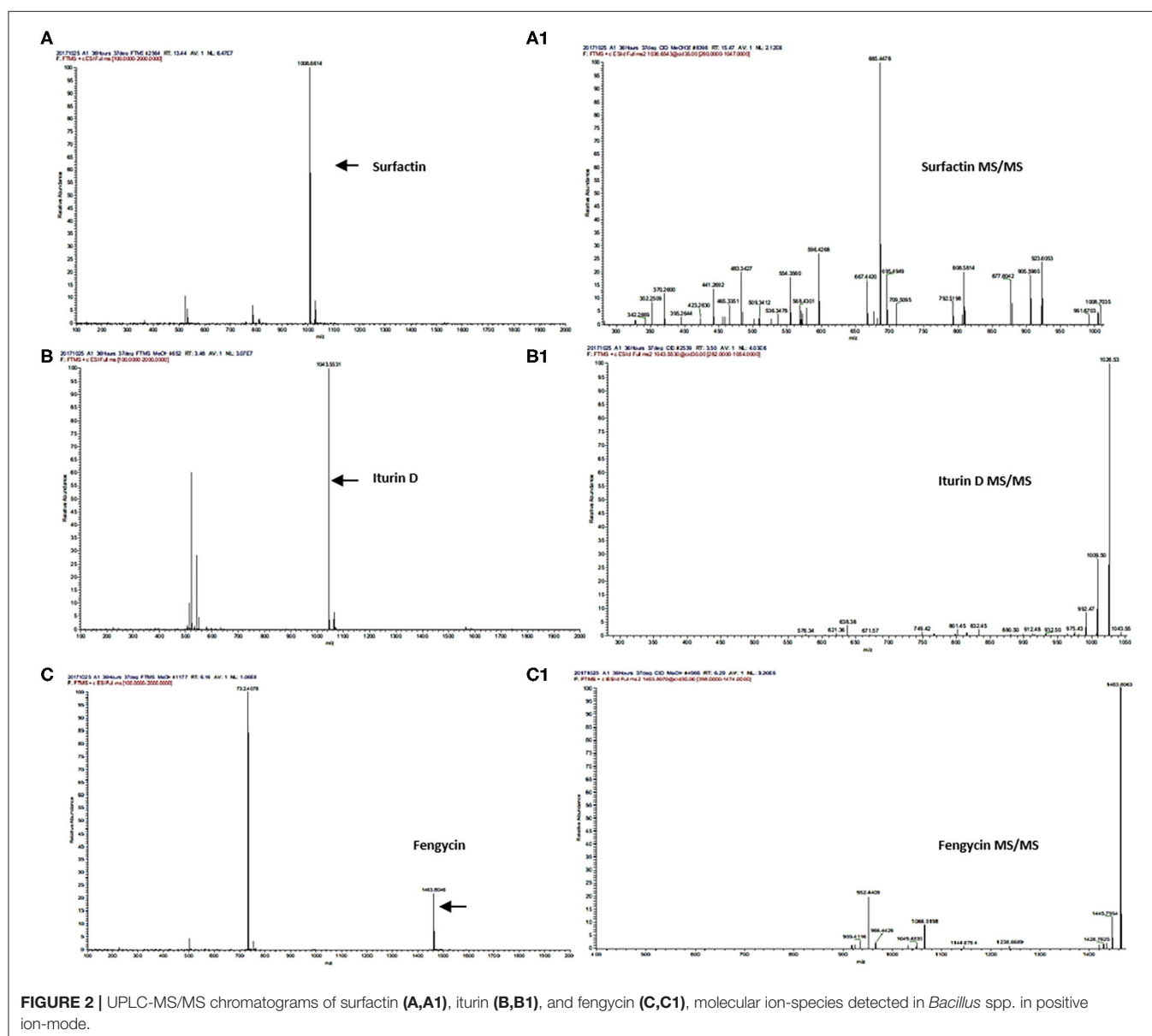


NCBI BLAST. Results confirmed the strain SB-1 as *B. amyloliquefaciens*, and A-2 as *B. subtilis* as they showed 100% homology with 16S rRNA sequences of these strains reported in database. Whereas, strain A-3 exhibited 100% homology with *B. tequilensis* strain (KU179328) and 99.79% homology with *B. subtilis* (KU179327). It was confirmed as *B. tequilensis* due to 99.86 and 99.79% homologies with other reported 16S rRNA sequences of *B. tequilensis* in database (Supplementary Table 7). Phylogenetic tree based on 16S rRNA gene sequences showed the homology of these strains with closely related strains (Supplementary Figure 2). Sequence lengths, accession numbers, and percent homology of each strain are summarized in Supplementary Table 3. There were a total of 1,388 positions in the final dataset of SB-1, 1,387 of A-2 and, 1,362 of A-3. Sequences were deposited in the GenBank database

and accession numbers were obtained (SB-1; MF171193, A-2; MF574398, A-3; MF574399).

## Mass-Spectrometry (LC-MS/MS) Analysis and Pseudo-Relative Quantification of Secondary Metabolites of *Bacillus* spp.

Secondary metabolites produced by *Bacillus* strains were first detected by LC/ESI-FTMS in the full-mass scan mode (Figures 3A,B). The first set of peaks observed belong to the surfactin cyclic-lipopeptide (CLPs) family, where strong signals corresponding  $m/z$   $[M+H]^+$  were recorded for surfactin C12-C17, i.e., C12  $m/z$   $[M+H]^+$  = 994.644, C13  $m/z$   $[M+H]^+$  = 1008.661, C14  $m/z$   $[M+H]^+$  = 1022.676, C15  $m/z$   $[M+H]^+$  = 1036.692, C16  $m/z$   $[M+H]^+$  = 1050.708, C17  $m/z$   $[M+H]^+$  = 1066.704, produced by all *Bacillus* strains (Figure 2,



**Supplementary Figures 3, 5A–K, Supplementary Table 8).** Weak signals for sodiated ions  $[M+Na]^+$  and deprotonated molecules  $[M-H]^-$  of surfactin variants were also recorded. Similarly, peaks for iturin homologs were apparent in both positive and negative ion-modes (**Supplementary Figure 3**). ESI-MS showed strong signals for iturin D  $m/z [M+H]^+ = 1043.553$ , iturin C  $m/z [M+H]^+ = 1044.536$ , and iturin A1  $m/z [M+H]^+ = 1029.538$  from *B. amyloliquefaciens* strain SB-1 (**Figure 2, Supplementary Figures 6A–8B, Supplementary Table 8**). The molecular weights of these iturin homologs and their variants were in agreement with iturin structures confirming the acyl chains of C14, C15, C16, and C17. Other than *B. amyloliquefaciens* strain SB-1, *B. subtilis* A-2, and *B. tequilensis* A-3 also demonstrated strong signals for iturin D. However, only weak  $[M+H]^+$  signals for iturin A1 and iturin C were recorded for these two strains whereas no  $[M-H]^-$  peaks for iturin A1 and iturin C were observed in the extracts of strains A-2 and A-3. Next family of CLPs observed was of fengycin where strong signals corresponding to fengycin  $m/z [M+H]^+ = 1463.804$  and fengycin A  $m/z [M+H]^+ = 1477.820$  were observed in all three bacterial extracts (**Figure 2, Supplementary Figures 9A–10B, Supplementary Table 8**). Moreover, equally strong sodiated ions  $[M+Na]^+$  i.e., fengycin  $m/z [M+Na]^+ = 1485.62$  and fengycin A  $m/z [M+Na]^+ = 1499.65$  were also noted for this class.

Next strong signals observed in sugarcane endophyte *B. amyloliquefaciens* strain SB-1 corresponded to iturin-like antifungal polypeptide bacillomycin D  $m/z [M+H]^+ = 989.494$ . Its sodiated ions were not observed, however, weak signals of deprotonated ions were recorded (**Supplementary Figure 11**). Production of bacillibactin, a catechol-based siderophore, was observed in all three *Bacillus* strains. Peaks corresponding to  $m/z [M+H]^+ = 883.262$  at retention time of 1.52 were detected in bacterial extracts giving the exact fragmentation pattern of daughter ions, confirming the bacillibactin production (**Figure 2,**

**Supplementary Figures 12A,B, Supplementary Table 8**). Next two molecular ions detected belonged to bacillaene  $m/z [M+H]^+ = 581.358$ , and bacilysocin  $m/z [M+H]^+ = 471.271$ , both of which are polyene and phospholipid antibiotics (**Figure 2, Supplementary Figures 13A–14B, Supplementary Table 8**). Strong signals for bacilysocin were detected in all three strains however, weak signals for bacillaene were observed in strains A-2 and A-3. Besides these, signals for lactone ring harboring macrolide compounds i.e., macrolactin A  $m/z [M+H]^+ = 403.247$ , macrolactin E  $m/z [M+H]^+ = 401.232$ , and macrolactin U  $m/z [M+H]^+ = 481.331$  were also noted and literature demonstrates their characteristic production from *Bacillus* spp. (**Figure 2, Supplementary Figures 15A–F, Supplementary Table 8**). The MS<sup>2</sup> fragmentation showed the exact pattern of daughter ions as reported in literature and identified molecules were structurally confirmed by LC-MS/MS. The MS/MS spectra obtained from ion fragmentation in HCD and CID mode were searched against the available MS/MS databases including CFM-ID (Allen et al., 2014), METLIN (Smith et al., 2005), and GNPS (Wang et al., 2016). The MS/MS spectra were also compared with previously published spectra in the literature, especially for those compounds still not entered into any database. Chemical formulas, monoisotopic neutral masses, observed  $m/z$  values, and retention times of these compounds have been described in **Table 2**.

All the extracts were analyzed in triplicates following the normalization of peak areas. Average of peak area values were plotted to visualize the comparative amounts of secondary metabolites in three different *Bacillus* strains. Surfactin homologs were abundantly produced by all *Bacillus* strains and amongst them, surfactin C13  $m/z [M+H]^+ = 1008.661$ , and C15  $m/z [M+H]^+ = 1036.692$  were observed in high amounts followed by surfactin C14  $m/z [M+H]^+ = 1022.676$  (**Figures 3C–E**). Minimum values were observed for surfactin C17  $m/z [M+H]^+$

**TABLE 2 |** Chemical formulas, monoisotopic neutral mass,  $m/z$  and observed peaks of detected metabolites.

Sr. No.	Metabolites	Chemical formula	Monoisotopic neutral mass	Monoisotopic $m/z$	Observed peaks $m/z$	Retention time	Bacterial strains		
				$[M+H]^+$	$[M+H]^+$		SB-1	A-2	A-3
1	Surfactin	C <sub>53</sub> H <sub>93</sub> N <sub>7</sub> O <sub>13</sub>	1035.683	1036.690	1036.692	14.95	+	+	+
2	Iturin A1	C <sub>47</sub> H <sub>72</sub> N <sub>12</sub> O <sub>14</sub>	1028.529	1029.536	1029.538	2.57	+	w+	w+
3	Iturin D	C <sub>48</sub> H <sub>74</sub> N <sub>12</sub> O <sub>14</sub>	1042.544	1043.552	1043.553	3.48	+	+	+
4	Iturin C	C <sub>48</sub> H <sub>73</sub> N <sub>11</sub> O <sub>15</sub>	1043.528	1044.536	1044.536	3.70	+	+	+
5	Bacillibactin	C <sub>39</sub> H <sub>42</sub> N <sub>6</sub> O <sub>18</sub>	882.255	883.262	883.264	1.52	+	w+	w+
6	Bacillaene	C <sub>34</sub> H <sub>48</sub> N <sub>2</sub> O <sub>6</sub>	580.351	581.358	581.358	6.06	+	+	+
7	Bacilysocin	C <sub>21</sub> H <sub>43</sub> O <sub>9</sub> P	470.264	471.271	471.271	10.06	+	+	+
8	Bacillomycin D	C <sub>45</sub> H <sub>68</sub> N <sub>10</sub> O <sub>15</sub>	988.486	989.493	989.494	1.77	+	-	-
9	Fengycin	C <sub>72</sub> H <sub>110</sub> N <sub>12</sub> O <sub>20</sub>	1462.795	1463.803	1463.804	6.16	+	+	+
10	Fengycin A	C <sub>73</sub> H <sub>112</sub> N <sub>12</sub> O <sub>20</sub>	1476.482	1477.820	1477.820	6.50	+	+	+
11	Macrolactin A	C <sub>24</sub> H <sub>34</sub> O <sub>5</sub>	402.240	403.247	403.247	2.82	+	nd	nd
12	Macrolactin E	C <sub>24</sub> H <sub>32</sub> O <sub>5</sub>	400.189	401.232	401.232	3.25	+	+	nd
13	Macrolactin U	C <sub>31</sub> H <sub>44</sub> O <sub>4</sub>	480.564	481.331	481.331	10.2	+	+	+

nd, not detected; W+, weak positive; +, positive; -, negative.

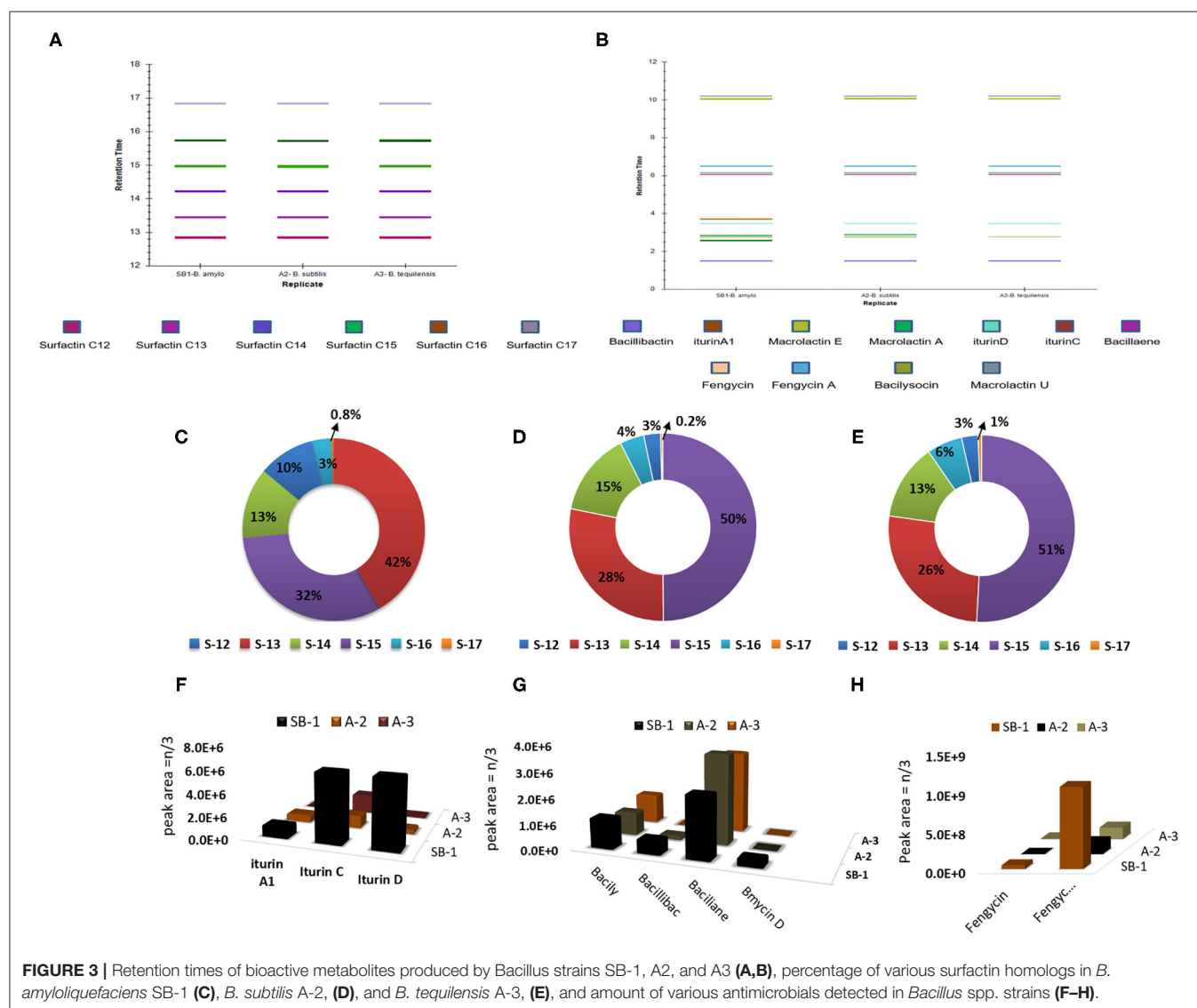
= 1086.704 which contributed 0.2–1% of the total surfactins produced by three strains. Likewise, maximum production of iturin variants was recorded for the strain *B. amyloliquefaciens* SB-1. The Highest amounts were noted for iturin C followed by iturin D in all three *Bacillus* strains, however, very minute amount of iturin A1 was detected for *B. subtilis* A-2 and *B. tequilensis* A-3 (Figure 3F).

Amongst other metabolites, all three *Bacillus* strains produced large amounts of polyene antibiotic bacillaene. Its maximum amount was observed in *B. subtilis* followed by *B. tequilensis* A-3. Moreover, all three strains produced phospholipid-based antibiotic bacilysocin and its production was prominently noted in strains *B. amyloliquefaciens* SB-1 and *B. tequilensis* A-3. Although the production of catechol-based siderophores was noted in all *Bacillus* strains, however, its maximum amount was seen in *B. amyloliquefaciens* SB-1. Production of Iturin-like polypeptide bacillomycin D was nevertheless, variable. Its production was seen only in sugarcane endophyte

*B. amyloliquefaciens* SB-1 and no peak was recorded with the same mass in other two strains (Figure 3G). *Bacillus* spp. were also compared for the production of cyclic-lipopeptide fengycins and demonstrated high values for fengycin A production. Maximum amounts of fengycin and fengycin A were seen by the strain SB-1 *B. amyloliquefaciens* followed by strains A-3 and A-2 (Figure 3H).

## Genes Encoding for Cyclic-Lipopeptides in Bacillus Strains

Gene *sfp* has been reported as the marker for identification of surfactin production by *Bacillus* spp. PCR amplification of *sfp* gene (675 bp) was shown by all three strains. Similarly, *ituD* gene amplification (1,200 bp) was also noted in all three *Bacillus* spp. which is the characteristic biomarker for iturin A production and encodes a putative malonyl coenzymeA transacylase. Amplification of 300 bp fragment with fengycin



**FIGURE 3 |** Retention times of bioactive metabolites produced by *Bacillus* strains SB-1, A2, and A3 (A,B), percentage of various surfactin homologs in *B. amyloliquefaciens* SB-1 (C), *B. subtilis* A-2, (D), and *B. tequilensis* A-3, (E), and amount of various antimicrobials detected in *Bacillus* spp. strains (F–H).

primers confirmed that all three *Bacillus* strains harbor the gene for production of fengycin lipopeptide. However, bacillomycin D showed the amplification of 375 bp only in *B. amyloliquefaciens* SB-1 (Figures 4A–D). Moreover, peaks corresponding to bacillomycin D  $m/z$   $[M+H]^+ = 989.494$  were only seen in SB-1 confirming that strain A-2 and A-3 do not produce this iturin-like polypeptide. Sequencing of PCR products were searched through BLAST search and confirmed homologies with respective gene sequences reported in database.

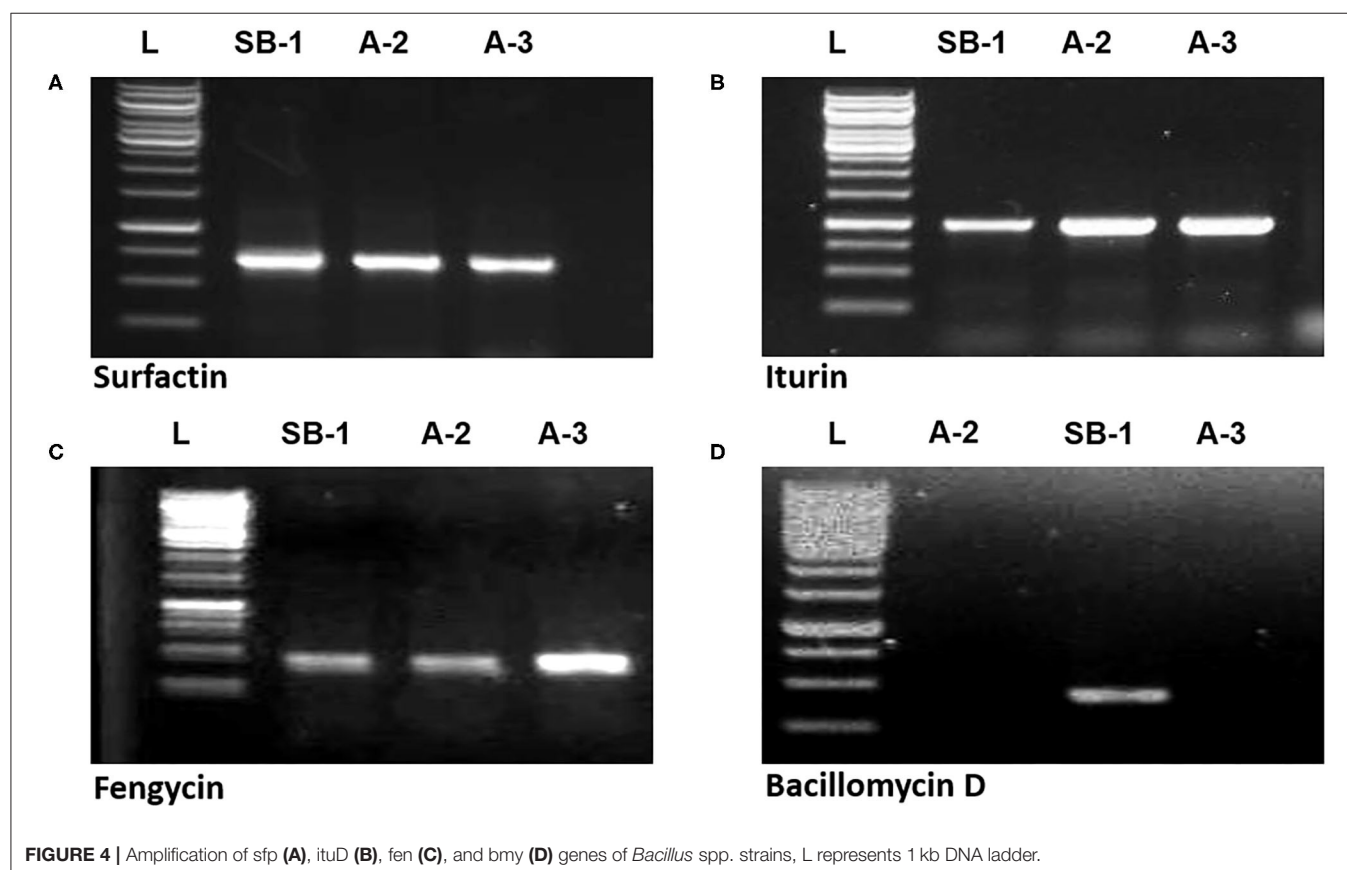
### Identification of Plant Growth Promoting Traits of *Bacillus* spp.

All three *Bacillus* strains produced plant-growth promoting auxin, indole-3-acetic acid. Highest amount of IAA, i.e.,  $0.147 \mu\text{M}$  was produced by *B. amyloliquefaciens* SB-1 followed by *B. tequilensis* A-3 which produced  $0.132 \mu\text{M}$ . Least amounts of IAA were noted by *B. subtilis* strain A-2 which produced  $0.067 \mu\text{M}$  of IAA. Furthermore, production of extracellular hydrolytic enzymes including protease, lipase, and cellulase was noted by all three *Bacillus* spp. in plate bioassays. None of the strains was positive for volatile HCN production, whereas, all three strains produced catechol-type siderophores in plate bioassays. When checked for insoluble mineral solubilization, only *B. amyloliquefaciens* SB-1 solubilized tris-minimal agar medium supplemented with  $\text{ZnCO}_3$  ( $2.1$

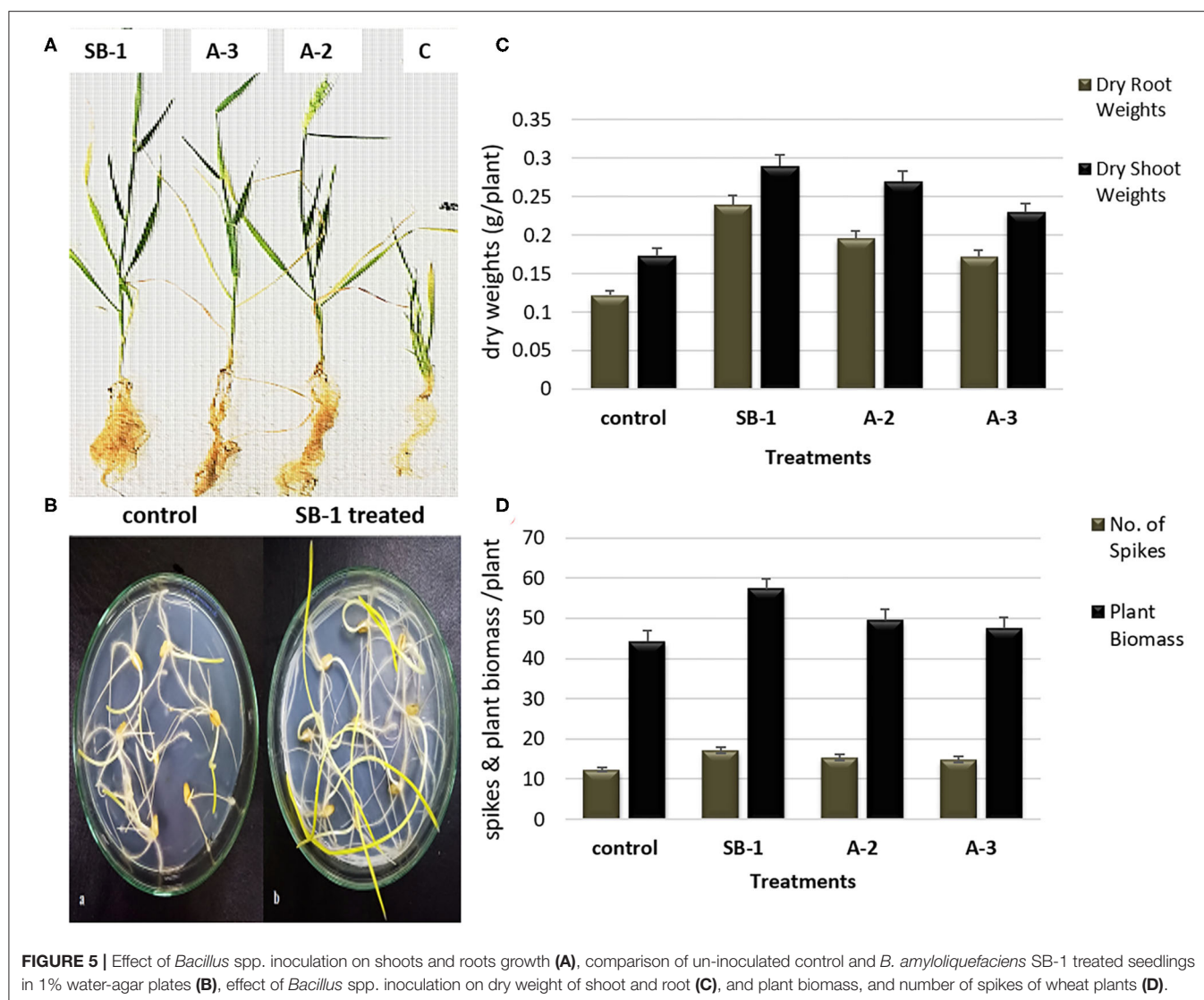
$\pm 0.18$  SI) and Pikovskya's agar, i.e.,  $23.2 \pm 0.21 \mu\text{g/mL}$  (Supplementary Table 9).

### Stimulation of Wheat Growth by *Bacillus* spp. Bioinoculants

Significant effects of *Bacillus* spp. bioinoculants were noted on the growth of wheat plants. Results manifested that bacterial inoculation stimulated plant growth as compared to the un-inoculated controls. Plants inoculated with *B. amyloliquefaciens* SB-1 demonstrated maximum shoot lengths followed by *B. tequilensis* A-3, and *B. subtilis* A-2, respectively. Maximum root lengths were however noted for *B. subtilis* A-2 inoculated plants followed by *B. tequilensis* A-3 (Figures 5A,B). When compared for dry weights of shoot and root, most significant difference was recorded for the *B. amyloliquefaciens* SB-1 inoculated plants followed by *B. subtilis* A-2. Likewise, *B. amyloliquefaciens* SB-1 substantially increased the overall plant biomass ( $\sim 42\%$ ) as compared to uninoculated controls. *B. subtilis* A-2 inoculated plants were next in the queue that showed considerable increase in overall plant biomass ( $\sim 31\%$ ). Although all the inoculated plants demonstrated more spikes in comparison to uninoculated ones, however, *B. amyloliquefaciens* SB-1 inoculated plants were notably significant (Figures 5C,D). Overall results suggested *B. amyloliquefaciens* SB-1 as the most successful bioinoculum for the wheat.







**FIGURE 5 |** Effect of *Bacillus* spp. inoculation on shoots and roots growth (A), comparison of un-inoculated control and *B. amyloliquefaciens* SB-1 treated seedlings in 1% water-agar plates (B), effect of *Bacillus* spp. inoculation on dry weight of shoot and root (C), and plant biomass, and number of spikes of wheat plants (D).

## DISCUSSION

*Bacillus* spp. are omnipresent in nature and constitute a major microbial community of rhizomicrobiome. Root-colonizing *Bacillus* spp. have been excessively characterized for their range of agricultural, environmental, and industrial applications and are regarded as eco-friendly substitutes of chemical fertilizers (Cardinale et al., 2015). Besides possessing diverse plant growth-stimulating factors, *Bacillus* spp. are also bestowed with the immense capacity of producing antimicrobial compounds active against broad range of plant-pathogens. Among these antimicrobials, the most significant are cyclic-lipopeptides which make them versatile tool for phytopathogenic biocontrol (Santoyo et al., 2012; Smith et al., 2017).

This study describes the isolation and polyphasic characterization of three antagonistic *Bacillus* spp. from different plant sources. Based on 16S rRNA sequencing, these strains were characterized as *B. amyloliquefaciens* SB-1, *B.*

*subtilis* A-2, and *B. tequilensis* A-3. Strain SB-1 showed 100% homology with reported database strains of *B. amyloliquefaciens*. Likewise, rice rhizosphere isolate *B. subtilis* A-2 demonstrated 100% homology with *B. subtilis* KX692273, and 99.93% homology with other reported *B. subtilis* strains accession nos.; KU551205, AB735984, and AY775778. Corn isolate A-3 however, showed 100% homology with one *B. tequilensis* strain (accession no. MF417796) and exhibited 99.79% similarity with two other reported strains of *B. tequilensis* (accession no. KU179328, KU179329). Additionally, this strain showed 99.86% homology with *B. subtilis* strain (accession no. KU179327). Close homologies with two different species can be further resolved with additional biomarkers including DNA-DNA based hybridization. These three *Bacillus* strains demonstrated broad-spectrum *in-vitro* antifungal activities against eight phytopathogens including *F. oxysporum*, *F. solani*, *F. moniliforme*, *C. falcatum*, *A. niger*, *A. flavus*, *Curvularia* sp. and *Rhizopus* sp. Among these, maximum antagonism was observed by

*B. amyloliquefaciens* SB-1 showing >85% inhibition of *F. oxysporum* and ~60–70% suppression of other fungal pathogens tested. Previous *in-vitro* studies have shown the ability of *Bacillus* spp. in inhibiting the growth of several fungal pathogens. For instance, *B. subtilis* was evaluated for the biocontrol of *C. gloeosporioides* OGC causing anthracnose disease of chili (Luna-Bulbarela et al., 2018). Similarly, *B. subtilis* strain BPR7 isolated from common bean rhizosphere demonstrated antagonism against several plant-pathogenic fungi including *M. phaseolina*, *F. oxysporum*, *F. solani*, *S. sclerotiorum*, and *Colletotrichum* sp. (Kumar et al., 2012). Likewise, *B. amyloliquefaciens* CNU114001 showed effective biocontrol of sclerotinia rot of cucumber fruit (Ji et al., 2013). However, biocontrol potential of *B. tequilensis* have been explored in recent years and one of the latest research studies evaluated the efficacy of *B. tequilensis* strain MML2476 in biocontrol of rhizome-rot disease of turmeric (Chenniappan et al., 2019).

The underlying mechanism of biocontrol of plant diseases (caused by aerial and soil-borne fungi), by *Bacillus* spp. is usually attributed to the production of various antibiotics by this genus. Suppression of plant-pathogenic fungi by *B. amyloliquefaciens* SB-1, *B. subtilis* A-2, and *B. tequilensis* A-3 suggested their secondary metabolites as potential biocontrol agents. When subjected to detailed ESI-LC-MS/MS analysis, all three *Bacillus* spp. strains showed the production of three main classes of cyclic-peptides (CLPs) including surfactins, iturins, and fengycins. These cyclic-lipopeptide bioactive metabolites have been well-evaluated for their biocontrol and pharmaceutical applications. Iturins constitute the group of iturin A~E, bacillomycins, and mycosubtilins which induct ion-conducting pores in the membranes of growing pathogens and damage mycelia (Zhang et al., 2013). Surfactins however, act by membrane disruption and solubilization and considered as the strong bio-surfactants. Many studies illustrate the synergistic effects of surfactins on broadening the biological properties of iturins and where two of them act in a coordinated manner (Fan et al., 2017). Similarly, fengycins have been demonstrated as key factors in antagonism of several *Bacillus* spp. which together with surfactins can elicit induced systemic resistance (ISR) in plants (Fira et al., 2018). Such as, these CLPs were characterized as the major functional bioactive metabolites of *B. amyloliquefaciens* C06 toward suppression of *M. fructicola* (Liu et al., 2011). Likewise, CLPs from *B. amyloliquefaciens* DH-4 were identified as the main antagonizing agents against citrus green mold *P. digitatum* (Chen et al., 2018). UV-MALDI-TOF-MS analysis of bioactive compounds of *B. subtilis* subsp. *subtilis* PGPMori7 revealed iturins, fengycins, and surfactins in triggering the immunity and microbial competition against *M. phaseolina* (Torres et al., 2016). Recently, genomic insights into the endophytic *B. tequilensis* 7PJ-16 strain displayed its biocontrol potential against mulberry fruit sclerotiniose (Xu et al., 2019).

The strains *B. amyloliquefaciens* SB-1, *B. subtilis* A-2, and *B. tequilensis* A-3 used in this study, were able to produce various homologs of surfactins, iturins, and fengycin compounds. Well-ionized peaks for surfactin C12-C17, m/z 994.644–1086.704, iturin A1, D, and C, and fengycin were noted in positive ion mode in all strains. Maximum amounts of these CLPs

were however noted for the strain *B. amyloliquefaciens* SB-1 as compared to other two *Bacillus* strains. These homologs were further confirmed through ESI-MS/MS and results were in accordance with previously published mass-spectrometry data for these compounds. Furthermore, the fragmentation patterns of daughter ions were also confirmed by latest databases CFM-ID and GNPS (Smith et al., 2005; Allen et al., 2014). Moreover, amplification of biosynthetic genes responsible for the synthesis of surfactins, iturins and fengycins confirmed the production of these metabolites by the strains under investigation. All strains showed 675 bp amplification for *sfp* gene coding for surfactin, 1.2 kb fragment for iturin D, and 300 bp fragment for fenD. Following amplification, PCR products were sequenced and compared with reported sequences in database.

Surfactins from *B. subtilis* has been evaluated for its role in root-colonization and protection against plant-pathogen namely *P. syringae* in *Arabidopsis thaliana* (Jourdan et al., 2009) and are known for their potential role in initiation of systemic resistance and stimulation of plant-defense (Bais et al., 2004; Hsieh et al., 2004). Likewise, iturin cyclic-peptides are strong antifungal CLPs produced by many of *Bacillus* strains and act by forming small pores in the membranes of the encountering phytopathogens (Zohora et al., 2016; Zhao et al., 2018). Iturins from *B. amyloliquefaciens* and *B. subtilis* were evaluated for effectively managing anthracnose disease of chili caused by *C. gloeosporioides* OGC1 (Ashwini and Srividya, 2014). Similarly, fengycin family homologs have been characterized for suppressing the growth of filamentous fungi by inducing reactive oxygen species (ROS) production and chromatin condensation (Zhang and Sun, 2018). Moreover, Romero et al. (2007) investigated the contribution of iturins and fengycins from four *B. subtilis* strains UMAF6614, UMAF6616, UMAF6639, and UMAF8561 in the suppression of powdery mildew of cucurbits caused by *Podosphaera fusca*.

Furthermore, iturin-like antifungal peptide, bacillomycin D was only detected in *B. amyloliquefaciens* SB-1 and its PCR analysis also revealed the 375 bp product coding for bacillomycin gene. The result was in accordance with LC-MS/MS results which showed the characteristic peak corresponding to bacillomycin m/z [M+H] = 989.494 in the extracts of strain *B. amyloliquefaciens* SB-1 only. Previous research studies manifest the production of bacillomycin D from *B. amyloliquefaciens* and its contribution in inhibiting spore germination. For instance, *B. amyloliquefaciens* strain 83 was extensively investigated for its potential to produce certain bacillomycin D homologs effectively inhibiting fungal spores and mycelia (Luna-Bulbarela et al., 2018). Detailed ESI-LC-MS/MS analysis of *Bacillus* strains also showed the production of a phospholipid- antibiotic, bacilysocin by all strains. Bacilysocin was characteristically reported from *B. subtilis* 168 and was highlighted for its potential competition against other microorganisms during spore germination (Tamehiro et al., 2002). However, genomic insights into the operational group *B. amyloliquefaciens* illustrate the presence of bacilysocin, iron-siderophore bacillibactin, and bacillaene polyene in several strains of *B. amyloliquefaciens*, *B. velezensis*, and *B. siamensis*,

characteristically produced by strains SB-1, A-2, and A-3 in this study (Fan et al., 2017).

Macrolactins produced by *Bacillus* spp. have been demonstrated for their efficient antibacterial properties and were recently found in reshaping the soil bacterial communities (Yuan et al., 2016). Production of macrolides A, E, and U was also noticed by the strains used in this study. *B. amyloliquefaciens* SB-1 showed the presence of all three macrolactin homologs whereas, *B. subtilis* A-2 showed the presence of two macrolactins; E and U. *B. tequilensis* A-3, however, showed the production of only macrolactin U which was further confirmed for its daughter ion fragmentation by MS/MS.

In addition to be efficient biocontrol agents, these strains also manifested to be strong biofertilizer candidates. When screening for plant growth-promoting traits, all strains produced IAA (0.067–0.147  $\mu$ M), lipase, cellulase, protease, and catecholate-based siderophore *in-vitro*. Several studies have shown the production these hydrolytic enzymes and siderophores by *Bacillus* spp. (Sayyed et al., 2004; Shaikh and Sayyed, 2014; Jadhav and Sayyed, 2016; Shaikh et al., 2018). Zinc and phosphorous solubilization was however exclusive to *B. amyloliquefaciens* SB-1 which solubilized  $23.2 \pm 0.21$   $\mu$ g/mL of insoluble phosphorus and zinc carbonate (SI =  $2.1 \pm 0.18$ ).

To be used as biofertilizers, the *in-vivo* impact of *Bacillus* spp. inoculation was also investigated in this study on the growth of wheat plants. Substantial increase in the overall wheat biomass, dry roots and shoots weight, and number of spikes was noted for the plants inoculated with *Bacillus* bio-inoculants as compared to un-inoculated controls. The most significant results were shown by *B. amyloliquefaciens* SB-1 as bioinoculant over un-inoculated control plants which caused  $\sim 42\%$  increase in the total biomass.

Conclusively, detailed physiological, genetic and metabolomic analyses of three *Bacillus* spp. strains explored their ability as biocontrol and biofertilizer agents. Comprehensive analysis of the secondary metabolites of these strains provided new insights about their adaptation to diverse environmental niches and their strain-specific metabolites. Among these, sugarcane endophyte *B. amyloliquefaciens* SB-1 showed ideal plant growth-promoting and biocontrol abilities and demonstrated all the attributes to be employed as user-friendly, single-strain inoculum. Presumably, its ability to solubilize phosphorous and zinc can convert more elements into the bioavailable forms

to stimulate plant growth and yield. Furthermore, in future, different consortia and single-strains bioinoculum of these plant growth stimulating *Bacillus* spp. can be used for other crops for the competitive suppression of phytopathogens and as biofertilizer agents.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

## AUTHOR CONTRIBUTIONS

IS performed experiments and wrote the manuscript. JH and CB conceptualized the idea. SH helped to prepare illustrations. KM provided workspace. SM edited the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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# Successful Plant Growth-Promoting Microbes: Inoculation Methods and Abiotic Factors

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Plant-microbe interactions have been the subject of several biotechnological studies, seeking sustainable development and environmental conservation. The inoculation of plant growth-promoting microbes (PGPM) in agricultural crops is considered an environmental-friendly alternative to chemical fertilization. Microbial inoculants are mainly inoculated onto seeds, roots and soil. PGPM improve plant growth by enhancing the availability of nutrients, the regulation of phytohormones, and by increasing plant tolerance against biotic and abiotic stresses. One of the main obstacles with PGPM research are the inconsistent results, which may be the result of inoculation methods and abiotic factors, such as soil (nutrient or heavy metal contents and pH), water availability, light intensity and temperature. This review addresses how the PGPM inoculants act on plant growth, what mechanisms they use to survive under stressful environmental conditions, and how inoculation methods and abiotic factors can interfere on the success of microbial inoculation in plants, serving as a basis for research on plants-microorganisms interaction.

**Keywords:** PGPM, PGPR, soil, light, pH, temperature, water

## INTRODUCTION

The growth rate of global population demands for increasing food production. However, in many situations, boosting agricultural productivity relies heavily on the use of chemical fertilizers, which are economically unavailable to many farmers throughout the world and can cause negative environmental impacts. In addition, environmental stresses may also be major constraints to plant growth and yield, causing low crop productivity, affecting global food security (Souza et al., 2015; Mimmo et al., 2018; Khan et al., 2019; Asghari et al., 2020). Therefore, to increase global agricultural production in a more economically and environmentally sustainable way, there is the need to use less chemical fertilizers and increase plant tolerance to abiotic stresses. The use of plant growth-promoting microbes (PGPM) is a potentially advantageous technique for improving crop productivity, food quality and security in more sustainable and eco-friendly agricultural systems (Souza et al., 2015; Abhilash et al., 2016; Mimmo et al., 2018; Asghari et al., 2020; Etesami, 2020).

Rhizosphere fungal and bacterial community can harbor beneficial organisms known as PGPM. These organisms have the ability to colonize plant roots providing benefits to their hosts, by modulating the production of phytohormones, increasing the availability of soil nutrients, and the resistance against pathogens. Besides, minimizing the use of chemical fertilizers, mitigating biotic

and abiotic stresses, and increasing plant production (Abhilash et al., 2016; Asghari et al., 2020; Etesami, 2020). The microorganisms used to increase agriculture productivity are *Azospirillum*, *Bacillus*, *Burkholderia*, *Enterobacter*, *Flavobacterium*, *Pseudomonas*, *Rhizobium*, *Frankia*, *Klebsiella*, *Clostridium*, *Trichoderma*, *Beauveria*, *Serratia* and *Streptomyces* (Abhilash et al., 2016; Oosten et al., 2017; Gouda et al., 2018).

PGPM act as biofertilizer, increasing the availability of nutrients, through bio-fixation of atmospheric nitrogen and solubilization of soil minerals, such as phosphorus and potassium. There are rhizobacteria that can facilitate the production of siderophores enhancing iron uptake (Bhat et al., 2019). They also directly promote plant growth as phytostimulator, influencing the phytohormones metabolism by enhancing auxin, cytokinins, abscisic acid, gibberellins production, and reduction of ethylene (Martínez-Viveros et al., 2010; Bhat et al., 2019; Khan et al., 2020). PGPM also act indirectly, as biopesticide or biocontrol agents increasing resistance against phytopathogens, through competition for nutrients, antagonism and induces systemic resistance (Abhilash et al., 2016; Bhat et al., 2019; Khan et al., 2020).

Considering all known factors that are involved in the PGPM activity, it is common to find numerous responses to inoculation with promising strains, which may be the result of the inoculation method and abiotic factors (Egamberdiyeva, 2007; Hernández-Montiel et al., 2017; Dutta and Bora, 2019; Fleming et al., 2019; Salwan et al., 2019; Etesami, 2020). To increase the success in the use of microbial biotechnology it is necessary to know how to improve plant-PGPM interaction, and how PGPM respond to changing environmental conditions, since terrestrial ecosystems are increasingly under anthropogenic influence. Therefore, this review addresses how the PGPM inoculants act on plant growth, what mechanisms they use to survive under stressful environmental conditions, and how inoculation methods and abiotic factors can interfere on the success of microbial inoculation on plant development.

## PGPM INOCULANTS ON PLANT GROWTH

Microbial inoculants combined or separate, can be inoculated into seed, leave, seedling roots, or soil. They colonize the rhizosphere or the interior of the plant, stimulating growth and plant tolerance against abiotic stresses. PGPM directly promote plant growth by enhancing the availability of nutrients, phytohormones regulation, and indirectly inducing systemic resistance (Abhilash et al., 2016; Bhat et al., 2019; Khan et al., 2020; Khoshru et al., 2020).

Under stress conditions plant growth is inhibited, mainly due to the increase in the production of reactive oxygen species, lipid peroxidation, accumulation of free radicals and high ethylene production, causing cell death. Hence, result in chlorosis, necrosis, leaf senescence, damage in photosynthesis apparatus, reduction in photosynthetic rates and chlorophyll content, and change in concentrations of metabolites. It also affects seed germination, seedling vigor, plant height, root development,

reduce the biomass and productivity of crop plants (Sharma et al., 2012; Khoshru et al., 2020).

On the other hand, beneficial microbes improve plant growth by enhancing the availability of nutrients, the regulation of phytohormones, and by increasing plant tolerance against biotic and abiotic stresses. Based on its effects mentioned above, PGPM increase the nutritional, auxin, gibberellin, cytokinin and ACC-deaminase concentrations. Beneficial microbes can also secrete volatile metabolites (VOC), which can induce disease resistance and abiotic stress tolerance. In addition, PGPM can also mitigate stress by increasing exopolysaccharides, osmoregulants and antioxidants, and reducing the oxidative stress (Varma et al., 2019; Khan et al., 2020; Khoshru et al., 2020). Thus, PGPM promote the increase foliage and leaf area, chlorophyll content, photosynthetic rates, seed germination, seedling vigor, plant height, root development, and biomass production (Figure 1).

## Nutrients

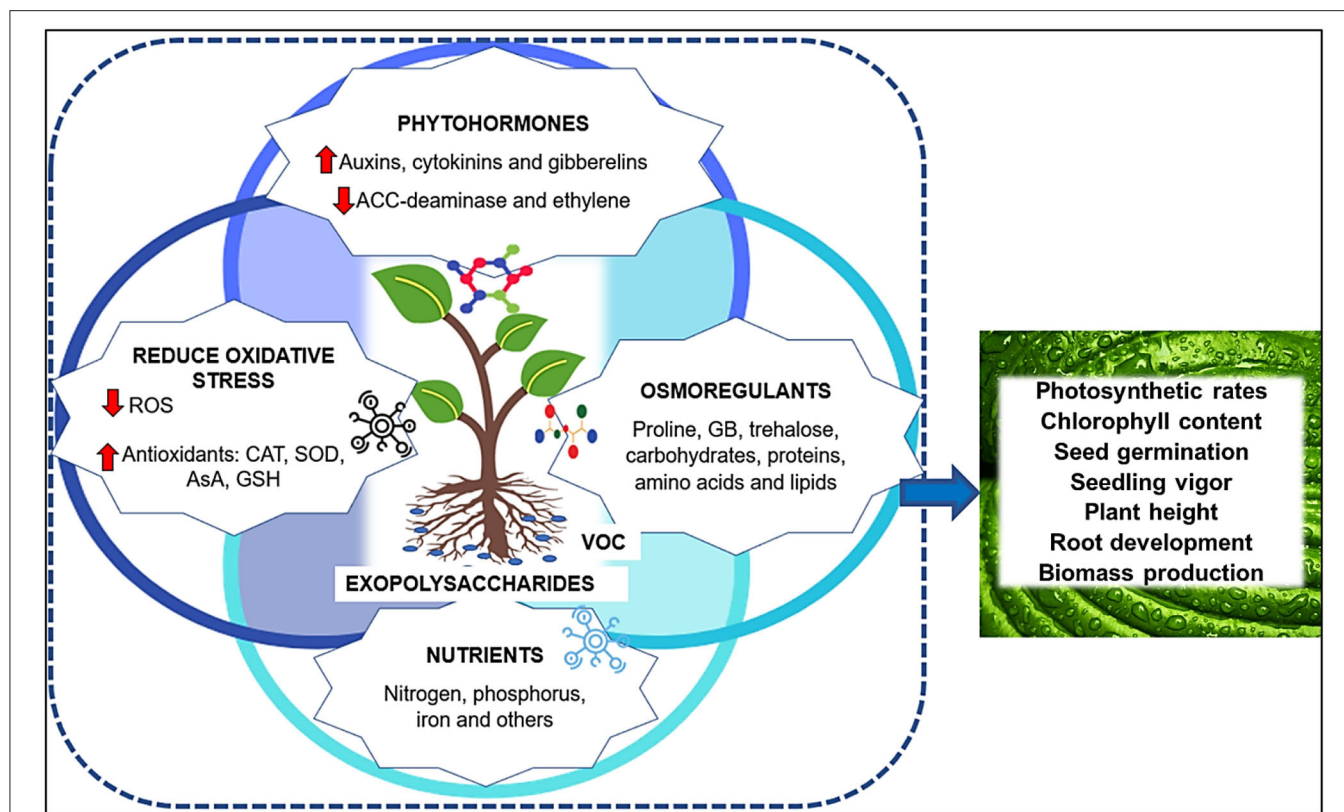
PGPM has been studied as biofertilizer which could enhance the supply of macro and micronutrients, promote plant growth and reduce the need of chemical fertilization. Nitrogen, phosphorus and iron are essential nutrients for plants. Hence, in PGPM selection test, the nitrogen fixation, phosphate solubilization and siderophore production capacity are usually investigated.

Nitrogen is an essential macronutrient for synthesis of proteins and nucleic acids. The microbes viz., *Azospirillum*, *Azotobacter*, *Achromobacter*, *Bradyrhizobium*, *Beijerinckia*, *Rhizobium*, *Clostridium*, *Klebsiella*, *Anabaena*, *Nostoc*, *Frankia* are biological nitrogen fixers through reduction of nitrogen gas ( $N_2$ ) to ammonia ( $NH_3$ ) (Souza et al., 2015; Bhat et al., 2019).

Phosphorus is an essential macronutrient for production of phospholipids, adenosine triphosphate (ATP), and increase the photosynthesis. Nonetheless, a large proportion of P in the soil is in insoluble forms, making it unavailable for the plants. PGPM changes the pH of the soil to solubilize inorganic phosphates. In alkaline soils, PGPM reduces pH by excretion of organic acids, such as gluconate, citrate, lactate and succinate, solubilizing  $Ca_3(PO_4)_2$ . In acid soils, PGPM increases the pH by production of protons, during the assimilation of ammonium ( $NH_4^+$ ), solubilizing  $AlPO_4$  and  $FePO_4$  (Martínez-Viveros et al., 2010). *Bacillus*, *Pseudomonas*, *Rhizobium*, *Achromobacter*, *Burkholderia*, *Micrococcus*, *Agrobacterium*, *Erwinia* sp., *Penicillium* sp. and *Aspergillus* sp. are capable of solubilizing inorganic phosphorus, transforming into forms capable of being absorbed by plants, such as monobasic ( $H_2PO_4^-$ ) or dibasic phosphate ( $HPO_4^{2-}$ ).

Iron is a micronutrient required to chlorophyll biosynthesis, photosynthesis and respiration. *Burkholderia*, *Enterobacter*, *Grimontella* and *Pseudomonas* are siderophore producers. Siderophores are chelator agents, with high specificity for binding iron, followed by the transportation and deposition of  $Fe^{3+}$  within bacterial cells. In this way, the excretion of siderophores improve plant nutrition and inhibit phytopathogens through iron sequestration from the environment (Souza et al., 2015; Varma et al., 2019).

Sulfur is an essential macronutrient found in cysteine and methionine. These amino acids are important in maintaining of enzymes and protein synthesis. Cysteine is important in



**FIGURE 1 |** Beneficial microbes improve plant growth by enhancing the availability of nutrients, the regulation of phytohormones, and increasing plant tolerance against stresses. PGPM act as biofertilizer, increasing macro and micronutrient availability. They increase the concentrations of auxin, gibberellin, cytokinin, ACC-deaminase, and reduce ethylene levels. Beneficial microbes also produce volatile metabolites (VOC), that may induce disease resistance and abiotic stress tolerance. In addition, PGPM mitigate stress by increasing exopolysaccharides, osmoregulators (such as glycinebetaine—GB), and antioxidants (including catalase—CAT, superoxide dismutase—SOD, ascorbate—AsA and glutathione—GSH), reducing the reactive oxygen species—ROS and oxidative stress. Thus, PGPM promote the increase of leaf area, chlorophyll content, photosynthetic rates, seed germination, seedling vigor, plant height, root development and biomass production of plants.

cell division, and methionine is a precursor of ethylene, responsible for fruit ripening (Taiz and Zeiger, 2017). *Bacillus* are producers of volatile compounds, such as dimethyl disulfide that provides sulfur for plants. In addition, *Bacillus* and *Aspergillus* produces organic and inorganic acids, acidolysis, chelation and exchange reactions which are capable of solubilize potassium (Varma et al., 2019).

## Phytohormones

Phytohormones are organic compounds responsible for the development of plants. There are PGPM capable of modulating phytohormones. The effects of stress on plants are mitigated by microbial inoculants, through the production of auxin, cytokinin, gibberellin, ACC deaminase, abscisic acid, jasmonates, brassinosteroids, and strigolactones (Saravanakumar, 2012; Oosten et al., 2017; Arora et al., 2020; Khan et al., 2020).

Auxin and ACC-deaminase are usually investigated in PGPM selection tests. This is because, auxin produced by microorganisms will increase auxin in the plant, and promote plant growth by enhancing nutrient and water uptake. Microbial auxins also beneficial in the regulation of cell division, shoot

growth, differentiation of vascular tissue, adventitious and lateral root, elongation and surface area of root. ACC-deaminase produced by microorganisms is a beneficial enzyme for reducing ethylene levels, mitigating stress in plants. High ethylene levels cause leaf chlorosis, necrosis, senescence, reduction in fruit yield, root development, leaf expansion, and photosynthesis (Souza et al., 2015). *Pseudomonas* sp. and *Bacillus* promote plants growth by increase auxin and ACC-deaminase (Samaddar et al., 2019; Danish et al., 2020; Khoshru et al., 2020).

PGPM can also promote plant growth by increasing gibberellin, improving seed germination, and the development of stem, leaves, flower and fruit. In addition, PGPM-induced cytokinin result in increased roots development, activity of vascular cambium, cell differentiation, and apical dominance (Gouda et al., 2018; Khan et al., 2020). Under stress conditions, PGPM inoculants are able to increase abscisic acid, jasmonates and brassinosteroids concentrations in plant. Under drought, cytokinin increase the abscisic acid, causing stomatal closure to reduce foliar water loss (Arora et al., 2020). Under drought or low temperature, jasmonates and brassinosteroids increase  $Ca^{2+}$  concentration in plant, acting intracellularly as secondary



messenger under stress conditions (Oosten et al., 2017; Gouda et al., 2018; Khan et al., 2020).

## Exopolysaccharides

Microbes are known to produce exopolysaccharides, forming a protective biofilm on root surface. This mechanism enhances water retention in soil particles and maintaining soil moisture in the root zone. In this way, it protects root cell against osmotic and ionic stress, regulating osmotic balance, under changing pH, saline stress, drought and temperature extremes.

To mitigate stress PGPM produce exopolysaccharides. This mechanism acts to stabilize the soil ionic balance, immobilizing  $\text{Na}^+$  under salinity stress. Exopolysaccharides are produced by *Bacillus* to increase its antimicrobial activity in the soil (Hashem et al., 2019).

## Antioxidants

PGPM inoculants also promote plant growth and tolerance to abiotic stresses by increasing antioxidants levels, reducing reactive oxygen species (ROS) and oxidative stress. Temperature, pH, heavy metal, water availability and UV-B radiation cause disruption of cellular homeostasis, increasing of reactive oxygen species, such as superoxide anion, hydroxyl radical, hydrogen peroxide and singlet oxygen. High concentrations of ROS is a primary result of abiotic stress, and are extremely harmful, causing oxidative stress in cell. Moreover, in chloroplasts, mitochondria, and peroxisomes, ROS induce oxidative damage to lipids, proteins, nucleic acid, enzyme inhibition and activation of programmed cell death (Sharma et al., 2012; Khoshru et al., 2020).

Microbial inoculants reduce the damaging effects of ROS, thus securing the cell, membranes, and biomolecules by increasing the production of antioxidants such as catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (AsA), glutathione (GSH), carotenoids, tocopherols and phenolics (Gouda et al., 2018; Arora et al., 2020). Environmental stresses cause either reduction of CAT activity and increase the hydrogen peroxide ( $\text{H}_2\text{O}_2$ ).

Catalase is also produced by microbes. Although it is relatively simple to examine whether microbes are catalase producers, this investigation is usually not performed on screening tests (Romeiro, 2007; Varma et al., 2019). The production of catalase by microorganisms must be carried out in screening tests, mainly with the objective of increasing the plant tolerance against abiotic stresses. This enzyme is efficient in  $\text{H}_2\text{O}_2$  degradation, reducing ROS and oxidative stress, consequently increase the plant tolerance to abiotic stress. This is the simple analyses in PGPM, which should be routine in the selection tests.

## Osmoregulators

Under stress conditions, microbial inoculants induce production of osmoregulators, such as carbohydrates, proteins, amino acids, lipids, proline, glycinebetaine, and trehalose (Oosten et al., 2017; Gouda et al., 2018; Khoshru et al., 2020). Thus, osmoregulation maintains the homeostasis, preventing membrane plasmolysis, increasing synthesis of heat shock proteins (HSPs) and regulating biological enzymatic mechanisms (Oosten et al., 2017; Khoshru

et al., 2020). Under salinity, osmoregulators stabilize the osmotic balance across the membrane, maintain the turgor pressure, and ensure the correct folding of proteins (Sharma et al., 2012; Khoshru et al., 2020). *Burkholderia* sp. increases plant tolerance against low temperature by modifying carbohydrate metabolism (Fernandez et al., 2012). Under water stress, *Pseudomonas fluorescens* promote plant tolerance by increasing the activity of catalase and peroxidase, and the accumulation of proline (Saravanakumar et al., 2011). Beneficial microbes improve tolerance in plants, increasing the accumulation of osmolytes in the plant cell cytoplasm. This maintains the cell turgor and contributes to improved stress tolerance in plants (Khoshru et al., 2020). The osmoregulation mechanism is important for plants to survive and improve tolerance under extreme conditions by reducing cellular damage caused by abiotic stress (Fernandez et al., 2012; Khoshru et al., 2020).

## FACTORS THAT AFFECT THE SUCCESS OF MICROBIAL INOCULATION

Soil represents a heterogeneous environment that allows the development of many microorganisms, which are in continuous interaction with other species, under conditions of symbiosis, antagonism, mutualism, parasitism and as saprophytes (Gouda et al., 2018; Bhat et al., 2019). In the soil, PGPM inhabits the rhizosphere zone, playing a symbiotic mutualistic relationship with plants (Gouda et al., 2018).

The knowledge on physiological characteristics of the microbial inoculant and the host plant is essential to decide the best inoculation method (Strigul and Kravchenko, 2006; Souza et al., 2015; Etesami, 2020). Successful microbial inoculation depends on inoculation method, inoculum density, root colonization, which varies with multiplication and distribution microbes through the rhizosphere, microbial antagonism, plant physiological state, soil humidity, pH, temperature, host, and root exudates (Venturi and Keel, 2016; Hernández-Montiel et al., 2017; Msimbira and Smith, 2020). Microbial inoculants are placed mainly into seeds (Romeiro, 2007; Souza et al., 2015; Arora et al., 2020). However, exudate compounds vary with plant genotype and age, and are determinants for microorganism colonization. Therefore, the effects of different inoculation methods should be evaluated, as it influences the PGPM colonization and the success in promoting plant growth (Romeiro, 2007; Souza et al., 2015; Hernández-Montiel et al., 2017; Arora et al., 2020).

Abiotic conditions, such as soil nutrients, pH, heavy metals, drought, flood, light intensity, and temperature can change the rhizosphere, affecting the survival, diversity of microbes, and PGPM potential for improving plant growth and health (Souza et al., 2015; Venturi and Keel, 2016; Hernández-Montiel et al., 2017; Mahmood et al., 2019; Etesami, 2020).

Temperature, water availability and light intensity can modify the soil compositions, structure and moisture, C and N transformation, metabolic processes and microbial survival. The soil fertilizer, composition, moisture and organic matter modify pH, thus influence the nutrients availability and mineral

toxicity, including iron and aluminum. Soil surface exposure to sunlight, more specifically gamma rays and ultraviolet light can cause mutations in the DNA of microorganisms or kill them (Mahmood et al., 2019; Varma et al., 2019; Etesami, 2020).

Soil conditions modify the microbiome diversity. In acidic soils, fungal activities are favored, while in alkaline soils are dominated by bacteria. Fungi disperse more intensively in the soil, while bacteria have access to smaller pore spaces only. Low soil moisture is detrimental mainly to bacteria, because they are single-celled organisms. On the other hand, fungal spores are more resistant, and survive in inactive states. High soil moisture, as in flooding environments, reduces oxygen, consequently reduces the redox potential of the soil and the mineralization of organic matter, resulting in lower biodiversity of microorganisms (Gouda et al., 2018; Etesami, 2020). Therefore, the changes in plant metabolism, composition of root exudates, and rhizosphere environment caused by abiotic factors, can negatively affect inoculation and necessities the reinoculation of microbes through soil drench.

## PGPM MECHANISMS TO SURVIVE IN DIVERSE CONDITIONS

The beneficial PGPM-plant interaction requires that the microorganisms are able to use root exudates for colonizing roots, quickly proliferate, compete with the native microbiota and adapt to environmental changes to mitigate abiotic stresses in plants (Souza et al., 2015; Hernández-Montiel et al., 2017; Mimmo et al., 2018; Msimbira and Smith, 2020).

To survive under stressful environments and promoting plant growth, the microbes developed several mechanisms. Some survive in low and high temperatures (psychrophiles and thermophiles), saline conditions (halophiles), and in acid and alkaline conditions (acidophiles and alkaliphiles) (Romeiro, 2007; Souza et al., 2015; Khoshru et al., 2020). Cell wall modification, alteration in metabolic responses and gene expression are also mechanisms against environmental stress (Sharma et al., 2012; Khoshru et al., 2020).

Quorum sensing is a communication system that enable the host colonization and the survival of microbe under stress conditions. It involves intercellular signaling and the regulating of microbe population. Some microorganisms, as *Bacillus*, secrete volatile metabolites (VOC), such as alkyl sulfides, indole, and terpenes. VOC can facilitate signaling across, microbial interactions by distributing easily through pores in the soil (Hashem et al., 2019; Varma et al., 2019).

Under low soil moisture, microbes accumulate amino acids, reducing their water potential, avoiding dehydration and death. Arbuscular mycorrhizal fungi modify the rhizosphere by amassing glomalin protein, increase the absorptive surface area and water holding capacity (Varma et al., 2019).

Under high light intensity, some microorganisms, such as *Bacillus* and *Serratia*, have pigmented that filter radiation and prevent DNA damage (Moeller et al., 2005; Zion et al., 2006; Varma et al., 2019). In pH extremes, microbes use proton transfer systems in their cytoplasm to maintain osmotic balance,

control metabolic activities, and their cellular vitality. Some microbes, such as *Azospirillum*, *Pseudomonas*, and *Bacillus* can influence soil micronutrient availability by solubilization, chelation, oxidation reduction reactions, and alter soil pH acidifying their surroundings, and inhibiting other microbes (Souza et al., 2015; Abhilash et al., 2016; Oosten et al., 2017).

## INOCULATION METHODS FOR THE SUCCESS OF PLANT GROWTH-PROMOTING MICROBES

Inoculation methods introduce PGPM to host plant and influence the establishment and persistence of microorganism populations in the rhizosphere and their growth promoting effects (Strigul and Kravchenko, 2006; Hernández-Montiel et al., 2017). Inoculation should be carried out as close as possible to the rhizosphere, as there is a certain inability of microorganisms to move from the inoculation site to the rhizosphere. This is because microorganisms are not usually very mobile in the soil and, therefore, it has been suggested that nematodes are vectors for spreading microorganisms around the rhizosphere (Strigul and Kravchenko, 2006; Hernández-Montiel et al., 2017).

Besides inoculum density and inoculation method, plant response to PGPM inoculation depends on root colonization, which varies with microorganism's multiplication and distribution through the rhizosphere, microbial antagonism, soil humidity, pH, temperature, host, root exudates, as well as the plant physiological state (Venturi and Keel, 2016; Hernández-Montiel et al., 2017; Msimbira and Smith, 2020). After inoculation, the decrease in microorganism population, may be related to difficulties in adapting to their new environment. However, root exudates play a significant role in microorganisms' growth. Several biotic and abiotic factors influence the structural and functional diversity of microorganism communities. In this way, it is necessary to evaluate and select microorganisms from site-specific plant associations. The objective is to optimize the inoculant for applications in plant production. The physiological characteristics of the inoculant organism determine to a great extent its fate and activity in the soil (Strigul and Kravchenko, 2006; Souza et al., 2015; Etesami, 2020).

Inoculation can be performed with a single isolate or with more than one, called co-inoculation. In co-inoculation, microorganisms interact synergistically, increasing the efficiency of inoculation, resulting in improved plant development. Lopes et al. (2018a) in an assay comparing individual inoculation and co-inoculation of *Pseudomonas fluorescens* and *Burkholderia pyrrocinia*, found that these rhizobacteria when co-inoculated, stimulated root development, and increased growth and productivity of the tropical forage grass *Brachiaria brizantha*. Co-inoculation of PGPM also increased growth and quality in *Triticum* spp. (Upadhyay et al., 2011), *Glycine max* L. (Bakhshandeh et al., 2020), *Capsicum annuum* L. (Samaddar et al., 2019) and *Mentha pulegium* L. (Asghari et al., 2020).

Positive results obtained with co-inoculations, reinforce the importance of additional research to elucidate the interactions between microorganisms, envisioning the production of

mixed inoculants, as an alternative of greater success of microbial biotechnology.

For the inoculation of plants with beneficial microorganisms, different techniques are being employed including seed, root, soil, and foliar inoculation (Figure 2; Table 1). The foliar inoculation is the least used, while seed inoculation are the most used methods (Romeiro, 2007; Souza et al., 2015; Arora et al., 2020). Nevertheless, variation in the composition and quantity of root exudates during plant growth and also environmental stresses, can influence the success of the microbe inoculants. It is therefore necessary to test different inoculation methods in the screening tests.

## Seed Inoculation

Seed inoculation method with PGPM is an alternative to chemical seed treatments. It consists in immersing the seed in a microorganism solution of known concentration (Romeiro, 2007; Lopes et al., 2018a). The seed germination process releases abundant carbohydrates and amino acids in the form of seed exudates (Ahemad and Kibret, 2014). In this way, these organisms introduced together with the inoculated seeds in the soil use the exudates as a nutritional source and colonize roots, as soon as they emerge (Ammor et al., 2008).

Interaction of PGPM with the plant roots modulate the level of phytohormones that are produced by plants. Phytohormones are organic compounds that modulate plant growth and are also capable of inducing tolerance in plants against various biotic and abiotic stresses (Khan et al., 2020). Microorganisms colonize plant tissues and synthesize phytohormones, such as gibberellin that improve germination (Bhat et al., 2019). PGPM is also capable of producing antimicrobial compounds that protect seeds against phytopathogens that cause seed rotting (Souza et al., 2015).

Seeds inoculation with *Rhizobial* and *Bacillus* sp. increased biomass production of *Oryza sativa* (Ullah et al., 2017)

and *Cicer arietinum* L. (Khan et al., 2019), respectively. Seed inoculation with mycorrhizal fungi and plant growth-promoting rhizobacteria was more effective in promoting growth and wood production in *Schizolobium parahyba*, as compared to the seedling inoculation (Cely et al., 2016). In seed inoculation method, the inoculum remains dormant in the soil, until activated by the growing root tips. Under field conditions, it is often necessary to reinoculated to maintain effective cell densities (Martínez-Viveros et al., 2010). Seed inoculation method with *Burkholderia phytofirmans* has also been successfully used in phytoremediation of organic pollutants such as hydrocarbons (Afzal et al., 2013). *Pseudomonas fluorescens* was also beneficial when inoculated in the seed for increasing the vigor, biomass and resistance to water stress of *Vigna radiata* (Saravanakumar et al., 2011).

## Root Inoculation

Root inoculation method consists of immersing roots in a microorganism solution (Romeiro, 2007). After inoculation, the seedling is planted on a proper substrate for its development. This method allows plant size standardization, as inoculation can be carried out on seedling of similar sizes. Another advantage of this inoculation method is that the inoculum is placed directly in contact with the host roots, improving root colonization (Ahemad and Kibret, 2014). This method can be preferentially used in plant species with asexual propagation, as PGPM have the ability to synthesize growth phytohormones such as auxin, that besides promoting plant growth, can also counteract phytopathogens that compromise plant survival after planting (Ahemad and Kibret, 2014; Gouda et al., 2018).

Root inoculation with *Burkholderia* sp. increased *Vitis vinifera* tolerance to low temperature, modified carbohydrate metabolism and increased plant growth and yield (Fernandez et al., 2012). In *Oryza sativa*, root inoculation with *Rhizobial* was more effective in increasing plant height and panicle length, as compared to the

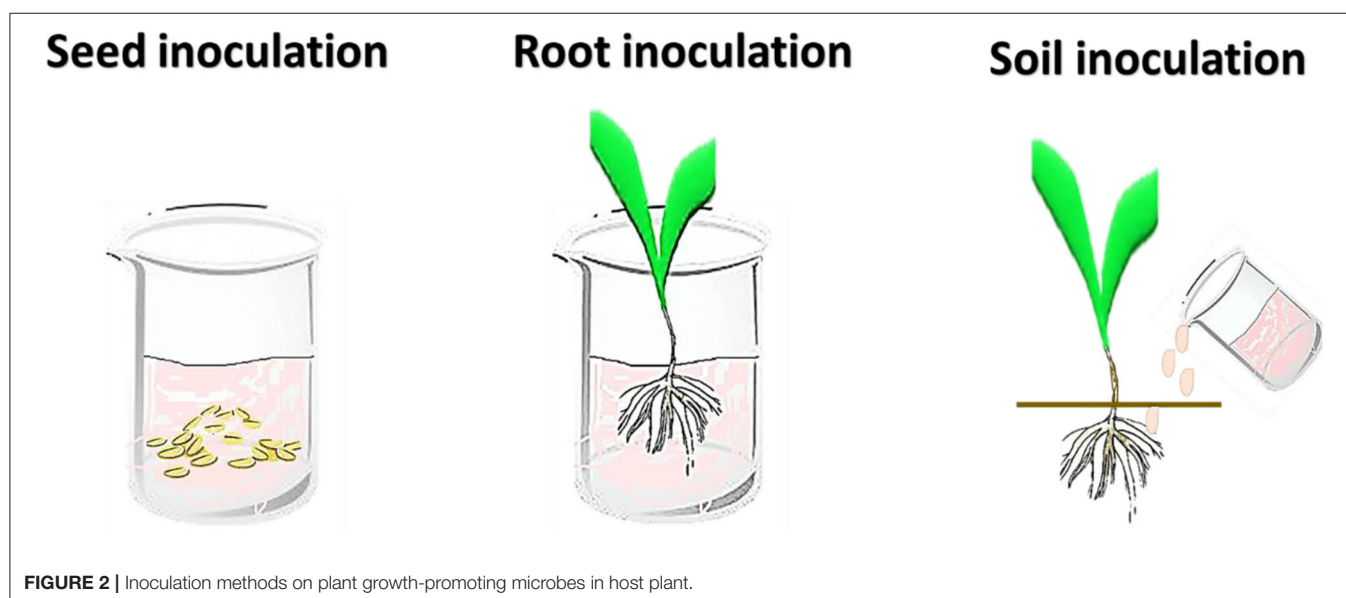


FIGURE 2 | Inoculation methods on plant growth-promoting microbes in host plant.

**TABLE 1** | Effect inoculation methods on plant growth-promoting microbes (PGPM) potential benefit.

PGPM	Plant	Inoculation	Effect inoculation	References
<i>Burkholderia phytofirmans</i>	<i>Ryegrass</i>	Seed Root Soil	Soil inoculation method was found to be the most efficient in improving plant biomass production, hydrocarbon degradation and phytoremediation	Afzal et al., 2013
<i>Pseudomonas</i> sp.	<i>Cicer arietinum</i>	Seed Soil	Soil inoculation method was more efficient to improve plant growth	Bhattacharjya and Chandra, 2013
<i>Pseudomonas putida</i>	<i>Lycopersicon esculentum</i>	Soil—microcapsules and liquid medium	Microcapsules inoculation on plants increased growth and yield	Hernández-Montiel et al., 2017
<i>Rhizobial</i>	<i>Oryza sativa</i>	Seed Root	Seed inoculation method was more efficient to increase plant growth	Ullah et al., 2017
<i>Pseudomonas Burkholderia</i>	<i>Brachiaria brizantha</i>	Seed Soil	Soil inoculation method was more efficient to increase plant growth	Lopes et al., 2018a

seed inoculation method (Ullah et al., 2017). These results also prove that inoculation method can influence the beneficial effect of microorganism in promoting plant growth.

## Soil Inoculation

Soil inoculation method consists of introducing PGPM directly into the soil, by drenching, soil incorporation (mixed in the substrate) or microcapsules (Romeiro, 2007; Hernández-Montiel et al., 2017; Prisa, 2020). In soil drenching, a microorganism solution is added as close as possible to the host roots (Romeiro, 2007; Lopes et al., 2018a). This is necessary because it is in the rhizosphere that the PGPM will be able to perform several critical functions for promoting plant development, such as phosphate solubilization, synthesis of siderophores and phytohormones (Gouda et al., 2018).

Inoculation of the forage grass (*Brachiaria brizantha*) with *Burkholderia pyrrhocina* and *Pseudomonas fluorescens* was not successful when carried out on seeds, but promote growth when inoculated by soil drenching 14 days after seedling emergence. This is because allelochemicals with negative allelopathic effects in these PGPM have been reduced over the growth stages of *B. brizantha* (Lopes et al., 2018a). In *Cicer arietinum* L., the soil inoculation of *Bacillus* resulted in better nodulation and growth than when inoculated on seeds (Bhattacharjya and Chandra, 2013).

Plant growth promoting rhizobacteria inoculated by soil incorporation, improved *Ranunculus asiaticus* growth, increasing the efficiency of nutrient and water absorption by roots (Prisa, 2020). *Burkholderia phytofirmans* was more efficient in improving *Lolium multiflorum* biomass production, when inoculated in soil. When inoculated in seeds, root or leaves, the indigenous microbiota made it difficult for inoculated bacteria to colonize successfully and promote plant growth (Afzal et al., 2013).

In *Lycopersicon esculentum*, growth and productivity were increased by soil inoculation with *Pseudomonas putida* delivered in microcapsules. According to Hernández-Montiel et al. (2017), soil inoculation with microcapsules offered greater protection and viability, since the release was gradual, improving adhesion, stability, and colonization of roots by PGPM.

## ABIOTIC FACTORS ON THE SUCCESS OF PLANT GROWTH-PROMOTING MICROBES

Microbial activity in the soil is influenced by plant roots, soil structure and particle size, mineral composition and agricultural practices (Doornbos et al., 2012; Hartman and Tringe, 2019). Due to the production of root exudates, most microorganisms are accumulated in the rhizosphere. The rhizosphere is the region of the soil connected to plant roots, where compounds are exuded by plant roots to attract organisms. These compounds, can be beneficial, neutral, or harmful to plants (Doornbos et al., 2012; Souza et al., 2015; Bhat et al., 2019; Mahmood et al., 2019). Host plant uses root exudation compounds to select specific microbes in its rhizosphere microflora, establishing plant species-specific rhizosphere communities (Doornbos et al., 2012; Hartman and Tringe, 2019). Root exudation also determines which organisms will produce mucilage in the root system, reducing the roots peeling and improving the contact between the roots and the soil solution (Doornbos et al., 2012; Venturi and Keel, 2016; Gouda et al., 2018).

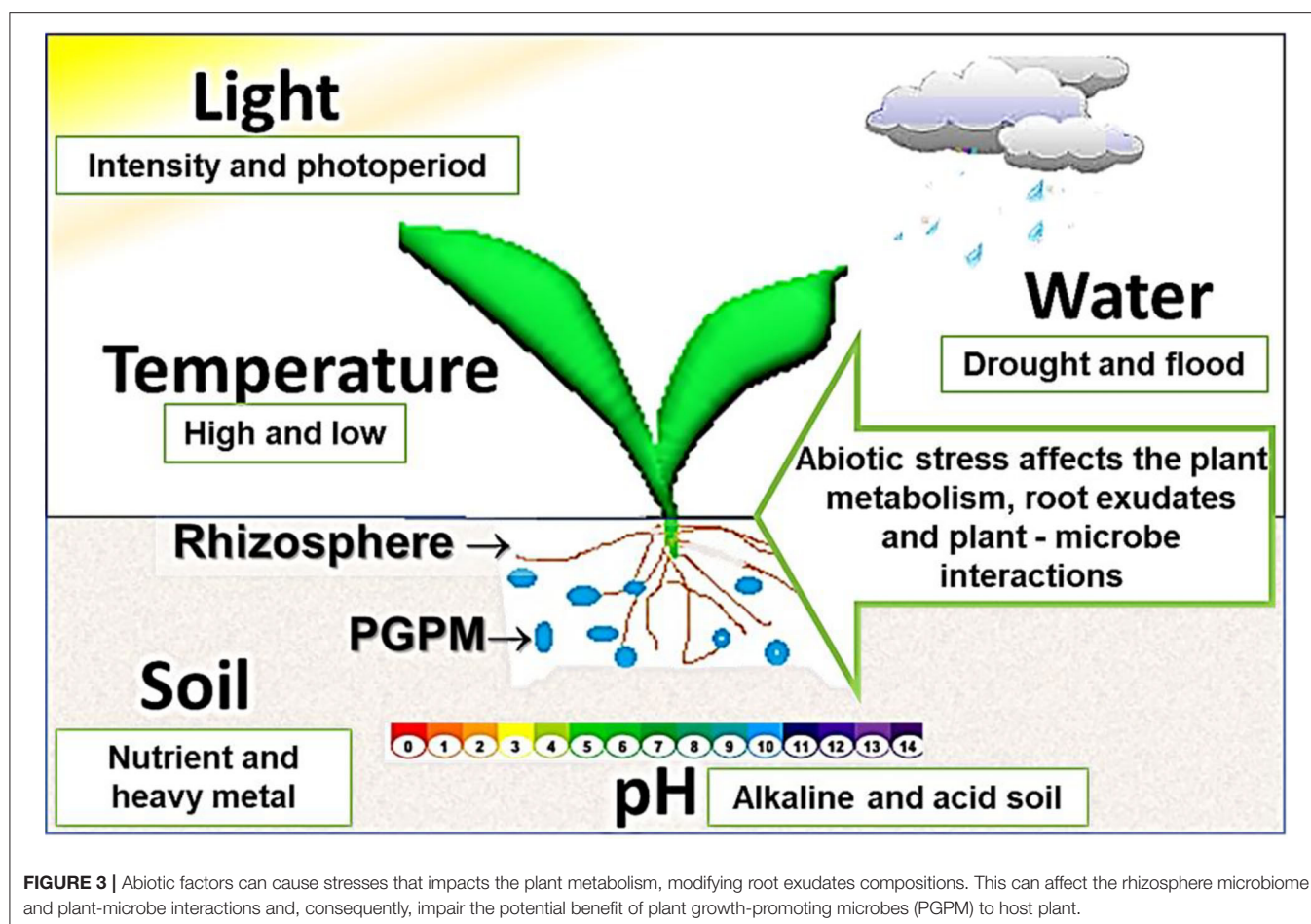
Abiotic factors can cause stress that interfere with plant metabolism, modifying root exudates compositions. This can affect the rhizosphere microbiome and plant-microbe interactions and, consequently, impair the potential benefit of PGPM to host plant (Figure 3). Abiotic stresses are responsible for most of the major losses in crop productivity. This is a serious problem to global food security. Utilization of PGPM may be an eco-friendly, sustainable, and cost-effective approach to overcome abiotic stresses in plants (Table 2).

However, if there is a change in the exudative pattern of the plant, the same isolate and the same plant genotype may interact differently. Such changes can lead to genetic changes in microorganisms causing then to lose the ability to colonize the rhizosphere and, consequently, their PGPM potential (Oosten et al., 2017; Enebe and Babalola, 2018; Hartman and Tringe, 2019).

## Soil

Soil pH is key for the solubility of different metal ions, nutrient availability and soil physical properties (Dutta and Bora, 2019;





**FIGURE 3 |** Abiotic factors can cause stresses that impacts the plant metabolism, modifying root exudates compositions. This can affect the rhizosphere microbiome and plant-microbe interactions and, consequently, impair the potential benefit of plant growth-promoting microbes (PGPM) to host plant.

Msimbira and Smith, 2020). High or low soil pH is a worldwide problem to agricultural productivity (Dutta and Bora, 2019; Salwan et al., 2019; Zerrouk et al., 2019). Salinity of agriculture soils is also a serious constraint to plant growth. This stress condition causes nutrient deficiency, ion toxicity, osmotic and oxidative stress, reducing yield of agricultural crops (Dutta and Bora, 2019; Salwan et al., 2019).

Under salinity conditions or in alkaline soils, the high pH, affects the bioavailability of nutrients, causing osmotic stress, nutrient deficiency and increase in the reactive oxygen species production (Dutta and Bora, 2019; Salwan et al., 2019). In acidic soils, the low pH and high aluminum ions concentration cause toxicity and formation of phosphoric acid complexes, which makes phosphorus unavailable to plants (Dutta and Bora, 2019; Zerrouk et al., 2019).

The pH range 5.5–6.5 is optimal for plant growth and increasing production of root exudates to microbes. Bacteria are favored by neutral pH, but the fungi are favored by acidic pH conditions (Msimbira and Smith, 2020). Therefore, pH interferes in plant metabolism, which can also disrupt biological activities, inhibiting the microorganisms that inhabit the rhizosphere (Salwan et al., 2019). Saline stress inhibits seed germination, causes stomata closure affects seedling growth, the onset of

flowering and fruiting set (Enebe and Babalola, 2018; Salwan et al., 2019). Aluminum toxicity reduces cell division, root growth, nutrient absorption, and phenolic metabolite production and increases reactive oxygen species production (Zerrouk et al., 2019; Msimbira and Smith, 2020).

Plant beneficial microorganism inoculation is a suitable strategy to improve plant tolerance to pH extremes, as reported to *Bacillus* and *Trichoderma* in *Glycine max* (Bakhshandeh et al., 2020), and *Rhizobium* and *Paenibacillus* increase pH tolerance in *Triticum* (El-Sayed and Hagab, 2020). *Pseudomonas* promote growth and increases tolerance to high salinity conditions in *Capsicum annuum* (Samaddar et al., 2019) and in *Zea mays* under salt and aluminum toxicity (Zerrouk et al., 2019) (Table 2).

Soil nutritional condition can also affect the PGPM efficiency. In soils with high nutrient profile there can be absence of root colonization; this is due to microorganism displacement to regions more abundant in nutrients (Egamberdiyeva, 2007; Bhat et al., 2019). Mathematical modeling indicates that PGPM inoculation is more efficient in nutrient poor soils, or stressed soils, because the development of the resident microflora is inhibited (Strigul and Kravchenko, 2006). Inoculation with *Pseudomonas*, *Bacillus*, and *Mycobacterium* are often more effective in promoting plant growth in

**TABLE 2 |** Abiotic factors on beneficial plant-microbe interactions.

PGPM	Plant	Inoculation	Abiotic factor	Effect	References
<i>Pseudomonas alcaligenes</i> , <i>Bacillus polymyxa</i> and <i>Mycobacterium phlei</i>	<i>Zea mays</i>	Seed	Soil	Increase plant growth and nutrient uptake more in nutrient-deficient soil than in fertile soil	Egamberdiyeva, 2007
<i>Pseudomonas aeruginosa</i> , <i>Alcaligenes faecalis</i> and <i>Bacillus subtilis</i>	<i>Brassica juncea</i>	Seed	Soil	Increase plant growth, metal tolerant and phytoextraction efficiency	Ndeddy Aka and Babalola, 2016
PGPR (CM2)	<i>Ranunculus asiaticus</i>	Soil	Soil	Improve plant growth, increasing the efficiency of root nutrient and water absorption	Prisa, 2020
<i>Pseudomonas</i>	<i>Cajanus cajan</i> and <i>Eleusine coracana</i>	Seed	Soil	Increase growth and grain yield in nutrient-deficient soil	Mathimaran et al., 2020
<i>Bacillus</i> sp. <i>Arthrobacter</i> sp.	<i>Triticum</i> spp.	Soil	Soil	Increase growth and high pH tolerance by activity of antioxidant enzymes	Upadhyay et al., 2011
<i>Pseudomonas</i> sp.	<i>Capsicum annuum</i>	Seed	Soil	Increase growth by increase ACC deaminase and reduce ethylene under salinity stress	Samaddar et al., 2019
<i>Pseudomonas</i> sp.	<i>Zea mays</i>	Seed	Soil	Phytostimulation and tolerance to salt and aluminum toxicity by increase ACC deaminase and IAA	Zerrouk et al., 2019
<i>Bacillus</i> sp. <i>Trichoderma</i> sp.	<i>Glycine max</i>	Seed	Soil	Improve germination, growth, potassium uptake under drought and salt stress	Bakhshandeh et al., 2020
<i>Rhizobium</i> sp. <i>Paenibacillus</i> sp.	<i>Triticum</i> spp.	Seed	Soil	Increase growth, yield, biochemical contents and high pH tolerance	El-Sayed and Hagab, 2020
<i>Klebsiella variicola</i>	<i>Glycine max</i>	Soil	Water	Improve plant growth and flood tolerance by inducing adventitious root	Kim et al., 2017
<i>Azotobacter chroococcum</i> and <i>Azospirillum brasilense</i>	<i>Mentha pulegium</i> L.	Seed	Water	Improve physiological, phytochemical parameters and drought tolerance	Asghari et al., 2020
<i>Pseudomonas</i> sp. <i>Azotobacter</i> sp.	<i>Cymbopogon citratus</i>	Soil	Water	Increase biomass, antioxidant potential and drought tolerance	Mirzaei et al., 2020
<i>Azospirillum</i> sp.	<i>Zea mays</i>	Seed	Water	Increase growth, drought and flood stress tolerance	Czarnes et al., 2020
<i>Achromobacter</i> sp. <i>Enterobacter</i> sp., <i>Leclercia</i> sp. <i>Pseudomonas</i> sp.	<i>Zea mays</i>	Seed	Water	Increase growth, ACC deaminase, nutrients concentrations and drought stress tolerance	Danish et al., 2020
<i>Pseudomonas fluorescens</i>	<i>Vigna radiata</i>	Seed	Water	Increase vigor, biomass, activity of catalase and peroxidase, accumulation of proline and water stress tolerance	Saravanakumar et al., 2011
<i>Bacillus</i> sp.	<i>Solanum lycopersicum</i> and <i>Capsicum</i> sp	Seed	Light	Increase growth in long-days	Kloepper et al., 2007
<i>Glomus</i> sp. <i>Paraglomus</i> sp. <i>Rhizophagus</i> sp. <i>Rhizobium</i>	<i>Phaseolus lunatus</i>	Seed	Light	Under high light, PGPM increase growth and seed production, but inhibited under shade	Ballhorn et al., 2016
<i>Burkholderia</i> sp. <i>Pseudomonas</i> sp.	<i>Brachiaria brizantha</i>	Soil	Light	Increase plant growth and shade tolerance	Lopes et al., 2018b
<i>Kaistobacter</i> sp. <i>Pseudomonas</i> sp.	<i>Ophiopogon japonicus</i>	Soil	Light	Increase plant growth and shade tolerance	Fu et al., 2020
<i>Pseudomonas</i> sp.	<i>Triticum</i> sp.	Seed	Temperature	Increase plant growth and high temperatures tolerance	Ali et al., 2011

(Continued)

TABLE 2 | Continued

PGPM	Plant	Inoculation	Abiotic factor	Effect	References
<i>Burkholderia</i> sp.	<i>Vitis vinifera</i>	Root	Temperature	Increase low temperatures tolerance, by modification of carbohydrate metabolism	Fernandez et al., 2012
<i>Arthrobacter</i> , <i>Flavimonas</i> , <i>Flavobacterium</i> , <i>Massilia</i> , <i>Pedobacter</i> <i>Pseudomonas</i>	<i>Solanum lycopersicum</i>	Seed	Temperature	Increase germination, plant growth and low temperature tolerance	Subramanian et al., 2016
<i>Bacillus</i> sp.	<i>Solanum lycopersicum</i>	Seed	Temperature	Increase plant growth and high temperature tolerance	Mukhtar et al., 2020

nutrient-deficient soils (Egamberdiyeva, 2007; Mathimaran et al., 2020).

Heavy metal contamination in soils is toxic to most organisms and can also inhibit the effectiveness of inoculants. Heavy metals reduce soil fertility, affect the rhizosphere microbial community, plant photosynthetic efficiency, causes nutrient imbalance, and reduce yields (Mimmo et al., 2018). Beneficial microorganisms, such as *Pseudomonas aeruginosa*, *Alcaligenes faecalis*, and *Bacillus subtilis* are an effective remediation strategy in contaminated soils, increasing plant tolerance to heavy metals, as reported in *Brassica juncea* (Ndeddy Aka and Babalola, 2016).

Soil affects inoculation efficiency and influences the rhizosphere microflora (Egamberdiyeva, 2007; Souza et al., 2015). After inoculation, cell numbers will undergo a rapid decline, especially on unsterilized soils. In autoclaved soils, because there is no competition with other microorganisms, inoculants remain in high cell densities for many weeks. In nonsterile soils, because there is great competition with the resident soil microbiome and predation by protozoa and nematodes, inoculants populations will decline rapidly, until the population reach an equilibrium (Martínez-Viveros et al., 2010; Varma et al., 2019). For this reason, research work screening PGPM must be carried out in nonsterile soils, since the possibility of testing the competition efficiency of the inoculated microorganisms against those already native to the soil will be greater.

## Water

Rainfall is the major water source for growing crops in many parts of the world (Enebe and Babalola, 2018; Danish et al., 2020). Water availability, lack (drought) or excess (flood), can result in abiotic stress, limiting crop production (Enebe and Babalola, 2018; Ipek et al., 2019; Danish et al., 2020). PGPM can improve plant tolerance to drought stress (Fleming et al., 2019; Asghari et al., 2020), as *Azotobacter chroococcum* and *Azospirillum brasilense* in *Mentha pulegium* L. (Asghari et al., 2020), and *Pseudomonas* sp. and *Azotobacter* sp. in *Cymbopogon citratus* (Mirzaei et al., 2020) and *Zea mays* (Danish et al., 2020). *Klebsiella variicola* and *Azospirillum* sp. can improve flooding stress tolerance by adaptations, such as the formation

of adventitious roots resulting from endogenous hormonal regulation, as reported in *Glycine max* (Kim et al., 2017) and *Zea mays* (Czarnes et al., 2020).

However, water stress can influence the plant-microbe interactions. Drought increases soil temperature, which can inhibit multiplication of beneficial microorganisms. Flooding reduces O<sub>2</sub> availability in soil, restricting microorganisms that are not capable of anaerobic respiration (Enebe and Babalola, 2018; Hartman and Tringe, 2019; Ipek et al., 2019). In addition, drought and flooding are stress that affect plant metabolism and photoassimilates production, interfering with production and composition of root exudates (Enebe and Babalola, 2018; Fleming et al., 2019; Hartman and Tringe, 2019; Danish et al., 2020). This will affect plant-microorganism interaction, which may inhibit the microorganism potential to promote plant growth. For this reason, it is important to know where the target plant species is usually adapted. Considering climate change, it is important to study how the excess and the lack of water would affect the interaction between plants and PGPM.

## Light

Light intensity influences plant metabolism, growth and production (Venturi and Keel, 2016). Light can interfere in plant-microorganisms interaction by modifying the amount and the chemical composition of root exudates (Venturi and Keel, 2016; Lopes et al., 2018b). Microbial inoculation demands carbohydrate allocation in exchange of nutrients delivered to plants. Beneficial microorganism inoculation can increase plant growth under limited light conditions, by increasing shade tolerance, as *Kaistobacter* sp. and *Pseudomonas* sp. in *Ophiopogon japonicus* and *Lolium perenne* (Fu et al., 2020).

However, under light-limited conditions (i.e., shade) the microbial root symbionts can create additive costs, inhibiting plant growth, as *Glomus* sp. *Paraglomus* sp. *Rhizophagus* sp. and *Rhizobium* in *Phaseolus lunatus* (Ballhorn et al., 2016). Aguilar-Chama and Guevara (2016) describe that a mycorrhizal inoculation had a positive effect on stem mass, root mass, and leaf nitrogen content in *Datura stramonium*, but only when light was not a limiting factor. This is because, under limited light conditions, photosynthesis is reduced, consequently,

carbohydrate production is also reduced, turning mutualistic microbes into parasites (Ballhorn et al., 2016).

As previously explained, during screening for PGPM, light conditions commonly experienced by the targeted plant species must be taken into account. This is because light intensity can interfere with the PGPM efficiency. For example, *Brachiaria brizantha* is a tropical forage grass grown either under full sun, or under moderate shade, as when cultivated in silvipastoral systems. Therefore, Lopes et al. (2018b) report that they tested the efficiency of rhizobacteria in promoting growth of this forage grass under both full sun and moderate shade. According to Lopes et al. (2018b), the PGPM efficiency varied with the type of microorganism and light intensity. When the bacteria were inoculated individually, plants under full sun showed the highest growth with *Pseudomonas fluorescens*, while under moderate shade *Burkholderia pyrrocinia* was more efficient in promoting plant growth (Lopes et al., 2018b).

## Temperature

High and low temperatures potentially caused by climatic change may become a major threat to global agriculture, reducing crop production, with drastic economic results (Ipek et al., 2019; Mukhtar et al., 2020). Beneficial microorganism inoculation is efficient in enhancing plant growth and mitigating adverse stresses caused by extreme temperature, as *Pseudomonas putida* in *Triticum* sp. (Ali et al., 2011) and *Bacillus cereus* in *Solanum lycopersicum* (Mukhtar et al., 2020) under high temperature. Fernandez et al. (2012) related that under low temperature *Burkholderia* sp. increased tolerance to low temperature by modification of carbohydrate metabolism and increased plant yield in *Vitis vinifera*.

Extreme temperatures are recognized stress in agriculture, reducing seed germination, seedling growth, yield and altering plant metabolism (Ipek et al., 2019; Mukhtar et al., 2020). Temperature impacts morphological, biochemical and physiological attributes of plants, interfering with plant-PGPM interaction by changing root exudation composition (Ali et al., 2011; Meena et al., 2015; Ipek et al., 2019). Therefore, for PGPM to be able to withstand environmental transformations that crop plants are exposed, it is necessary to isolate these microorganisms from different rhizosphere environments, under diverse environmental conditions, such as prevalent high and low temperatures (Etesami, 2020). This is because rhizobacteria that persist under change temperatures have the ability of improving plant growth and productivity on these adverse environmental conditions (Meena et al., 2015).

## CONCLUSION AND FUTURE PERSPECTIVES

The use of PGPM inoculants is a potential tool to increase plant growth and crop yields in a more environmentally sustainable way, by reducing the need of chemical inputs and providing tolerance against abiotic stresses. The reviewed literature has shown that inoculation method and abiotic factors, associated

climatic conditions, are essential for the success of the interaction between PGPM and plants.

Inoculation methods have a great effect on the establishment and persistence of microorganism in the rhizosphere and on their growth-promoting effects on host plants. Decision on which inoculation method to use must be based on knowledge of the plant growth stages, if they produce the allelochemicals with negative allelopathic effects, and its morphological characteristics. For species, that exude more allelopathic compounds in germination, and these compounds are reduced throughout the development of the plant, the ideal inoculation method would be in the root or soil. On the other hand, in species with pivoting roots, the cell wall of the roots is denser, this could hinder the adhesion and colonization of PGPM. In these species, the ideal inoculation method would be in seeds.

Climatic changes influence the abiotic factors, resulting in plant stresses, besides affecting the success of its interaction with PGPM. Abiotic factors such as soil (nutrient, heavy metal, pH, and salinity), water availability, light intensity, and temperature can influence the plant-microbe interactions, because they alter plant metabolism, root exudates composition and rhizosphere biology. This is because, if there is a change in the exudative pattern of the plant, the same isolate and the same plant genotype may interact differently.

In screening studies, it is recommended to use non-sterile soil, test different inoculation methods and different abiotic stresses, and even select microorganisms from plants under abiotic stress. Therefore, in screening experiments for the selection of PGPM it is necessary to know about the environmental requirements of the target plant and the environmental conditions on which it will be cultivated. In addition, as the PGPM depend on the exudates provided by the plant, this causes a specific relationship, so, the ideal scenario is using, in selection tests, microorganisms from the rhizosphere site-specific plant associations.

In this respect, it is worth noting that in addition to auxin, ACC-deaminase, nitrogen fixation capacity, phosphate solubilization, and siderophore production, we suggest that the ability of the microorganisms to produce catalase should also be considered in screening tests. Microbial production of catalase is a simple test, low-cost and indicates the potential of the microorganism to reduce oxidative stress in the plant.

The future perspectives of microbial applications must include the improvement of screening techniques, such as the quantification of antioxidant enzymes and others that can benefit plant development. Also, screening tests should be carried out under different temperatures, soil nutrient, water and pH. In addition, it is important to evaluate the ability of PGPM to promote plant growth under contrasting light intensities. Future studies must include improved methods for inoculation, detailing the rhizosphere microbiome of each species studied, and the interaction between plants and PGPM. In the future, it is expected the development of software to indicate the ideal PGPM to benefit a specific plant species, the best inoculation method and its action under different abiotic factors. Thus, there will be a more efficient selection of microorganisms, resulting in increased



plant growth, leading to agricultural sustainability and environmental preservation.

## AUTHOR CONTRIBUTIONS

ML designed the project. ML, MD-F, and EG wrote the manuscript. ML and MD-F edited the manuscript.

All authors contributed to the article and approved the submitted version.

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# Microbial-Based Technologies for Improving Smallholder Agriculture in the Ecuadorian Andes: Current Situation, Challenges, and Prospects

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As in other Andean countries, smallholder agriculture is the base that supports food and nutrient security in Ecuador. Ironically, in spite of their importance, the development of context-appropriate technologies for these farming systems remains still at its infancy. Today, most smallholders in the Ecuadorian Andes practice a type of hybrid agriculture that merges traditional local practices and modern technologies. This mixture of traditional and “modern” conventional technologies does not always result in resource-efficient sustainable practices. Although they represent only part of a global solution, microbial-based technologies offer a great potential to improve the functioning of smallholder farms in the Ecuadorian Andes. From nutrient cycling to biocontrol and plant growth promotion, microbial technology applications match existing needs for technology development in these systems; however, as in many cases, knowledge gaps and context-adapted implementation are some of the challenges that slow down the spreading and efficient use of these technologies. Here we offer a review of the efforts made as of today to characterize, develop and test microbial-based technologies that could boost smallholder Andean agriculture with a particular focus in the Ecuadorian context. We also propose potential lines of action to increase or accelerate the impact of these technologies.

**Keywords:** family farming, development, sustainability, microbes, mountain, biodiversity

## INTRODUCTION

Smallholder farming plays a pivotal in the economy and, more importantly, the food security of Ecuador. However, in spite of its relevance, this sector has not received enough attention in terms of developing technological innovations that could improve their sustainability, of which, microbial technologies are among the most promising due to the high diversity of microbial resources in Ecuador. Furthermore, several microbe-based technologies have already been adopted by some smallholders, offering a platform from where to launch new innovations. Nonetheless, Ecuadorian smallholders are a very heterogeneous group, which precludes the proposal of one-size-fits-all innovations demanding instead solutions tailored to each biophysical and social context that will require a better characterization of existing microbial diversity. In this minireview, we have focused our attention in the group that Berdegue and Escobar (2002) define as “subsistence

and transitional smallholder farms” since innovations in these farms show the greatest potential to generate life-changing impacts (Berdegué and Fuentealba, 2011). Furthermore, we have focused this review in subsistence and smallholder farms in the mountainous region of the country, since they represent the majority of smallholders in Ecuador and grow a greater diversity of products intended for internal consumption, thus having a significant contribution to the country’s food and nutrient security.

## SMALLHOLDER FARMING IN THE ECUADORIAN ANDES: SETTING THE CONTEXT

Due to the extreme diversity of smallholdings (Figure 1), a simple definition of these system is unrealistic; however, a common feature of these systems in Latin America, as proposed by Berdegué and Fuentealba (2011) is the strong reliance of these systems on family administration and labor, alongside a relative small size for their local context, in other words, the definition of “smallholder farming” overlaps considerably that of “family farming” in the Latin American context. Based on the endowment of assets of smallholder farms, Berdegué and Escobar (2002) classified these farms three different groups, namely, subsistence-, transitional- and consolidated smallholder farms. This classification is practical and useful to describe the actual diversity of smallholdings in the Ecuadorian context. Using this classification, and data from the last agricultural census in Ecuador, ~88% (739,952) of all farms in Ecuador could be considered as smallholder farms (Soto Baquero et al., 2007). Most of these farms (58%) were located in the Andean region, with almost 99% of them falling within the category of transitional (~33%) or subsistence (~66%) farms. Succinctly, the main characteristic of these two types of farm is that both show some level of limitation in terms of their resource base (e.g., soils, topography, and weather) that affects their productivity, being this limitation more marked in subsistence farms. It is noteworthy to mention that, even though subsistence farms have been the focus of many agricultural development programs; it is actually transitional farms, the ones that show greater potential for agricultural development programs to boost sustainable livelihoods and inclusive economic growth (Berdegué and Fuentealba, 2011).

The production of smallholdings in the Ecuadorian Andes consist primarily on vegetables, fruits, roots/tuber crops, grains, pulses, and pastures (Soto Baquero et al., 2007). Typically, these farms are diversified systems in which horticultural, agronomic, forest, and medicinal species are co-cultivated. The diversity in some of these farms is so rich, that they contribute significantly to the conservation of agrobiodiversity (Wong and Ludeña, 2006; Oyarzun et al., 2013). Nonetheless, it must be noted, that often this agrobiodiversity is largely composed of many introduced species, with native crops occupying only a small share of the cultivated area in these farms (Oyarzun et al., 2013). Furthermore, space is preferentially allocated to a few dominant, commercial crops such as potatoes, pulses, or corn,

with much less space devoted to traditional or native crops (Oyarzun et al., 2013; Córdova et al., 2018). Smallholder vegetable farms are commonly managed intensively using polycultures (3–4 simultaneous crops) with almost no fallow periods (Zea et al., 2020). Some smallholder farms consist almost exclusively of passively ventilated greenhouses, with even more intense management and greater dependence on external inputs.

Geographically, smallholder agriculture is distributed along production belts of different altitudes, with fruit and vegetable farms located near the bottom of valleys, and grain, pulses and tuber/root crops farms located at the highest altitudes up to 3,800 as described by Harden (1988) for the Ambato river basin, an agriculturally important basin in Ecuador. In terms of technology, smallholder farms are highly heterogeneous, although a syncretism of traditional and “modern” technology is a common feature in most cases. The adoption of “modern” production technologies is represented primarily by the use of synthetic inputs (fertilizers, pesticides) and mechanization. Interestingly, a revival of the use of traditional, and introduction of alternative technologies has been apparent in recent years.

## BIOPROSPECTING POTENTIALLY USEFUL MICROBES FOR MOUNTAIN AGRICULTURE

In the Andean context, several native crops and soils have been prospected for useful plant-growth promoting microorganisms (PGPM) (Table 1). Among those, potato is -by far- the most studied. This tuber has been grown for millennia by Andean people and is the most important staple crop in the region. Unfortunately, although in recent decades chemical fertilizers and pesticides have been applied to counteract the effect of unfavorable soil conditions and fungal pathogens, potato production yields remain low in the region (Aubron et al., 2009). In view of this situation, several quests have been conducted to identify potato-beneficial microbes, of which, perhaps the most important have been those conducted by the International Potato Center and the VALORAM Consortium (Oswald et al., 2007, 2010; Vélez et al., 2008; Calvo et al., 2009, 2010; Oswald and Calvo, 2009; Calvo and Zúñiga, 2010; Ghyselinck et al., 2013; Velivelli et al., 2015).

For instance, a study conducted by Velivelli et al. (2015) in Ecuador, showed that inoculation of potato fields with native soil bacteria significantly raised yields of potato by increasing the number of tubers per plant. Some strains identified as *Pseudomonas palleroniana*, *Bacillus* sp., *Paenibacillus* sp., and *Bacillus simplex* also showed antagonism against *Rhizoctonia solani* *in vitro*, even though this phenotype could not be clearly related to the incidence or severity of disease symptoms on tubers.

Another two studies conducted in the Ecuadorian Andes by members of the VALORAM consortium evaluated the inoculation with *Rhizophagus irregularis*, an arbuscular micorrhizal fungus widely used as a biofertilizer (Berruti et al., 2014), on potato. In the first of these studies, Loján et al. (2016) found that several rhizobacteria isolated from the





**FIGURE 1 |** Diversity in smallholder farms in the Ecuadorian Andes. Typical landscapes of regions where smallholder farming is practiced: **(A,B)** Farms at high elevations (potato, grains, pulses, pastures, other tuber/root crops), panel **(B)** shows a typical rotation consisting in pasture (background), potato, fallow and fava beans (foreground), **(C)** Farms in lower mountain slopes and valleys (vegetables, fruits, greenhouse horticulture), **(D)** Urban and periurban farms (vegetables). Panels **(E–I)** show representative subsistence **(E)** and transitional **(F)** systems, greenhouse **(G)** and diversified agroecological **(H)** systems and the combination of traditional (oxen plow) and modern technologies (greenhouses) in some of these farms **(I)**.

potato rhizosphere behaved as antagonists to the establishment of *R. irregularis* mycorrhizal associations with potato, although one isolate (namely *Pseudomonas plecoglossicida* R-67094) promoted *R. irregularis* growth, during the pre-symbiotic phase of the fungus. The same strain, entrapped within alginate beads, behaved as a mycorrhiza-helper bacteria, inducing *R. irregularis* sporulation while improving potato root colonization. In the second study (Loján et al., 2017), four commercial products containing *R. irregularis* were inoculated in potato under field; however, none of them had any effect on potato

yields, reportedly due to poor establishment of the AMF in the rhizosphere of potato plants.

Other native Andean tuber crops (ATC) have also been prospected for rhizosphere-associated microbes. Recently, Chica et al. (2019) used high throughput sequence analysis of 16S rRNA genes to describe the bacterial diversity of rhizosphere soils associated to oca (*Oxalis tuberosa*), ullucu (*Ullucus tuberosus*), and mashua (*Tropaeolum tuberosum*). Unfortunately, this study was not followed by plant-growth promotion assays.

**TABLE 1** | Studies reporting results of the prospection and evaluation of microbial technologies in the Ecuadorian Andes.

Crop	Microorganisms inoculated or treatment used	Results reported	Reference
<b>Potato</b>	Native <i>Beauveria</i> sp. and <i>Metarhizium anisopliae</i> isolated from potato fields	Biocontrol of <i>Premnotypes vorax</i>	Barriga, 2003
	<i>Beauveria bassiana</i>	Biocontrol of <i>Premnotrypex vorax</i>	Guapi, 2012
	Baculovirus strain JLZ9f and <i>Bacillus thuringiensis</i> subsp. <i>Kurstaki</i>	Biocontrol of <i>Tecia solanivora</i> , <i>Phthorimaea operculella</i> and <i>Symmetrischema tangolias</i>	Suquillo et al., 2012
	Native <i>Pseudomonas palleroniana</i> , <i>Bacillus</i> sp., <i>Paenibacillus</i> sp., <i>Bacillus simplex</i> isolated from soil	Increased yield per plant and antagonism against <i>R. solani</i> <i>in vitro</i>	Velivelli et al., 2015
	Native <i>Pseudomonas plecoglossicida</i> and <i>Rhizophagus irregularis</i> (AMF) from the Glomeromycota <i>in vitro</i> collection (Belgium)	Promotion of <i>R. irregularis</i> growth, sporulation, and colonization in potato roots	Loján et al., 2016
	<i>R. irregularis</i> (commercial inoculant)	Not different than controls	Loján et al., 2017
	Digestates	Increased yield and tuber weight	Guerrero, 2017
	Digestates and phosphate-solubilizing bacteria	Increased yield per plant	Flores, 2019
<b>Tamarillo</b>	Native AMF	Increased seedling growth, improved acclimation and protection against <i>M. incognita</i>	Espín et al., 2010
	Native <i>Pseudomonas fluorescens</i> and AMF	Antagonism against <i>M. incognita</i> , <i>M. java</i> and <i>M. hapla</i> ; increased root and shoot development	Orrico et al., 2013
	Native AMF and <i>Pseudomonas putida</i>	Antagonism against <i>Meloydogine</i> spp. and plant growth promotion	Ramírez et al., 2013
	Native AMF and <i>P. fluorescens</i>	Plant growth promotion and increased N and P absorption	Echeverría et al., 2013
<b>Common bean</b>	Native <i>Rhizobium</i> spp.	Increased nodulation, biomass increase	Granda Mora et al., 2016
	34 <i>Rhizobium</i> spp. isolates	Increased nodulation	Torres-Gutiérrez et al., 2017
<b>Oca, ullucu, mashua</b>	Rhizobacteria	Rhizobacteria diversity	Chica et al., 2019
<b>Corn</b>	<i>Beauveria</i> sp. and <i>Metarhizium</i> sp. strains	Biocontrol of <i>Macrodactylus</i> sp. in the field	Ayala, 2006
<b>Blackberry</b>	<i>Trichoderma asperellum</i>	Growth promotion, increased fruit weight and yield	Viera et al., 2019
<b>Strawberry</b>	Consortia of microorganisms	Increased root growth and leaves per plant	Alvarez et al., 2018; Alvarez-Vera et al., 2018, 2019
<b>Pepper</b>	Digestates	Not different than controls	Cobo, 2012
<b>Broccoli</b>	Digestates	Higher nutrient absorption	Manosalvas, 2012
<b>Avocado</b>	<i>Trichoderma harzianum</i> and <i>Glomus iranicum</i> var. <i>Tenuihypharum</i>	Plant growth promotion and improved nutrient absorption	Sotomayor et al., 2019
<b>In vitro assay</b>	Soil <i>Azospirillum</i> spp. isolates from corn fields	Isolate growth characterization	Sangoquiza Caiza et al., 2018

Another important crop, native to the Andean region is *Phaseolus vulgaris* L., the common bean (Tohme et al., 1995). This legume contributes minerals and vitamins to the human

diet, and also constitutes a major source of dietary protein (Sathe, 2002; Broughton et al., 2003). Since *P. vulgaris* is commonly associated to root-nodule forming bacteria (i.e., rhizobia), it

fixes atmospheric nitrogen biologically (López-Guerrero et al., 2012). In the Andean region, some taxonomic studies have been conducted by Ribeiro et al. (2013) allowed the discovery of three novel *Rhizobium* lineages in Ecuador, with one of them dominant in beans from this country. Further, the genetic diversity of *Agrobacterium* strains colonizing the nodules of this legume has been recently shown to be high, and it was proposed that members of this bacterial genus might contribute to plant growth (Delamuta et al., 2020).

Even though the symbiotic association between *Rhizobium* spp. and *Phaseolus* has been thoroughly studied in the past, there is still much to learn concerning the possibility of using these bacteria to promote growth and development of the legume. Toward this aim, Granda Mora et al. (2016) isolated six native strains of *Rhizobium* from *P. vulgaris* (cultivar Mantequilla) in Southern Ecuador, and showed their promoting effect on legume nodulation, biomass, nitrogen fixation, and symbiotic efficiency in a greenhouse experiment. These results encouraged the authors to propose the possibility of using these strains to develop bioinoculants for *P. vulgaris* bioinoculants. One year later, the same group isolated, characterized and identified 34 *Rhizobium* isolates from plants grown in Southern Ecuador (Torres-Gutiérrez et al., 2017). The strains belonged to nine species and were both phenotypically and genetically diverse; most of them promoted nodulation and nitrogen fixation, but the results were highly variable. Several strains did also produce high amounts of indolacetic acid, a well-known auxin involved in plant cell division/differentiation and vascular bundle formation (Theunis et al., 2004). Once again, the authors claimed on the utility of such native *Rhizobium* strains to develop biofertilizers, but insisted on conducting more trials.

Corn is a very important cereal grown in the Andean mountains. In 2018, Sangoquiza Caiza and et al. reported on some phenotypic and physiological characteristics of three *Azospirillum* strains isolated from the rhizosphere of corn plants grown at more than 2,000 m.a.s.l. in the Ecuadorian mountains. The authors claimed on the biotechnological utility of such strains, without presenting any further details.

In order to prospect for potentially useful PGPM for agricultural purposes, Alvarez et al. (2018), Alvarez-Vera et al. (2018, 2019) tried a different experimental approach. Instead of monitoring the promoting effect of one microbial isolate at a time, they assembled together several consortia of beneficial microorganisms, originally isolated from different organs (stems, leaves, and roots) of native plant species in Southern Ecuador. The list of species included coffee (*Coffea arabica* L.), plantain (*Musa paradisiaca* L.), chamomile (*Matricaria chamomilla* L.), mugwort (*Artemisia vulgaris*), and rue (*Ruta graveolens* L.), among several others. The plants—and their corresponding microbes—were grouped following the altitude at which they were grown. The microbial strains included yeasts (*Saccharomyces* sp., *Kloeckera* sp., and *Rhodotorula* sp.), bacilli and lactobacilli (*Bacillus subtilis/amyloliquefaciens*, *Lactobacillus delbrueckii*, and *L. plantarum*), and streptomycetes (*Streptomyces sanglieri*, *S. lushanensis*, *S. griseorubens*, *S. thermocarboxydus*, and *S. bungeensis*). Different consortia were prepared by combining isolates from each altitude, and inoculated to strawberries

(*Fragaria* sp.) grown in the field. The results were highly heterogeneous: whereas some consortia increased in a significant way the number of leaves per plant, as well their root growth, others did not produce any detectable effect. However, it became evident that the approach followed by the researchers was effective in isolating potentially useful PGPM.

Studies aimed at improving fruit tree growth are really scarce in the Andean context with only a few reports on Tamarillo (*Solanum betaceum*), commonly known as tree tomato. This species, native to the Andes, is grown nowadays worldwide in “exotic” countries like Australia, New Zealand, and India (Bohs, 1989; Carrillo-Perdomo et al., 2015). Tamarillo is a rich source of vitamins and organic acids, and is also consumed as a potent antioxidant (Vasco et al., 2009; Acosta-Quezada et al., 2015). The tree is usually grown in small orchards following traditional management systems; unfortunately, its productivity is frequently challenged by diseases like anthracnose and powdery mildew (Tamayo, 2001), but also by herbivore nematodes (Prohens and Nuez, 2000).

To fight against the deleterious effect of such pathogens, some Ecuadorian researchers focused on antagonistic bacterial and fungal species, native to these lands. For instance, native Ecuadorian isolates of arbuscular mycorrhizal fungi (AMF) were shown to promote Tamarillo plantlet development and acclimation, in addition to protecting them from *Meloidogyne incognita* infection (Espín et al., 2010). Further, Orrico et al. (2013) reported on the protection of Tamarillo tree by native *Pseudomonas fluorescens* strains and AMF. The strains, isolated from organically grown trees were mixed in a biopesticide formulation, and shown to antagonize *Meloidogyne incognita*, *M. java* and *M. hapla*, in addition of reducing the formation of root-knots in the trees. Similar results were published the same year by Ramírez et al. (2013) and by Echeverría et al. (2013), but this time by either combining native strains of both AMF and *Pseudomonas putida*, or native AMF and *P. fluorescens*, respectively.

## EXPERIENCES WITH MICROBIAL TECHNOLOGIES IN SMALLHOLDER ANDEAN AGRICULTURE

Adoption of microbial technologies among Ecuadorian smallholders is not widespread. Some of the probable causes for this are (i) the greater perceived convenience, habituation, and immediate effectiveness of synthetic inputs, (ii) mixed experiences with microbial technologies tested, (iii) more complex management skills required for their implementation, and (iv) the limited availability of microbial based products along with their price. In spite of their current limited adoption, interest in these technologies has been growing steadily aided by the work of government agencies, farmer's associations and NGOs. While adoption of microbial technologies by smallholders, as of today, has been focused mostly on a few well-known groups of microorganisms and microbe-derived products, a great potential for developing new microbe-based



technologies from local biodiversity remains high as this diversity has barely been explored (Castillo Carrillo, 2020).

As mentioned earlier, some smallholder farmers have already included several microbe-related technologies into their production systems. Unfortunately, evaluation of these experiences remain largely confined to “gray” literature or await independent validation. Smallholder access to microbe-based technologies comes through both commercial products and homemade preparations. A large catalog of commercial microbe-based products is available from local vendors. These products fall mainly in three categories: (i) biocontrol agents (e.g., *Trichoderma* spp., *Beauveria bassiana*, *Bacillus thuringiensis*, *Bacillus amyloliquefaciens*, *Bacillus subtilis*, other *Bacillus* spp., *Paecilomyces* spp., *Arthrobotrys* spp., *Lecanicillium* spp., *Pseudomonas fluorescens*, *Acremonium butyri*, *Metarhizium* spp.); (ii) plant growth promotion agents and digestates (e.g., products based on *Acalulospora* spp., *Glomus* spp., *Sclerocystis* spp., *Pseudomonas fluorescens*, *Bacillus amyloliquefaciens* or their extracts); and, (iii) biofertilizers (e.g., *Rhizobium* spp., *Azotobacter* spp., mycorrhizae, composts and different types of digestates). While many of these products are imported, several local companies have been producing their own microbial formulations for some time already (Castillo Carrillo, 2020). In spite of their easy availability, the price of these products has hampered their wider adoption by smallholder farmers.

In contrast, homemade microbial preparations have been more rapidly adopted due to their relative ease of implementation and almost ubiquitous training from different actors (i.e., NGOs, farmer’s associations, and government agencies). Of these preparations, manure digestates (known locally as “biols”), compost (including “bokashi compost”), and some inoculants (e.g., “effective microorganisms” and native biocontrol agents such as *Trichoderma* spp., *Beauveria* spp., and *Bacillus thuringiensis*) are the most commonly used. Some of the effects reported for these microbe-derived products are: growth promotion in blackberry and avocado by *Trichoderma* spp. (Sotomayor et al., 2019; Viera et al., 2019), biocontrol of *Tecia solanivora*, *Phthorimaea operculella*, *Symmetrischema tangolias* in potato based on *Bacillus thuringiensis* (Ayala, 2006; Suquillo et al., 2012), biocontrol of *Premnotrypes vorax* in potato and *Macrodactylus* sp. in corn based on *Beauveria brogniartii* and *Metarhizium anisopliae* (Barriga, 2003; Guapi, 2012), and growth promotion by digestates in several crops (Cobo, 2012; Manosalvas, 2012; Guerrero, 2017; Flores, 2019). However, replication and independent evaluation are common limitations in most of these reports.

## CURRENT CHALLENGES AND PERSPECTIVES IN THE ECUADORIAN CONTEXT

Microbial technologies offer great potential to increase the sustainability of smallholder agriculture in the tropical Andes

(Yarzabal and Chica, 2017); however, key challenges must be overcome. Perhaps, the main challenge could be finding a way to balance the reliability and proven effectiveness seen in commercial formulations, with the ease and self-sufficiency characteristics of homemade formulations and interventions. Merely focusing future work on new formulations of bioproducts does not seem to be compatible with the dynamic of smallholdings in the Ecuadorian Andes; plus, it risks falling in an input substitution trap that could threaten long term sustainability goals (Rosset and Altieri, 1997). On the other hand, continuation of the existing transfer model for homemade formulation based on a few, barely-characterized products, precludes building sound data-based foundations to support the development of effective and proven microbial technologies. Thus, future microbial technologies amenable for adoption in smallholder farms in Ecuador would need to be effective and as self-sustaining as possible. To do this, it will be necessary to shift the focus from producing specific biocontrol/biostimulant/biofertilizer agents to produce engineered microbial communities and to integrate these microbial formulations with production practices such a tillage, crop rotations, soil amendments, and the selection of less disruptive synthetic inputs. Ideally, these emerging microbial technologies would be based on the local biodiversity in order to avoid introduction of exotic microorganisms and to improve the chances of stabilizing these communities. For the Ecuadorian context, it would be key to increase country-wide collaborations from multiple disciplines to tackle these challenges. These collaborations would necessarily include not only researchers, but also farmers, development organizations and regulators. The engagement of the latter is of paramount importance, due to their role in designing processes that foster biodiscovery and innovations in microbial technologies while avoiding unsustainable, depleting, or unfair use of native microbial resources. Also, to facilitate visibility and attract new collaborations, more effort should also be put on finding better avenues for dissemination of the acquired knowledge, to minimize the amount of experiences that remain shaded in the gray literature. Combined, these efforts would allow to put forward the idea of ecological agroecosystems engineering as a way to increase the sustainability of smallholder farms and consequently protect the livelihoods of these families.

## AUTHOR CONTRIBUTIONS

Both authors have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Thuricin17 Production and Proteome Differences in *Bacillus thuringiensis* NEB17 Cell-Free Supernatant Under NaCl Stress

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*Bacillus thuringiensis* strain NEB17, produces a bacteriocin, thuricin17 (Th17) and is known to promote the growth more effectively under salt stress conditions. In this study, bacterial salt stress tolerance screening and the possible changes in its secretome under two levels of NaCl stress was evaluated. The salt tolerance screening suggested that the bacterium is able to grow and survive in up to 900 mM NaCl. Thuricin17 production at salt levels from 100 to 500 mM NaCl was quantified using High Performance Liquid Chromatography (HPLC). Salt stress adversely affected the production of Th17 at levels as low as 100 mM NaCl; and the production stopped at 500 mM NaCl, despite the bacterium thriving at these salt levels. Hence, a comparative proteomic study was conducted on the supernatant of the bacterium after 42 h of growth, when Th17 production peaked in the control culture, as determined by Liquid Chromatography - Tandem Mass Spectrometry (LC-MS/MS). Optimal (salt free) bacterial culture served as a control and 200 and 500 mM NaCl as stress conditions. As salt levels increased, the major enzyme classes, transferases, hydrolases, lyases, and ligases showed increased abundance as compared to the control, mostly related to molecular function mechanisms. Some of the notable up-regulated proteins in 500 mM NaCl stress conditions included an S-layer protein, chitin binding domain 3 protein, enterotoxins, phosphopentomutase, glucose 6-phosphate isomerase and bacterial translation initiation factor; while notable down-regulated proteins included hemolytic enterotoxin, phospholipase, sphingomyelinase C, cold shock DNA-binding protein family and alcohol dehydrogenase. These results indicate that, as the salt stress levels increase, the bacterium probably shuts down the production of Th17 and regulates its molecular functional mechanisms to overcome stress. This study indicates that end users have the option of using Th17 as a biostimulant or the live bacterial inoculum depending on the soil salt characteristics, for crop production. The mass spectrometry proteomics data have been deposited to **Mass Spectrometry Interactive Virtual Environment (MassIVE)** with the dataset identifier **PXD024069**, and doi: 10.25345/C5RB8T.

**Keywords:** *Bacillus thuringiensis* NEB17, Thuricin17, bacteriocin, NaCl stress, proteins

## INTRODUCTION

Terrestrial plants have been co-evolving in symbiotic association with microbes for more than half a billion years (Knack et al., 2015). This association has been extensively studied with respect to the rhizosphere, which is a signal exchange center for both plants and microbial establishment (Desbrosses and Stougaard, 2011). Global agriculture is profoundly affected by overuse of fertilizers and harmful pesticides, and the growing adverse effects of climate change. In order to develop climate-resilient agriculture systems, appropriate microbes have been a sustainable resource as biopesticides and biofertilizers. Bacterial inoculum or isolated signal compounds from plant-associated microbes have been used in this context and newer compounds with new modes of action are constantly being recognized (Lyu et al., 2020).

Bacteriocins are small peptides secreted by most families of bacteria, either of plasmid or chromosomal origin synthesized at various stages of bacterial growth and under various environmental conditions (Daw and Falkner, 1996). *Bacillus* species were first reported to produce bacteriocins in 1976 and the low-molecular-weight bacteriocins of the Gram-positive bacteria were reported to have bactericidal activity against certain closely related Gram-positive bacteria (Tagg et al., 1976). Based on their peptide characteristics, they are grouped into distinct classes and show substantial diversity in structure and function (Abriouel et al., 2011; Subramanian and Smith, 2015).

*Bacillus thuringiensis* NEB17, isolated from the rhizosphere of soybean plants at the Emile Lods Agronomy Research center of the Macdonald Campus, McGill University, produces a bacteriocin, thuricin17 (Th17), that has been purified and partially sequenced. Thuricin17 (Th17) was isolated from *Bacillus thuringiensis* NEB17 (*Bt* NEB17), a putative endophytic bacteria of soybean root nodules. It is a low molecular weight peptide of 3.162 kDa, and belongs to class IId of bacteriocins (Gray et al., 2006a,b; Lee et al., 2009). It has been reported to show functional similarities and anti-microbial activities with bacthuricin F4 (Jung et al., 2008a). The protein also has plant growth promoting activities and the bacteria when co-inoculated with *Bradyrhizobium japonicum* under nitrogen free conditions, improved soybean growth, nodulation and grain yield (Bai et al., 2002, 2003). Application of Th17 as a leaf spray and root drench, has positive effects on soybean and corn and stimulated plant growth. The leaves of 2-week-old soybean sprayed with Th17 showed increased activities of lignification-related and antioxidative enzymes and their isoforms suggesting that Th17 acts as an inducer of plant defense mechanisms (Jung et al., 2008a, 2011; Lee et al., 2009).

Recent research on Th17 has highlighted its plant growth promotion and abiotic stress alleviation properties. *A. thaliana* responded positively to treatment with Th17 in the presence of salt stress (up to 250 mM NaCl). At this level of salt, chloroplast proteins and proteins of photosystems I and II that are generally strongly and negatively affected by salt stress and PEP carboxylase, Rubisco-oxygenase large subunit, and pyruvate kinase, were some of the noteworthy proteins enhanced by Th17 application, along with other stress related

proteins (Subramanian et al., 2016b). Canola cultivar 04C111 however did not respond favorably to Th17. Seeds treated with  $10^{-11}$  M Th17 were negatively affected under high temperature stress under greenhouse conditions and the plants from this treatment produced fewer seeds than the untreated controls (Schwinghamer et al., 2016). Application of Th17, under water stress conditions, to 1-month-old soybean plants increased plant biomass by 17%, root biomass by 37% and root nodule biomass by 55%, and also the amount of abscisic acid in soybean roots by 30% (Prudent et al., 2014). Application of Th17 to soybean seeds (variety Absolute RR) caused accelerated seed germination under salt stress of up to 150 mM NaCl, with the best response seen at 100 mM NaCl. The up-regulation of PEP carboxylase and a marked down-regulation of  $\alpha$ - and  $\beta$ -subunits of conglycinin, glycinin, as compared to the control treatment, is indicative of efficient storage protein utilization in conjunction with thioredoxin; and in organ maturation and transition from one stage to another in a plant's lifecycle (Subramanian et al., 2016a). While Th17 increased soybean seed germination at both 15 and 22°C, under field conditions, the lower concentration of  $10^{-11}$  M showed increased plant height, number of trifoliolate and nodules (Gautam et al., 2016). In the biofuel crop switchgrass, Th17 contributed to increase in plant height especially in the post-establishment years in the field (Arunachalam et al., 2018).

Our studies using Th17 in plant growth promotion, especially under salt stressed conditions, suggests that the bacteriocin is capable of alleviating NaCl stress in both *Arabidopsis* and soybean. Hence our interest in this part of the research was to study the response of the bacteria to NaCl stress and how that might affect the production of Th17 and other proteins in the cell-free supernatant. This attempt to understand the bacterial response to salt stress was also to allow one to decide between using live bacterial inoculum or Th17 in specific formulations for crop production.

## MATERIALS AND METHODS

Stock cultures of *Bacillus thuringiensis* NEB17 isolated from soybean rhizosphere, have been maintained in King's B medium as previously described (Gray et al., 2006a).

### *Bacillus thuringiensis* NEB17 Salt Stress Screening

*Bacillus thuringiensis* NEB17 was grown overnight in King's B medium in an orbital shaker set to 28°C and 120 rpm (Forma Scientific Inc., Model 4580) and diluted to an OD<sub>600</sub> nm of 0.01 corresponding to approximately  $10^7$  cfu mL<sup>-1</sup>. King's B medium supplemented with NaCl at 100 mM increments up to 1,000 mM was used to grow the bacteria in 96-well plates (Fisher, Canada) for 48 h. The bacterial growth was recorded every 2 h in a Cytation Gen 5 imaging reader (BioTek Instruments, Inc., USA) and a growth graph plotted as indicated by the OD<sub>600</sub>nm. The experiment was repeated three times to confirm the NaCl stress effects on bacterial growth kinetics.



## Thuricin17 Quantification Using HPLC

Once the bacteria were assessed for their capacity to tolerate NaCl stress, they were grown in 100 mL King's B medium in 250 mL conical flasks under optimal and 100 mM NaCl stress increments up to 500 mM NaCl, in an orbital shaker set to 28°C and 120 rpm. The experiment was conducted three times with every treatment having two replicates per experiment. Samples of 1 mL were taken from the flasks, centrifuged at 10,000 rpm for 10 min to pellet the cells. The clear supernatant was centrifuged again and the top clear liquid was collected to quantify Th17 using HPLC (following Gray et al., 2006a) every 6 for 48 h. In brief, the analyses of Th17 were performed using a Waters HPLC system equipped with a Waters 2487 Dual  $\lambda$  Absorbance Detector, Waters 1525 Binary HPLC pump and Waters717plus Autosampler. The conditions of the chromatography were as follows: column—Vydac C<sub>18</sub> reversed-phase column (0.46 × 25 cm; 5 $\mu$ ) (catalog no.218TP54; Vydac, Hesperia, CA, USA), temperature −25°C, flow rate −1 mL/min, detector wavelength—214 nm, gradient from 18 to 95% acetonitrile during the 18 min run. The peak corresponding to Th17 was identified by comparing with the retention time of a purified Th17 standard.

## Protein Profiling

### Protein Extraction

Bacterial cultures at 42 h of growth were centrifuged at 10,000 rpm for 10 min. at room temperature to pellet the cells and collect the supernatant. The supernatant was further purified by removing any remaining cells with a 0.22  $\mu$ m filter under vacuum and then subjecting it to TCA precipitation (25% v/v) at 4°C overnight in a shaker set to 100 rpm. These samples were centrifuged at 10,000 rpm for 10 min. at 4°C to obtain total protein precipitates as pellets. The TCA precipitation was carried out for the three independent experimental setups. Each experiment was comprised of three replicates per treatment and the TCA precipitated proteins were pooled to have enough protein for further steps of purification. The pellets were further treated with 1 mL ice cold methanol (Cat no. 15468–7, Sigma-Aldrich Co., St. Louis, MO, USA), vortexed, incubated in −20°C for 20 min. and centrifuged (Micro12, Fisher Scientific, Denver Instrument Co., USA) at 12,000 rpm for 7 min. at 4°C. The supernatant was discarded and the procedure was repeated twice, followed by a similar incubation in acetone (Cat. no. 179124, Sigma-Aldrich, Co., St. Louis, MO, USA); both steps were conducted in order to remove phenolics and secondary metabolites that might otherwise interfere with LC-MS/MS analysis. The total proteins were then dissolved in 2 M urea. The proteins were diluted and quantified using the Lowry method, and samples of 10  $\mu$ g in 20  $\mu$ L of 1M urea were submitted to the Institut de recherches cliniques de Montréal (IRCM) for label free proteomic analysis using LC-MS/MS.

### Proteome Profiling

The total proteins extracted were then digested with trypsin and subjected to LC-MS/MS using a Velos Orbitrap instrument (Thermo Fisher, MA, USA). Tandem mass spectra were extracted, and all MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.3.02). Mascot was set up to

search the *Bacillus thuringiensis* database assuming the digestion enzyme trypsin. Mascot searched with a fragment ion mass tolerance of 0.60 Da and a parent ion tolerance of 15 PPM. Carbamidomethyl of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification.

### Criteria for Protein Identification

Scaffold (version Scaffold 4.8.3), Proteome Software Inc., Portland, OR), was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability, as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained a minimum of two identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003).

## Data Analysis

Experiments were structured following a completely randomized design. The SAS statistical package 9.4 (SAS Institute Inc., Cary, NC, USA.) was utilized for data analysis. The Proc Mixed procedure and Tukey's multiple means comparison were used to determine differences among means at the 95% confidence level for bacterial growth and Th17 quantifications, at every time point.

Scaffold 4 was used to analyze the proteomics data for fold change and Fisher's exact test of the identified proteins, after subjecting the quantitative value of the spectra to the embedded normalization. The FASTA file generated was analyzed using Blast2GO-Pro 3.1.3 (Conesa et al., 2005; Götz et al., 2008, 2011), for the functional annotation and analysis of the protein sequences. Apart from these, Enzyme code (EC), KEGG maps and InterPro motifs were queried directly using the InterProScan web service. The mass spectrometry proteomics data have been deposited to Mass Spectrometry Interactive Virtual Environment (MassIVE), with the dataset identifier PXD024069 and doi: 10.25345/C5RB8T.

## RESULTS

The bacteriocin thuricin17 has the ability to help plants withstand abiotic stressors such as salt (NaCl) and drought for both *Arabidopsis* (Subramanian et al., 2016b) and soybean (Prudent et al., 2014; Subramanian et al., 2016a). While the isolation of Th17 is rather straightforward, the use of the bacteria *Bacillus thuringiensis* NEB17 as the inoculum could also be a very useful method in agriculture systems. Hence the bacteria were subjected to salinity stress and the amount of thuricin17 quantified to formulate an application method.

### *Bacillus thuringiensis* NEB17 Salt Stress Screening

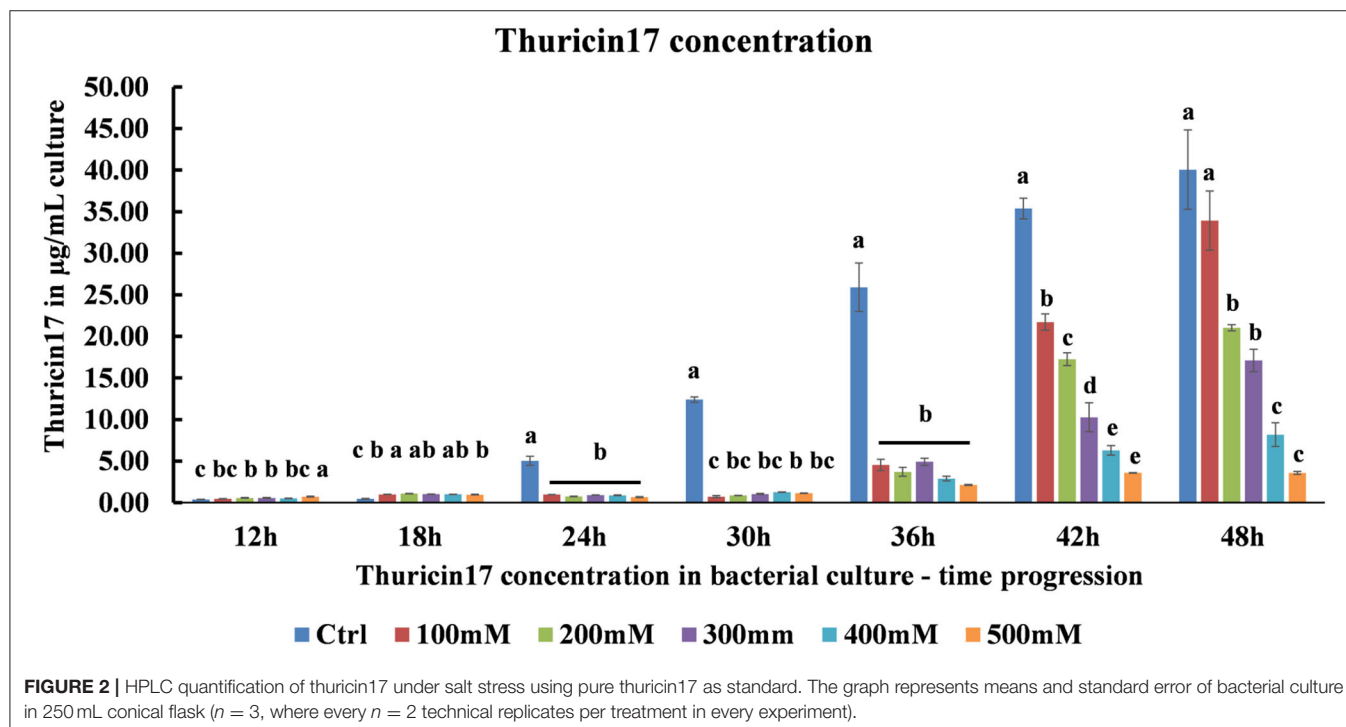
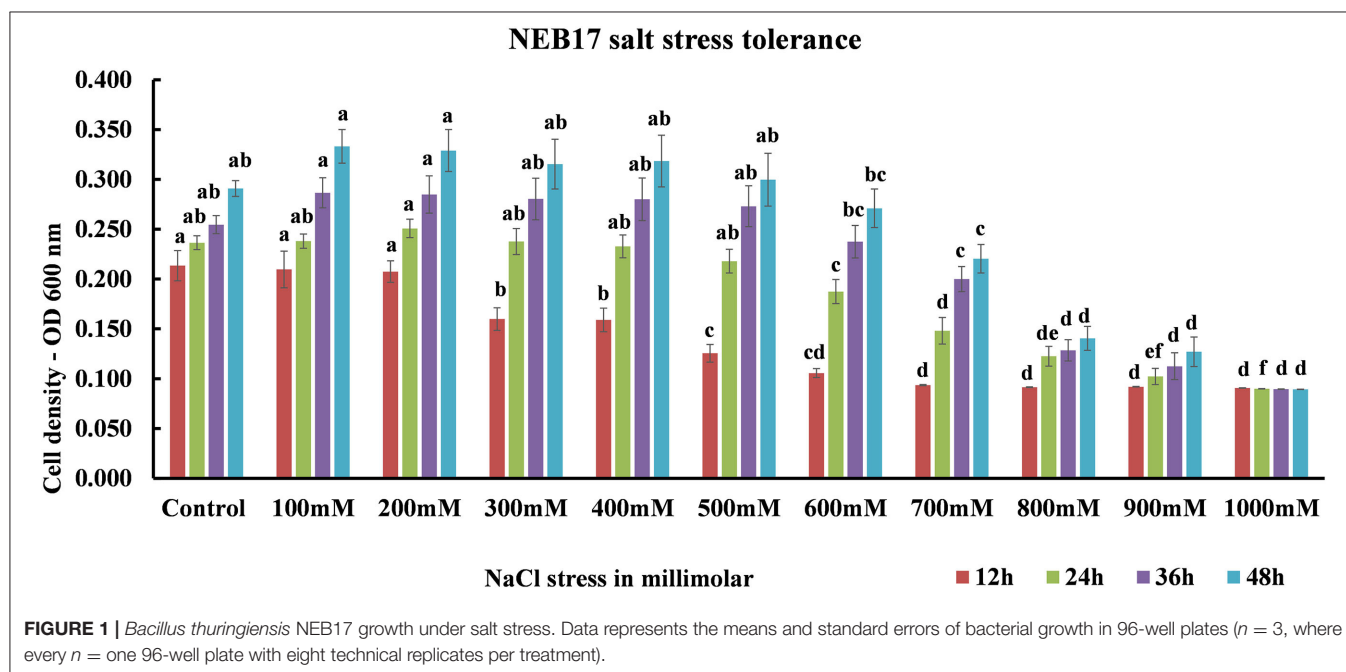
Salt stress screening suggested that the bacteria were capable of growing very well under salt stress until 600 mM; the growth started to be affected from 700 mM declining drastically at 900 mM. The bacteria ceased to grow in 1,000 mM NaCl

(Figure 1; Supplementary Table 1). This suggests that the *Bacillus thuringiensis* NEB17 is a salt tolerant strain.

### Thuricin17 Quantification Using HPLC

Using a standard graph for Th17, the samples from optimal, 100–500 mM NaCl-stressed cultures were quantified for the capability of the bacteria to produce Th17 under salt stress. As the level of salt stress increased, the amount of Th17 decreased

significantly (Figure 2). It was observed that in the 42 h culture, the amount of Th17 produced showed a steady decline in each of the NaCl stress levels and was statistically significant between every salt treatment (Supplementary Table 2). Hence, the 42 h cultures were further studied for the protein profiling to suggest differences in bacterial supernatant protein when the bacteria were subjected to 200 and 500 mM NaCl stress keeping the optimal conditions as control. Since Th17 sequence can only be



partially characterized using trypsin, the LC/MS trypsin-based digestion was performed to understand the general bacterial physiology related to salt stress tolerance and the changes that reflect in the cell-free supernatant proteome.

Protein Profiling

The results from LC-MS was analyzed using Scaffold and Blast2GoPro (Supplementary Material 1). The total proteins identified and the associated spectra are mentioned in Table 1; and the differences in the protein profile, total unique peptides and spectra are represented as Venn diagrams (Figure 3). Overall, in comparison with bacteria grown under optimal conditions, cultures in 200 mM NaCl showed an increase in response to stress, regulation of cellular, catabolic, metabolic and biosynthetic processes (Figure 4A). In 500 mM NaCl-stressed culture, response to stress, intracellular organelle parts and organelles, ribonucleoprotein complexes and non-membrane bound organelles were negatively affected. At both the salt levels, increased enzyme activities of ligases, hydrolases, lyases, and transferases were observed that profoundly affected the molecular function responses (Figure 4B; Table 2).

Further analysis, such as the Fisher test and fold change between contrasts 1 control and 200 mM, 2 control and 500 mM, and 3 200 and 500 mM NaCl were performed to compare the protein responses affected in the bacteria. Based on Fishers test (Supplementary Material 2), the

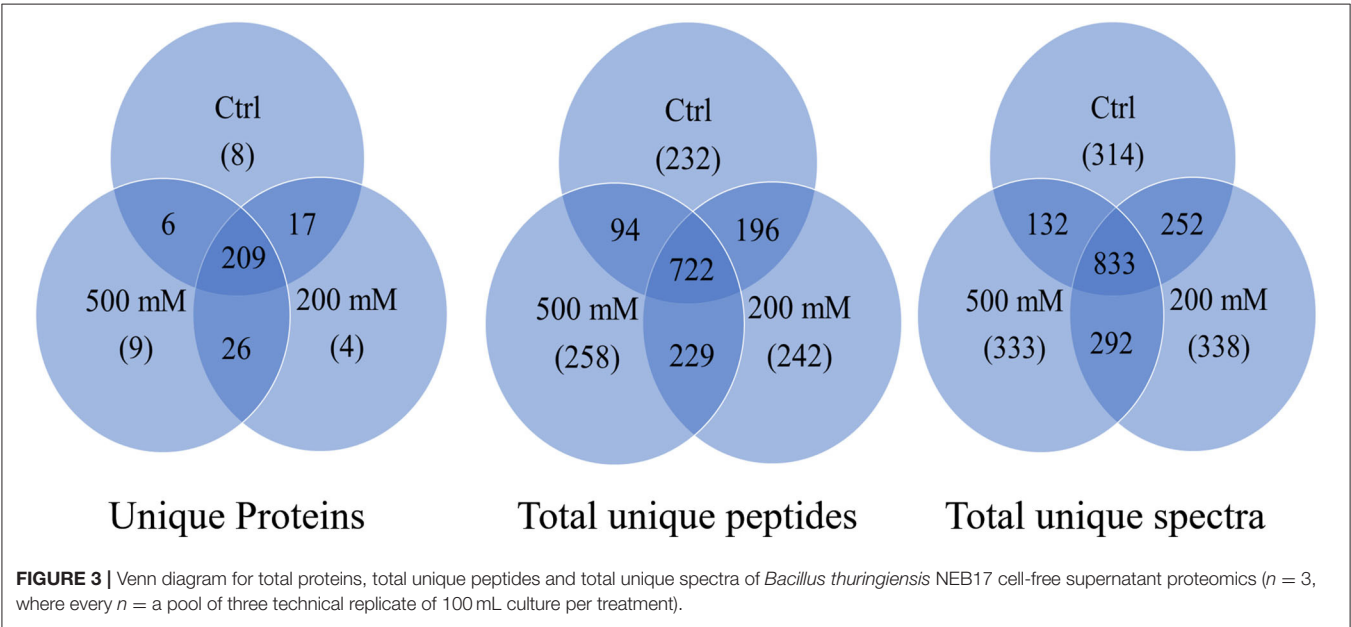
contrast of bacterial control with 200 mM NaCl saw an increase in proteins such as two isoforms of cold-shock DNA-binding protein family, Hbl L2 protein, collagenase and pyruvate dehydrogenase complex E2 component. In contrast control vs. 500 mM NaCl, a 3D domain protein, elongation factor G, 2-oxoisovalerate dehydrogenase beta subunit, phosphopentomutase, dihydrolipoyl dehydrogenase, S-layer protein, ornithine carbamoyltransferase, collagenase isoforms and a conserved hypothetical protein were up-regulated and statistically significant. The hemolytic enterotoxin, an cold-shock DNA-binding protein isoform, glycerol phosphate lipotechoic acid synthase, sphingomyelinase C, alveolysin, extra-cellular solute binding protein family 5, sulfatase and a universal stress protein were down-regulated. In contrast, 200 vs. 500 mM NaCl salt stress, except for a 3D domain protein, isoforms of cold-shock DNA-binding, hemolytic enterotoxin, a 50S ribosomal protein L15, phospholipase C, sphingomyelinase C, alveolysin and LSU ribosomal protein L16P were all down-regulated in 500 mM NaCl-stressed supernatant. This indicates that as the salt stress level increases, some of the bacterial pathogenicity factors are being affected.

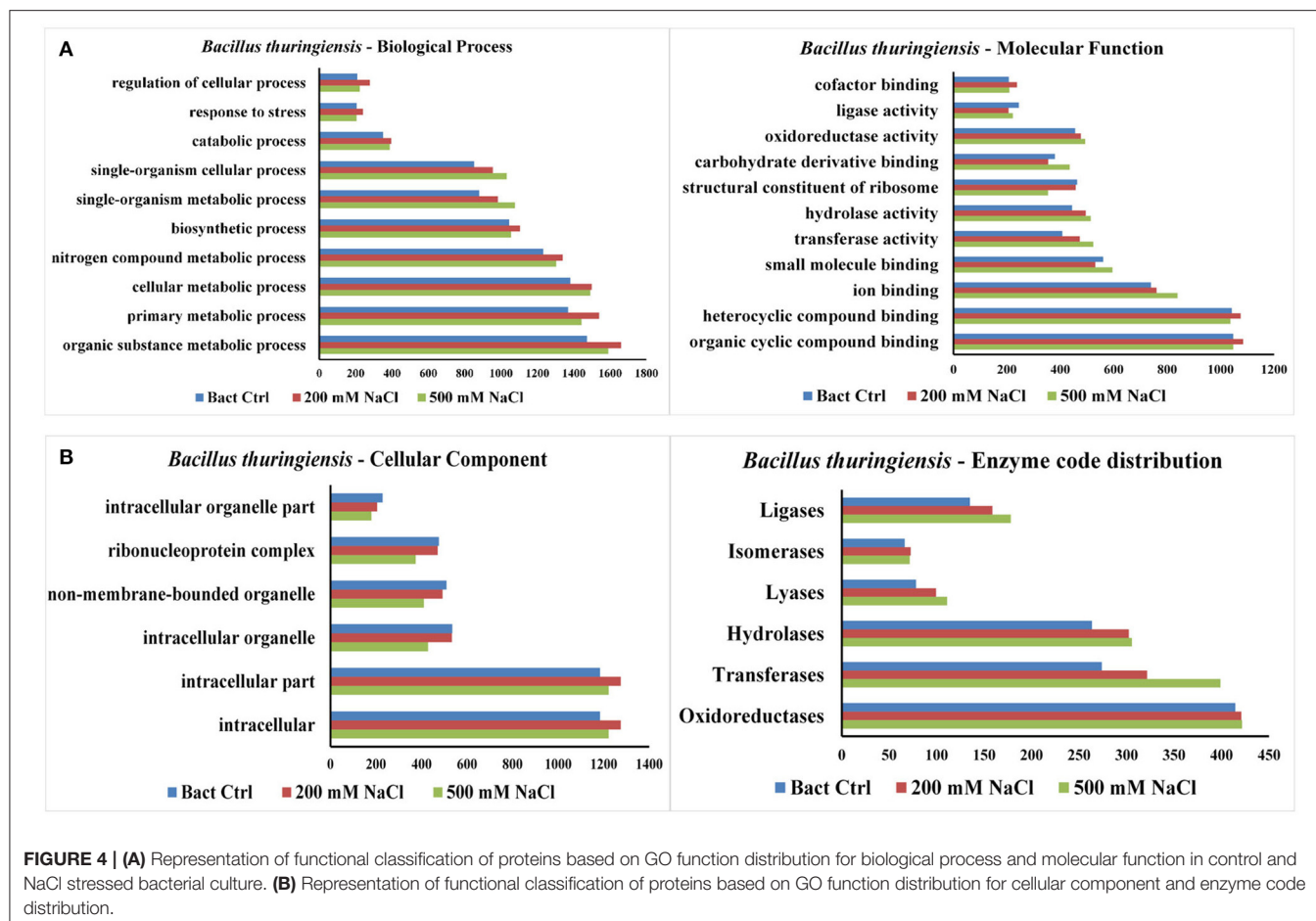
Protein data (quantitative spectra) of two or more-fold change was considered for biological interpretation (Supplementary Material 3) along with those identified with Fishers test. In contrast 1. control vs. 200 mM and 2. control vs. 500 mM, 59 proteins each and in contrast 3. 200

TABLE 1 | Total number of proteins identified at 95% protein probability and total spectra at 95% peptide probability, with minimum of two peptides.

Treatments	BC1	BC2	BC3	B21	B22	B23	B51	B52	B53
Proteins	216	127	181	167	184	209	177	206	173
Spectra	3408	2256	2739	2854	3031	3521	2922	3150	2782

The table represents data from every biological replicate analyzed.





**TABLE 2 |** Enzyme code distribution differences as compared to control in the cell-free supernatant of *Bacillus thuringiensis* NEB17 under salt stress.

Enzyme classes	BC (Ctrl)	B2 (200 mM NaCl)	B5 (500 mM NaCl)
Oxidoreductases	415	421 (↑ 1.44%)	422 (↑ 1.68%)
Transferases	274	322 (↑ 17.51%)	399 (↑ 45.62%)
Hydrolases	264	302 (↑ 14.39%)	306 (↑ 15.90%)
Lyases	78	99 (↑ 26.92%)	111 (↑ 42.30%)
Isomerases	66	73 (↑ 10.06%)	72 (↑ 9.09%)
Ligases	135	159 (↑ 17.77%)	178 (↑ 31.85%)

vs. 500 mM, 26 proteins that were above two-fold change were identified. In contrast 1. the two isoforms of cold-shock DNA-binding protein family had fold changes of 12.8 and 14.7, as compared to control. In contrast 2, dihydrolipoyl dehydrogenase, phosphopentomutase, two isoforms of 3D domain protein, 2-oxoisovalerate dehydrogenase beta subunit, elongation factor G and ornithine carbamoyltransferase were detected with more than a two-fold increase. Contrast 3 however had only the 3D domain protein with 2.3-fold change as compared to 200 mM NaCl, in keeping with the Fishers test data (Table 3).

## DISCUSSION

Since thuricin17 was first isolated and characterized, its potential use in agriculture has been tested on a range of plants as a plant growth promoter. In our studies, spanning over a decade, Th17 as a leaf spray on soybean, or as a root drench in soybean and corn (Jung et al., 2008a; Lee et al., 2009), in association with abiotic stress for Arabidopsis (Subramanian et al., 2016b), soybean (Prudent et al., 2014; Subramanian et al., 2016a) and under field conditions for soybean (Gautam et al., 2016) and switchgrass (Arunachalam et al., 2018) have demonstrated positive plant growth promotion and yield. *Bt* NEB17 when co-inoculated with *Bradyrhizobium japonicum* 532C under nitrogen free conditions, promoted soybean plant growth, nodulation, and yield (Bai et al., 2002). But, so far *Bt* NEB17 has not been evaluated for its capability to tolerate any stress or to determine if Th17 production is affected if the bacterial culture is subjected to any stress. Hence, in this study, *Bt* NEB17 was subjected to NaCl stress and the cell-free supernatant was evaluated to study the commonness or differences the bacteria undergo during their first 48 h of growth. Also, Th17 was quantified to determine if a stress environment caused an increase or decrease in its production. While the bacteria grew well up to 600 mM NaCl and the effect of salt stress started to be manifested from 700 mM



**TABLE 3 |** Proteins identified in both fold change and Fishers test in all the contrasts.

#	Contrast Ctrl Vs. 200 mM NaCl	Accession number	Molecular weight	Fold change and (Fisher)	BC	B2
53	Pyruvate dehydrogenase complex E2 component, dihydrolipoamide acetyltransferase [ <i>Bacillus thuringiensis</i> str. Al Hakam]	gi 118418407 (+21)	46 kDa	3.3 (0.03773)	5.0	16.0
13	Cold-shock DNA-binding protein family [ <i>Bacillus thuringiensis</i> str. Al Hakam]	gi 118418013 (+20)	7 kDa	12.8 (0.00034)	47.3	70.0
29	Cold-shock DNA-binding protein family [ <i>Bacillus thuringiensis</i> str. Al Hakam]	gi 118419204 (+16)	7 kDa	14.7 (0.01607)	34.3	36.0
#	Contrast Ctrl Vs. 500 mM NaCl	Accession number	Molecular weight	Fold change and (Fisher)	BC	B5
19	Dihydrolipoyl dehydrogenase [ <i>Bacillus thuringiensis</i> IBL 4222]	gi 228902475 (+13)	49 kDa	2.3 (0.01009)	23.7	47.0
33	Phosphopentomutase [ <i>Bacillus thuringiensis</i> serovar huazhongensis BGSC 4BD1]	gi 228922839 (+5)	42 kDa	3.1 (0.00996)	10.3	31.0
13	Cold-shock DNA-binding protein family [ <i>Bacillus thuringiensis</i> str. Al Hakam]	gi 118418013 (+20)	7 kDa	3.7 (0.00016)	47.3	11.3
115	3D domain protein [ <i>Bacillus thuringiensis</i> serovar huazhongensis BGSC 4BD1]	gi 228921618	56 kDa	4.9 (0.03397)	2.7	11.3
41	3D domain protein [ <i>Bacillus thuringiensis</i> serovar kurstaki str. T03a001]	gi 228951290	48 kDa	5.4 (0.00293)	6.7	36.0
67	2-oxoisovalerate dehydrogenase subunit beta [ <i>Bacillus thuringiensis</i> IBL 4222]	gi 228902675 (+11)	36 kDa	5.7 (0.00566)	4.7	19.3
17	Elongation factor G [ <i>Bacillus thuringiensis</i> BMB171]	gi 296500941	76 kDa	6.8 (0.00433)	20.0	49.3
61	Ornithine carbamoyltransferase [ <i>Bacillus thuringiensis</i> str. Al Hakam]	gi 118415344 (+7)	38 kDa	8.5 (0.01141)	3.7	16.0
#	Contrast 200 mM Vs. 500 mM NaCl	Accession number	Molecular weight	Fold change and (Fisher)	B2	B5
41	3D domain protein [ <i>Bacillus thuringiensis</i> serovar kurstaki str. T03a001]	gi 228951290	48 kDa	2.3 (0.02773)	15.7	36.0

NaCl, the production of Th17 started to decline from 100 mM NaCl and was half the amount at 200 mM NaCl. The amount declined further and by 500 mM NaCl, the production almost stopped. In all probability, the bacteria were utilizing their resources to deal with and thrive under the salt stress rather than invest energy in Th17 production. Also, because in a closed environment like a conical flask where they do not need to ward off closely related *Bacilli* for rhizospheric competition (Jung et al., 2008b), their resources could be allocated more for their survival. Bacterial populations display growth and colony size heterogeneity as a mechanism to adapt to stress. In this study, as the level of salt increased, the cell density of the cultures decreased, particularly at very high NaCl concentrations. In previous studies, *Bacillus cereus* ATCC 14579 displayed a low population variability under mild salt stress (2.5% NaCl stress) and the colony sizes were highly heterogeneous under severe salt stress (5% NaCl stress) which affected the cell density in liquid cultures (Den Besten et al., 2007). Whole genome expression analyses suggest an overlap of salt stress gene expression, in both these conditions, that involved genes that are part of general stress responses (Den Besten et al., 2009).

Proteins and secondary metabolites play key roles in metabolic processes of an organism. They also play key roles in the rhizosphere, enriching and establishing beneficial partners. From

a bacterial point of view, nutrient cycling, degradation of root exudates for enriching and attracting beneficial partners to their niche, etc., all have intricate evolutionary patterns. The label free proteomics approach to determine what the bacteria changed to accommodate to NaCl stress is interesting to consider.

Among the *Bacillus cereus sensu lato* group, survival in salt stress is particularly relevant to the food industry since salt is added as an additive and/or preservative. Cross protection between salt, heat, hydrogen peroxide, acid, and ethanol is established in *Bacillus* spp., in the context of food processing, where some of these are regular food contaminants. For example, *Bacillus cereus* NCIMB 11796 produced both general and stress specific (salt, heat) proteins that were accumulated differentially between stressed and unstressed cells (Browne and Dowds, 2001). The *Bacillus* group manifests a group of proteins that have unspecific but protective function regardless of stress exposure, and these are referred to as general stress proteins (GSPs). Many of these general stress proteins are seen at the onset and during the stationary phase. A significant amount of work has been done with *B. cereus* and model bacterium *B. subtilis* with respect to the cellular responses to stress. For example, *Bacillus subtilis* strains IS58 and 168 (*trpC2*) were reported to have about 50 such GSPs and about 40% of the cells translational

capacity was devoted to producing them. Also, the  $\sigma^B$ -dependent stress response varied in intensity, between the strains and the mutants in this study (Bernhardt et al., 1997). A comprehensive microarray and proteomics of *Bacillus subtilis* 168 (*trpC2*) wild-type strain was studied for very early (10–120 min. after exposure to NaCl stress) transcriptome and proteome changes in response to salt stress (6% NaCl). A sudden change in osmolarity caused the bacterial membrane proteins to increase by 20% in the first 10 min. of exposure. The immediate response was the activation of  $\sigma^B$  regulon that confers broad stress resistance to the cells (Hahne et al., 2010).

However, from an agriculture perspective, salt stress is detrimental to both crop production and to the microorganismal populations in soils. Studies at high salt stress levels was reported to favor the transfer of plasmid pAW63 from *Bacillus thuringiensis* subsp. *kurstaki* to other *Bt* strains. While the salt tolerance in *Bt* varies between strains, some strains alter their phenotype by modifying their mobility, reductions in cell number, cell clumping, and formation of filaments (Beuls et al., 2012). A study of *Bt* in rice fields suggests that the plasmids of various strains studied did not have any correlation with crystal morphology or salt tolerance of the strains (Das and Dangar, 2007). Since *Bt* NEB17 was isolated from Macdonald campus soil, Quebec, it is highly likely that the bacterium is a psychrotropic and a psychrotolerant strain. For cold adaptation, *Bt* strains use major cold-shock proteins (Csps) to survive and grow under low temperature (Francis et al., 1998). Natural cold adapted isolates of *Bacillus thuringiensis* have various combinations of *nhe*, *hblA*, and *cytK* genes that are toxigenic by nature and *cspA* that confer psychrotolerance to the bacteria (Bartoszewicz et al., 2009). While Csps are mostly associated with cold stress adaptation, they are also reported to be present during the early exponential phase of cell differentiation and proliferation under optimal conditions, and their levels are non-detectable in the late exponential phase. Culture dilutions and additional nutrient availability also play important roles in Csp induction (Brandi et al., 1999; Yamanaka and Inouye, 2001; Ermolenko and Makhatadze, 2002). Some members of the Csp family in *Bacillus subtilis* are reported to be involved in several cellular processes (Graumann et al., 1997), and general stress adaptation responses such as osmotic, oxidative, pH, starvation, ethanol, and host cell invasion (Keto-Timonen et al., 2016) and can hence be termed multi-functional (Lindquist and Mertens, 2018). In this study, two isoforms of cold-shock DNA-binding proteins (7 kDa) were seen to be up-regulated at 200 mM NaCl stress but down-regulated at 500 mM NaCl stress. As in cold temperature, these protein isoforms also help when the bacteria are affected by salt stress and probably function as general stress response proteins.

While insecticidal toxins are wide spread in *Bt*, most of these are seen in isolates from invertebrate samples as compared to water, soil, or plant samples (Espinasse et al., 2003). This suggests an evolutionary significance in the bacterial colonization dependent on the ecological niche they have evolved in. Repeated culturing of *Bt* in rich culture medium can alter some genetic, metabolic, pathogenic, and structural determinants over time, as compared to fresh isolates from plant tissues. Some of these

changes include complete loss of  $\delta$ -endotoxins and  $\beta$ -exotoxin production, changes in fatty acid profiles and mutation of cry genes, thereby altering the virulence determinants (Bizzarri et al., 2008). Of the several enterotoxins identified in *Bt*, two of the most important that contribute to food poisoning are hemolytic (Hbl) and non-haemolytic (Nhe) toxins. While these toxins were studied more in *B. cereus*, it is now evident that they are more prevalent in *Bt* populations (Gaviria Rivera et al., 2000) as are other associated toxins (Kim et al., 2015).

HBL is the best characterized three-component hemolysin enterotoxin. It is comprised of two lytic components  $L_1$  and  $L_2$  and a binding component B all of which are involved in hemolysis, vascular permeability and dermal damage (dermonecrotic). Many *Bt* strains do not contain  $L_2$  and B components but can still be cytotoxic when they cross react with Nhe (Prüß et al., 1999). The SinR regulon harbors genes coding for Hbl enterotoxin. This along with the main virulence regulator PlcR, controls Hbl expression (Fagerlund et al., 2014). In this study, the Hbl  $L_2$  protein component was not affected by 200 mM NaCl. But the hemolytic enterotoxin was significantly and negatively affected at 500 mM NaCl. Also, phospholipase C was down-regulated at 500 mM NaCl. PlcR regulator controls the transcription of genes encoding extracellular proteins including phospholipase C, proteases and enterotoxins (Hbl and Nhe). The *plcR* gene is activated during the onset of the stationary phase and acts as a quorum sensing effector. The disruption of this gene greatly reduces the haemolytic activity of *Bt* and thereby insecticidal virulence (Gominet et al., 2001).

Likewise, sphingomyelinase C and alveolysin were affected by 500 mM NaCl. Cereolysin B, or sphingomyelinase (*cerB*), is also controlled by *plcR* and is involved in pathogenesis (Kim et al., 2015). Alveolysin is a thiol-activated toxin first identified in *Bacillus alvei* that colonizes bee-hives. This protein shares homology with other toxins such as listeriolysin O, perfringolysin O, pneumolysin, and streptolysin O, all of which have a single cysteine in their conserved peptide sequence ECTGLAWWWWR (Geoffroy et al., 1990). It is an exoprotein that has a lethal membrane damaging and cardiotoxic nature when it binds to membrane cholesterol and withdraws its interaction with other membrane lipids (Thelestam et al., 1981). It is possible that *Bt* NEB17 being a rhizospheric bacteria uses this toxin as a defense to prevent other *Bacilli* from colonizing the same niche space and this capability is lost under salt stress.

## CONCLUSION

In this study, the effect of NaCl on bacterial growth, production of the bacteriocin thuricin17 and characteristics of the bacterial culture cell-free supernatant were determined, to study the changes in bacterial physiology in response to salt stress. The comparison of two levels of salt stress is another step in understanding the mechanism of the bacterial tolerance to salt and how they manipulate their external environment in order to maintain a healthy population under adverse conditions. Salt stress adversely affects the bacterial defense mechanisms but does not affect growth until the NaCl levels are

700 mM or greater. This study is another step to understanding bacterial growth under salt stress that can help the end user use either the live inoculum or Th17 as a biostimulant, for crop production.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: CCMS MassIVE, Accession No: MSV000086823.

## AUTHOR CONTRIBUTIONS

SS designed, performed experiments, data analysis, and wrote the manuscript. AS contributed to HPLC quantification of thuricin17. AS and DS contributed to procuring reagents/materials/analysis tools/funding. DS provided the conceptual context for the work. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.630628/full#supplementary-material>

**Supplementary Table 1** | Bacterial growth under salt stress data with statistical analysis.

**Supplementary Table 2** | Thuricin17 quantification under salt stress data with statistical analysis.

**Supplementary Material 1** | Protein data Blast2GoPro for all the treatment replicates of *Bt* NEB17 and under salt stress.

**Supplementary Material 2** | Fishers test data between contrasts of *Bt* NEB17.

**Supplementary Material 3** | Fold change data between contrasts of *Bt* NEB17.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Spatial and Temporal Distribution of Soil Microbial Properties in Two Shrub Intercrop Systems of the Sahel

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The Sahel is an ecologically vulnerable region where increasing populations with a concurrent increase in agricultural intensity has degraded soils. Agroforestry offers an approach to remediate these landscapes. A largely unrecognized agroforestry resource in the Sahel are the native shrubs, *Piliostigma reticulatum*, and *Guiera senegalensis* that to varying degrees already coexist with row crops. These shrubs improve soil quality, redistribute water from the deep soil to the surface (hydraulic lift), and can improve crop growth. However, little information is available on whether these shrubs affect spatial and temporal dynamics of microbial communities. Therefore, the objective of this study was to determine microbial composition and activity in the wet and dry seasons of soil in the: shrub rhizosphere (RhizS), inter-root zone (IntrS), and outside the influence of shrub soil (OutS) for both *G. senegalensis* and *P. reticulatum* in Senegal. A 3 × 2 factorial field experiment was imposed at two locations (490 and 700 mm annual rainfall with *G. senegalensis* and *P. reticulatum*, respectively), that had the soil sampling treatments of three locations (RhizS, IntrS, and OutS) and two seasons (wet and dry). Soils were analyzed for: microbial diversity (DGGE with bacterial 16S or fungal 28S rRNA gene sequences phospholipids fatty acid, PLFA); enzyme activities; microbial biomass carbon (MBC); and nitrogen (N) mineralization potential. For the DGGE profiling, the bacterial community responded more to the rhizosphere effect, whereas, the fungal community was more sensitive to season. PLFA, MBC, enzyme activities and inorganic N were significantly higher in both seasons for the RhizS. The presence of shrubs maintained rhizosphere microbial communities and activity during the dry season. This represents a paradigm shift for semi-arid environments where logically it would be expected to have no microbial activity in the extended dry season. In contrast this study has shown this is not the case that rather the presence of shrub roots maintained the microbial community in the dry season most likely due to hydraulic lift and root exudates. This has implications when these shrubs are in cropped fields in that decomposition and mineralization of nutrients can proceed in the dry season. Thus, enabling accumulation of plant available nutrients during the dry season for uptake by crops in the rainy season.

**Keywords:** microbial diversity, soil, shrub-intercropping, DGGE, PLFA

## INTRODUCTION

The Sahel is an ecologically vulnerable region where increasing populations with a concurrent increase in agricultural intensity has degraded soils. Agroforestry offers an approach to remediate these landscapes. A largely unrecognized agroforestry resource in the Sahel are the native shrubs, *Piliostigma reticulatum*, and *Guiera senegalensis* that to varying degrees already coexist with row crops. These two species dominate over any other shrub species and are coppiced in the spring prior to the cropping/rainy season. Subsequently, the shrubs regrow during the long dry season.

Previous recommendations were to remove shrubs from cropped fields because of perceived competition of shrubs with crops for nutrients and water, and reduced crop yield (Somarrriba, 1988; Kater et al., 1992). However, a preliminary study in Niger in farmers' fields showed millet growing within the influence of *G. senegalensis* rhizospheres had greater growth than when grown outside the influence of the shrub (Wezel et al., 2000). Long-term statistically valid field studies have shown that *G. senegalensis* (Dossa et al., 2012) and *P. reticulatum* (Dossa et al., 2013; Bright et al., 2017) in Senegal can increase yields for groundnut (*Arachis hypogaea* L.) and millet [*Pennisetum glaucum* (L.) R. Br.] with or without inorganic fertilizer.

These pedo-ecological benefits by woody species have been shown in natural, semi-arid desert environments. This phenomenon has been characterized as soil fertility and hydrologic resource islands that develop beneath woody species and influence the biogeochemistry and decomposition of uncultivated desert environments (Schlesinger et al., 1996; Schlesinger and Pilmanis, 1998; Rango et al., 2006; Schade and Hobbie, 2006). Brooker et al. (2016) proposed that plant-plant interactions hold potential to facilitate sustainable crop production and address food insecurity in developing countries where subsistence farmers use limited external inputs.

Rhizospheres produce organic root exudates and affect soil moisture, which changes the numbers and activities of microbial communities living under the influence of plant roots (Lynch and Whipps, 1990; Diedhiou et al., 2009). A large proportion of the C released by roots is in the form of water-soluble substances such as sugars, organic acids and amino acids. The differences in type and quantity of C available in different root zones thereby select for distinct rhizospheric community structures (Young, 1998; Yang and Crowley, 2000). In addition, these communities may vary with respect to plant species (Westover et al., 1997).

An important finding for *G. senegalensis* and *P. reticulatum* is that their roots redistribute water from the subsoil to the surface, a phenomenon known as hydraulic redistribution (Kizito et al., 2007, 2012). Hydraulic redistribution is the movement of water from regions of higher soil water potential (subsoil) to regions of lower soil water potential (surface soil) via plant roots, and is typically characteristic of semi-arid to arid environments (Richards and Caldwell, 1987) as well as mesic environment during drought (Dawson, 1993; Caldwell et al., 1998). The amount of water redistributed can go as high as 0.1 mm day<sup>-1</sup> for *P. reticulatum* and 0.2 mm day<sup>-1</sup> for *G. senegalensis* (Kizito et al., 2007). Hydraulic redistribution could be important

to maintain microbial communities and drive biogeochemical processes in shrub rhizosphere soil during the long dry season of semi-arid or arid environments (Saul-Tcherkas and Steinberger, 2011). Previous research has shown that soil beneath canopies of *P. reticulatum* or *G. senegalensis* increased decomposition, microbial biomass C, and enzyme activities over soil outside the influence of these shrubs. Also, Debenport et al. (2015) found that root zone soil of pearl millet [*Pennisetum glaucum* (L.) R. Br.] grown within the influence of *P. reticulatum* or *G. senegalensis* had increased diversity and abundance with some genera in both bacterial and fungal communities over soil outside the influence of shrubs. However, little information is available on whether shifts in microbial communities remain during the dry season due to hydraulic lift.

Therefore, the hypothesis was that the presence of shrubs will shift soil microbial communities with the objective to determine microbial composition and activity in the wet and dry seasons of shrub (RhizS), inter-root soil (IntrS), and outside the influence of shrub soil (OutS).

## MATERIALS AND METHODS

### Experimental Design

The study was conducted in two agro-ecological zones of Senegal within the major cropping region known as the Peanut Basin. The first site, Keur Matar Arame (KMA) is located at Thies (N14° 46' W16° 51') where *G. senegalensis* is the main shrub species found in farmers' fields. The soil is a sandy, ferruginous Oxisol (FAO, 1998). The KMA site has low rainfall with ~300 mm per annum with a water table depth of ~10 m. The second site is located at Nioro (N13° 45' W15° 47'), predominantly occupied by *P. reticulatum* shrubs on a sandy, lateritic area classified as an Oxisol (FAO, 1998). The area has unimodal rainfall, 700 mm per annum and a mean annual temperature of 32°C. The water table is at ~8 m. The sites were both in farmers' fields that had been under long-term cropping of millet [*Pennisetum glaucum* (L.) R. Br.] and groundnuts (*Arachis hypogaea* L.) that included coppicing of aboveground biomass each spring. The coppiced residues were normally burned or occasionally some portion was removed and taken back for household use. Both sites in the rainy season were under millet cultivation which were planted in a 1 × 1 m pattern.

Soil samples from rhizosphere soil (RhizS), inter-root soil (IntrS), and outside the influence of shrub soil (OutS) were collected in relation for both shrub species (0–15 cm depth) during the dry season (March 2005), and the wet season (August 2005). At each experimental site, soils were sampled randomly under six plants at three locations: (1) shrub rhizosphere soil RhizS (2) soil beneath shrub canopy but between roots or inter-root soil (IntrS); and (3) samples collected two meters away from the shrubs which was outside the influence of shrub (OutS). The OutS were taken between millet plants at a distance of 0.5 m from millet plants. The rhizosphere soils was obtained by peripheral excavation of surface soil followed by careful excising the roots from the larger crown or tap roots. The excised roots were gently shaken and then the soil retained on the roots was collected for analysis which is defined as rhizosphere soil. The soil samples of

three pairs of shrubs for each species were combined (resulted in 3 field replicates for each shrub species), homogenized and then crushed to pass through a 2 mm mesh screen. The soil samples were maintained at field moisture and stored at 4°C until analysis. Soil extractions for nucleic acid and fatty acid analyses outlined below to characterize soil microbial communities were done within 2 days and other analyses were done within a week after sampling. Soils from old and new roots were pooled for all analyses except the for the DGGE analysis.

## Microbial Biomass C, Enzyme Activities, and Inorganic N

Microbial biomass was determined by chloroform–fumigation extraction (CFE) following the method of Amato and Ladd (1988) with some modifications. Briefly, 10 g of the moist soil was fumigated with chloroform (ethanol free) and then incubated for 10 days. Fumigated samples and unfumigated control samples were extracted with 2 M KCl solution for 60 min on a rotator shaker. After filtration, 2 ml of the filtrates were mixed with 0.5 ml of 0.4 M sodium citrate solution. Ninhydrin-reactive N was determined colorimetrically at 750 nm (Schinner et al., 1996) (Evolution II, Alliance-Instrument, France). Microbial biomass C (MBC) was estimated by multiplying by 21 the gain in ninhydrin-reactive N after fumigation (Amato and Ladd, 1988). Results were expressed as  $\mu\text{g C g}^{-1}$  of dry soil.

Soil inorganic-N species were quantified in the same extract, colorimetrically in KCl extracts (2 M KCl) using the method of Bremner (1965). Ammonium was quantified using the reaction of Berthelot modified with the indophenol blue at 660 nm. For the nitrate, nitrite is mixed with the sulfanilamide to form a diazo complex and the absorbance was read at 525 nm. Inorganic nitrogen was assessed as the sum of ammonium and nitrate, and the results were expressed as  $\mu\text{g N g}^{-1}$  of dry soil.

All soil enzyme analyses were performed on field moist soil. The  $\beta$ -glucosidase and chitinase activities were measured using a modified method originally described by Hayano (1973) and Ndour et al. (2001). Fresh soil samples (100 mg) were incubated for 2 h at 37 °C, with 100  $\mu\text{l}$  of 5 mM *para*-nitrophenyl  $\beta$ -D-glucopyranoside (pNP) for  $\beta$ -glucosidase and 5 mM *para*-nitrophenyl *N*-acetyl glucosaminide for chitinase as the substrates. The pNP released was measured 15 min after stopping the reaction at 400 nm for both enzymes. The results were expressed as  $\mu\text{g pNP released g}^{-1} \text{ h}^{-1}$ . Phosphatase activity was determined following the method described by Tabatabai and Bremner (1969). The method by Kandeler and Gerber (1988) was used to determine the urease activity. Two analytical replicates and one control were analyzed for each soil sample.

## Phospholipids Fatty Acids Analysis

Phospholipids fatty acids (PLFA) were extracted from the soil by Bligh and Dyer (1959) method as modified by Schutter and Dick (2000). Briefly, lipids were extracted from 3 g of soil samples using a chloroform-methanol-phosphate buffer solvent. PLFA were separated from neutral lipids and glycolipids using silicic acid columns (Supelco, Bellefonte, PA, USA). The phospholipid fractions were then converted to methyl-esters by alkaline methanolysis. PLFAs from all samples were extracted in

triplicates. PLFAs were analyzed by gas chromatography (GC) (Agilent Ultra 2 column; temperature ramping from 120 to 260°C at a rate of 5°C per min) using helium as the carrier gas, and peaks were detected by flame ionization detector (Frostegård and Bååth, 1996).

Individual fatty acid methyl esters (FAME) were identified and quantified using the MIDI Sherlock Microbial Identification System (MIDI, Newark, Delaware, USA) and with the mixture of 37 FAME (FAME 37 47885-4; Supelco, Inc), 24 bacterial FAME mixture (P-BAME 24 47080-U; Supelco, Inc.). Each individual fatty acid was expressed as a percentage of the total amount of fatty acids (mol%) found in a given sample. PLFA data with < 0.5% of the total relative abundance were not included in the data set. PLFA biomass was estimated by adding the amount of all fatty acids detected and was expressed in nano moles of PLFA per g of dry weight of soil ( $\text{nmol g}^{-1} \text{ dw}$ ) (White et al., 1979; Frostegård et al., 1991; Bossio et al., 1998). For each microbial group and total PLFA (PLFA<sub>tot</sub>), the total amount of PLFA identified was calculated to represent their contribution to the total microbial biomass.

A total of 23 PLFA markers out of 27 identified (91%) were used for the multivariate analyses for *G. senegalensis*, while a total of 23 PLFA out of 29 identified (94%) were used for multivariate analysis for *P. reticulatum*. The combined masses of FAMES reported as typical of fungi (18:2 $\omega$ 6c, 18:1 $\omega$ 9c) (Frostegård and Bååth, 1996; Olsson, 1999), Gram-negative (GN) bacteria (18:1 $\omega$ 7c; 17:0cy; 19:0cy) (Wilkinson, 1988), Gram-positive (GP) bacteria (15:0i; 15:0a; 16:0i; 17:0i; 17:0a) (O'Leary and Wilkinson, 1988) and actinomycetes (ACT) (16:0 10-Me; 17:0 10-Me; 18:0 10-Me) (Kroppenstedt, 1992) were used as signatures for these microbial groups. The summation of individual fungal or bacterial fatty acids were used to calculate fungal to bacterial ratios (FUN/BACT) as an indicator of the general change in the soil microbial community structure (Bardgett et al., 1998; Olsson, 1999; Zelles, 1999; Fierer et al., 2002). The ratio of 19:0cy to 18:1 $\omega$ 7c was calculated and is primarily an indicator to water stress (Guckert et al., 1986; Lundquist et al., 1999). The ratio of saturated to monounsaturated fatty acids (SAT/MONO) was calculated and is an indicator nutrient deprivation (Bossio and Scow, 1998; Larkin, 2003).

## Denaturing Gradient Gel Electrophoresis (DGGE)

Changes in soil microbial community structure were analyzed by DGGE profile of 16S rRNA and 28S rRNA gene sequences for bacteria and fungi, respectively. The method by Porteous et al. (1997) was used to extract soil DNA from 0.5 g of soil samples in triplicates.

Bacterial 16S rRNA was amplified using universal bacterial DGGE primers 338f-GC clamp and 518r primers (Muyzer et al., 1993). Amplification was performed by 5 min of denaturation at 94°C, 30 cycles of 45 s each at 94°C, 45 s at 52°C and 1 min at 72°C, followed by a final extension at 72°C for 10 min using a PTC-100 thermal cycler. The primers 403f-GC (U1) and 662r (U2) were used to amplify the 28S rRNA of the fungal population (Sandhu et al., 1995). The following cycle was used to amplify

fungal 28S rRNA: 5 min of denaturation at 94°C, 35 cycles of 30 s at 94°C, 1 min at 52°C and 2 min at 72°C, followed by a final extension at 72°C for 10 min using the same thermal cycler. The PCR tag “Ready- to-go” (Amersham Biosciences, USA) was used to amplify both the 16S and the 28S rRNA. PCR products were confirmed on 1.0% agarose gels after staining with ethidium bromide solution (0.25 µg ml<sup>-1</sup>). PCR products were then quantified using a spectrophotometer to measure the optical density.

Equivalent quantities of PCR products were resolved in an 8% polyacrylamide gel (37.5:1 acrylamide:bisacrylamide) in 0.5× TAE buffer (20 mM Tris-HCl, 10 mM acetate, 0.5 mM EDTA) and denaturants (100% denaturant contains 7 M urea and 40% deionized formamide). A gradient of denaturants ranged from 40 to 70% for bacterial communities and 35–65% for fungal communities. Electrophoresis was performed on an Ingeny apparatus (Ingeny phorU, Netherlands) at a constant voltage of 75 for 16 h. The software Bio-profile Biogene program (Vilber Lourmat) was used to analyze the DGGE profile. The detected bands were used to construct a matrix indicating presence or absence of bands in each sample.

## Statistical Analysis

Spatial and temporal shifts in the composition of PLFA profiles were analyzed by principal components analysis (PCA) using the PC-ORD package (MjM Software Design, Gleneden Beach, OR) (McCune and Grace, 2002) after converting the PLFA to mol% of peak totals. Scores of samples on the first two axes were then analyzed by ANOVA. For the DGGE analysis,

matrices of presence and absence of bands was then subject to ordination analyses to identify groups of similar samples, or structure in the dataset (Fromin et al., 2002). A second matrix containing summed values for PLFA or functional measurements were used to construct joint plots that overlaid on the PCA plots. The joint plots were used to visualize the relationship between a set of variables (in this case, taxonomic groups, enzyme activities, and stress indicators) and PCA scores. The angle and length of a line indicate the direction and strength of the relationship (McCune and Grace, 2002). The enzyme activities of the samples were analyzed using ANOVA analysis on SAS software (SAS Institute, 1996) to assess spatial and temporal effects. In addition, correlations between PLFA and enzyme activities were investigated using the Mantel test (McCune and Grace, 2002).

Diversity indices were calculated as below: the Shannon-Wiener index,  $H' = -\sum (ni/N) \ln(ni/N)$  (Shannon, 1948); the Simpson dominance index,  $\lambda = \sum (ni/N)^2$  (Simpson, 1949); and the evenness index,  $e = H'/\ln S$  (Pielou, 1966); where  $S$  is the total number of PLFA peaks,  $ni$  is the area of  $i$ th PLFA peak, and  $N$  is the sum of the area of all PLFA peaks for each sample.

## RESULTS

### Soil Inorganic N, Total Microbial Biomass, Enzyme Activities, and Moisture

Inorganic N consistently for both shrub species and seasons, the OutS had the lowest levels but it was not significant at  $P < 0.05$ .

**TABLE 1** | Soil inorganic N, MBC, PLFA<sub>tot</sub> and moisture of soil inside and outside the influence of *G. senegalensis* or *P. reticulatum* during the wet and dry season.

	Season	Soil Sampling Location		
		Outside shrub	Inter-root	Rhizosphere
<i>G. senegalensis</i>				
Inorganic N	Dry	6.7 bA <sup>†</sup>	12.0 aA	14.2 aA
(μg NO <sub>3</sub> -N + NH <sub>4</sub> -N g <sup>-1</sup> )	Wet	8.2 bA	14.7 aA	15.2 aA
MBC (μg g <sup>-1</sup> )	Dry	8.1 cB	24.5 bB	37.7 aB
	Wet	12.1 cA	34.3 bA	45.7 aA
PLFA <sub>tot</sub> (nmol g <sup>-1</sup> )	Dry	9.7 bA	12.9 bB	32.8 aA
	Wet	11.8 bA	37.7 aA	38.5 aA
Soil moisture (%)	Dry	0.49 cB	0.99 bB	2.64 aB
	Wet	5.14 cA	6.47 bcA	11.46 aA
<i>P. reticulatum</i>				
Inorganic N	Dry	8.0 bA	15.0 aA	15.6 aA
	Wet	9.0 bA	16.6 aA	17.1 aA
MBC	Dry	5.8 cB	27.0 bB	45.0 aA
	Wet	15.8 cA	36.9 bA	47.0 aA
PLFA <sub>tot</sub>	Dry	10.0 bA	11.6 bB	28.8 aB
	Wet	12.1 bA	40.7 aA	41.43 aA
Soil moisture	Dry	0.48 cB	1.46 bB	2.41 aB
	Wet	7.84 cA	9.07 bA	11.53 aA

<sup>†</sup>The lower case letters represent means separation between sampling locations within a row and upper case letters represent means separation between seasons for the same soil location; where values followed by the same letter are not significantly different at  $P < 0.05$ .



PLFA<sub>tot</sub> and MBC are indexes of the total microbial biomass and did not show the same treatment effects, except that they both had the RhizoS consistently being highest within a season and shrub species (Table 1). MBC consistently showed a stepwise decrease of: RhizoS>IntS>OutS within a season and shrub species. It also showed consistently higher levels in the wet season over the dry season at all sampling locations and for both species (Table 1).

Overall, both shrub species showed differences in PLFA<sub>tot</sub> between RhizS and OutS (Table 1) in both seasons with InterR being similar to RhizS in the wet season but intermediate in the dry season. During the dry season, PLFA<sub>tot</sub> was significantly higher ( $P < 0.05$ ) for RhizS than IntrS and OutS of both shrub (Table 1).

Acid phosphatase,  $\beta$ -glucosidase and chitinase activities were higher for the rhizosphere soil than the bulk soil (data not shown); the IntrS soil had significantly higher activities than did the non-rhizosphere soil. This is true for both seasons for those enzymes except the chitinase activity which during the dry season was the same whether it was a bulk soil or a non-rhizosphere soil. For urease, activity was significantly higher in rhizosphere soil than in bulk and non-rhizosphere soil only during the dry season. During the wet season, this activity was the same in the bulk and rhizosphere soil but significantly different from the non-rhizosphere soil.

Soil moisture was greatly reduced in the dry season. However, the RhizS soil had significantly higher levels than the other two sampling locations in the dry season for both shrub species.

## Soil Microbial Community Composition

### PLFA Profiling

Consistently, across all the PLFA functional groups in the dry season, RhizS was significantly higher than IntrS and OutS (Figure 1). On average for the dry season, fungal PLFA was 80% greater in the RhizS compared to IntrS for both shrub species (Figure 1). Conversely, in the wet season the fungal PLFAs in RhizS were only about 20% higher than the IntrS and often statistically showing no differences. Similar levels of increase were observed in the RhizS soil for the Gram+ bacterial PLFAs (65%) when compared to IntrS during the dry season. The wet season resulted in a significant increase of all microbial PLFAs in both shrubs, which was more prominent in the case of IntrS compared to those in dry season.

Comparison of the major functional groups measured by PLFA (Gram+, Gram-, fungi, and actinomycetes) showed no major differences among these relative to soil sampling location. That is, for Gram+, Gram-, fungi, and actinomycetes, patterns of each of the sampling locations within a season were quite similar for both shrub species—which was that RhizS remained high between seasons, OutS was always low, and IntrS tended to be not significantly different from the RhizS in the rainy season, with IntrS dropping significantly in the dry season. The most striking effect was on the PLFAs of fungi at the *G. senegalensis* site, which for OutS and IntrS became extremely low in the dry season but then for IntrS showed a dramatic increase in the wet season (1,495% increase). The other general outcome was that averaging across all sampling sites and seasons for both shrub

species showed that, on a nmol basis, Gram+ was higher than Gram- bacteria.

Principal component analysis of soil microbial community had a total variance of 65% explained by the first two axes, with the first axis explaining 47% of the total variance in PLFA community composition (Figure 2). Plant species did not show marked differences for the microbial community structures. The strongest factor in structuring the communities was the location of the soil sample; OutS vs. RhizS ( $p < 0.001$ ). PCA showed that the communities from the IntrS during the wet season were similar to those of the RhizS. Samples from the OutS clustered, and within this cluster they were separated primarily based on the season and then by the shrub species.

The FUN/BACT PLFA ratio was also affected by the presence of shrubs, which was highest in RhizS, followed by IntrS and OutS for both shrubs during both wet and dry seasons (Figure 3), except for *P. reticulatum* in dry season, where it was similar between IntrS and OutS. The FUN/BACT ratio was significantly higher in all soil locations of both shrubs during the wet season, except for OutS of *G. senegalensis*, where the ratio was higher in the dry season.

## DGGE Profiling

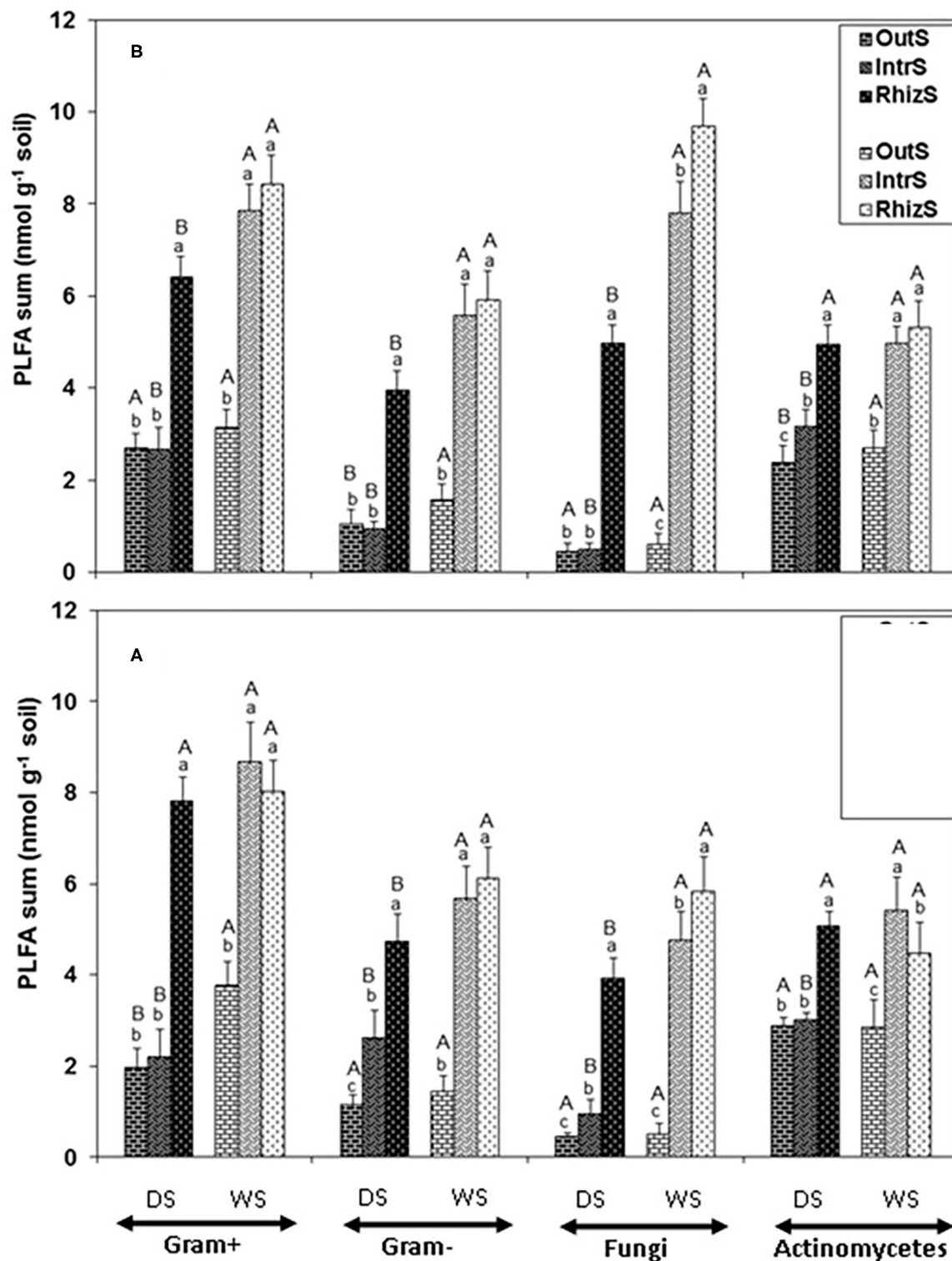
Analysis of DGGE banding data from 16S DNA bacteria profile (Figure 4A) using PCA (Figure 4B) explained a total of 33% for the first two axes. Samples from the RhizS clustered and separated from the IntS and OutS (ANOVA  $p < 0.01$ ), whereas there was minimal separation due to season for the DGGE bacterial profile (Figure 4B). The comparison of the DGGE bacterial banding between new roots and old roots for both species (Figure 4A) showed that the number of bands were higher and more intense in soil associated with new roots than soil associated with old roots during the wet season. During the dry season, intensity of bands was similar, and there was a small but non-significant difference for the number of bands between those two communities.

For the fungal community, the first two axes of PCA of bands explained 33% of the data (Figure 5). Samples clustered for the dry season but not based on sampling location. This was the opposite of bacterial samples where there was a weak seasonal effect but strong soil sampling location effect. During the dry season, fungal community in the RhizS was similar to IntS. The opposite was observed during the wet season where there was a separation between RhizS and IntS.

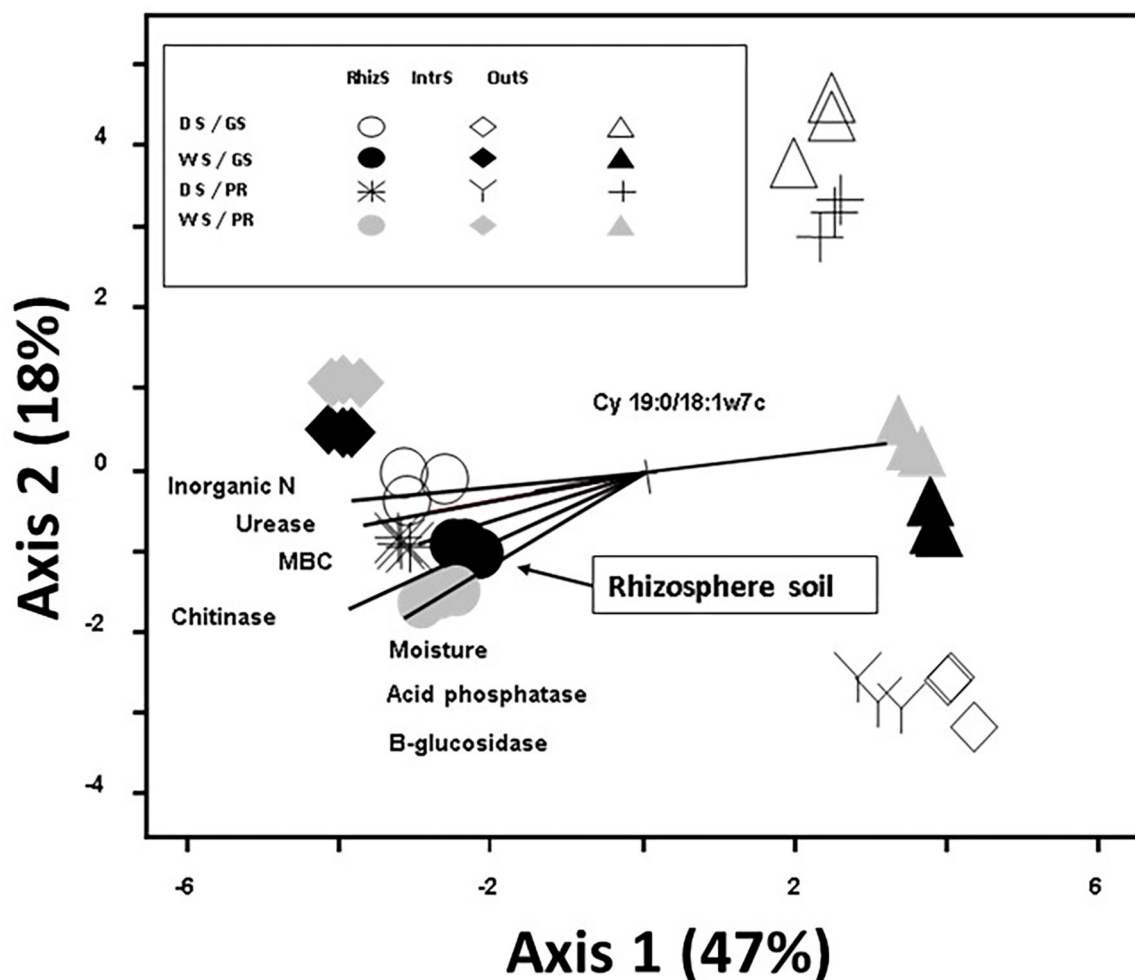
## Stress Ratio Indicators

The stress indicators, Cy 19:0/18:1 $\omega$ 7c and SAT/MONO, were lowest in RhizS followed by IntrS and OutS during both the dry and wet season in the case of both shrubs (Figure 3). The only exception was that SAT/MONO during the wet season for *G. senegalensis* was similar between RhizS and IntrS.

Both Cy19:0/18:1 $\omega$ 7c and SAT/MONO in OutS did not differ significantly between dry and wet seasons. However, Cy19:0/18:1 $\omega$ 7c and SAT/MONO in RhizS and IntrS were significantly higher in dry season than in the wet season of both shrubs, except for SAT/MONO in RhizS of *G. senegalensis*, which was similar between dry and wet season.



**FIGURE 1 |** Summation of PLFAs representing Gram+, Gram-, fungi and actinomycetes microorganisms extracted from soil associated with **(A)** *G. senegalensis*, and **(B)** *P. reticulatum*. Soil samples (RhizS, shrub rhizosphere soil; IntrS, inter-root soil; and OutS, outside the influence of shrub soil) are from the dry season (DS) and wet season (WS). Bars are standard deviations. The lower case letters represent means separation between sampling locations and upper case letters represent mean separation between seasons for the same soil location.



**FIGURE 2 |** Principle Component Analysis based on 23 phospholipid microbial markers from soil associated with *G. senegalensis* (GS) and *P. reticulatum* (PR) which are shrubs that co-exist with row crops in the Sahel. The vectors are joint plots of enzyme activities. Soil samples (RhizS, shrub rhizosphere soil; IntrS, inter-root soil; and OutS, outside the influence of shrub soil) are from the dry season (DS) and wet season (WS).

## Diversity Indices

The Shannon-Wiener diversity index ( $H'$ ) of PLFA was highest in RhizS for both shrubs in both seasons (Table 2). In the dry season  $H'$  index of IntrS and OutS were statistically similar, whereas, in wet season  $H'$  index of Intr S was statistically similar to Rhiz S in both shrubs. The evenness scores, although lower than  $H'$  index, demonstrated the same pattern as  $H'$  index for both shrubs in both seasons. The dominance index ( $D$ ), in contrast, was highest for OutS and lowest for RhizS in dry season of both shrubs. In the wet season, the  $D$  index in *G. senegalensis* was highest for RhizS, with OutS showing no statistically significant differences between RhizS and IntrS. Whereas, in the case of *P. reticulatum*, IntrS demonstrated the highest dominance index, followed by RhizS and OutS.

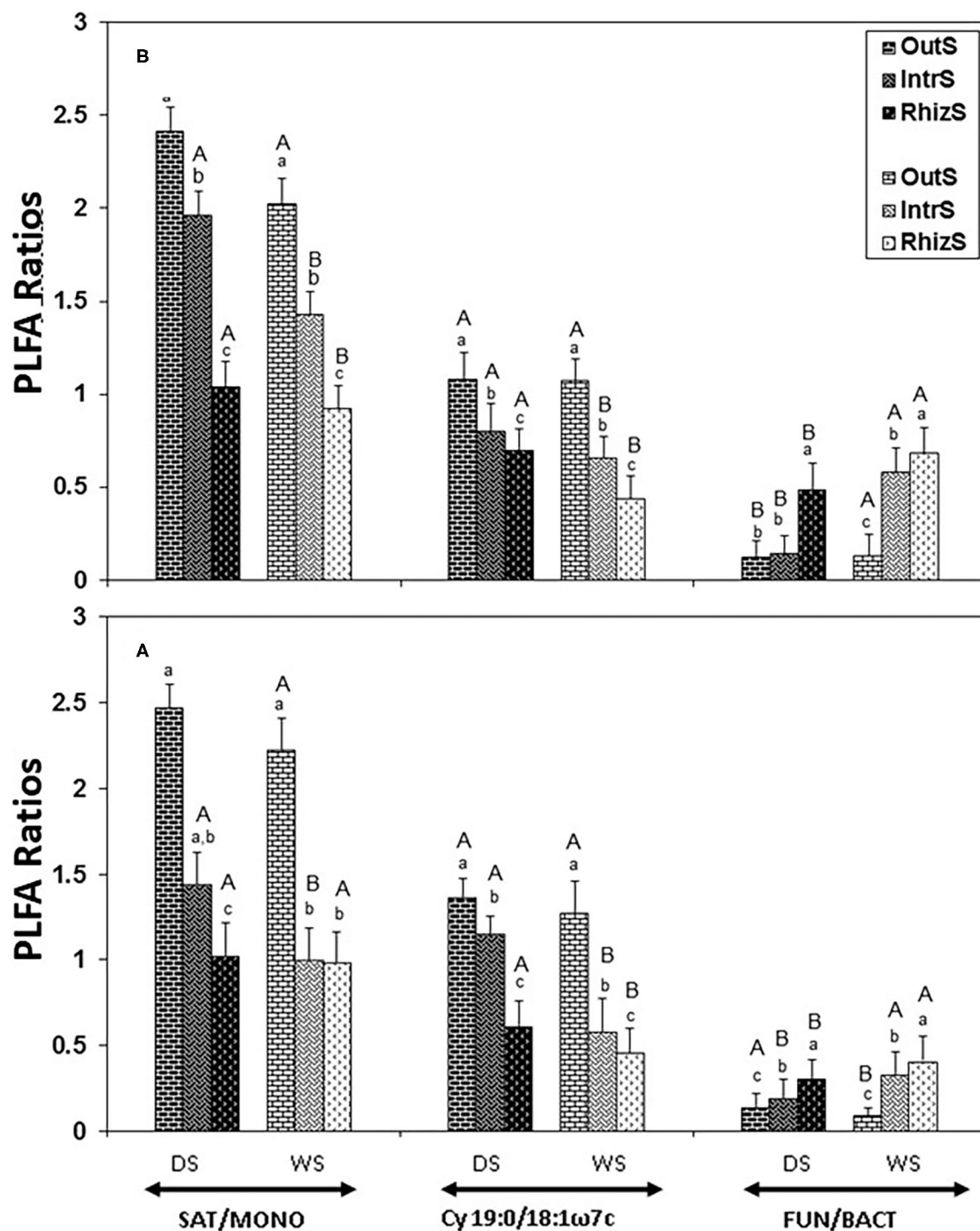
The microbial diversity in OutS did not change between seasons based on diversity indices analysis, as  $H'$ ,  $E$  and  $D$  indices remained statistically similar between dry and wet seasons in both shrubs (Table 2). The Shannon and evenness index in RhizS

also stayed the same between the dry and wet seasons, whereas the dominance index in RhizS was higher in the wet season than in the dry season in both shrubs. The IntrS exhibited higher  $H'$ ,  $E$  and  $D$  indices values in the wet season than in the dry season in both shrubs, except for dominance in *G. senegalensis*, which was higher in the dry season.

## DISCUSSION

### Microbial Community and Enzyme Activities

Both PLFA<sub>tot</sub> and MBC showed that RhizS maintained the highest microbial biomass over soil of the IntS and OutS locations in both wet and dry season (Table 1). Similarly, diversity analysis using PLFAs showed that RhizS had greater microbial diversity than these other sampling locations, again in both seasons (Table 2). Similar to our findings, several studies on crop systems have shown that rhizospheric soil has higher population densities

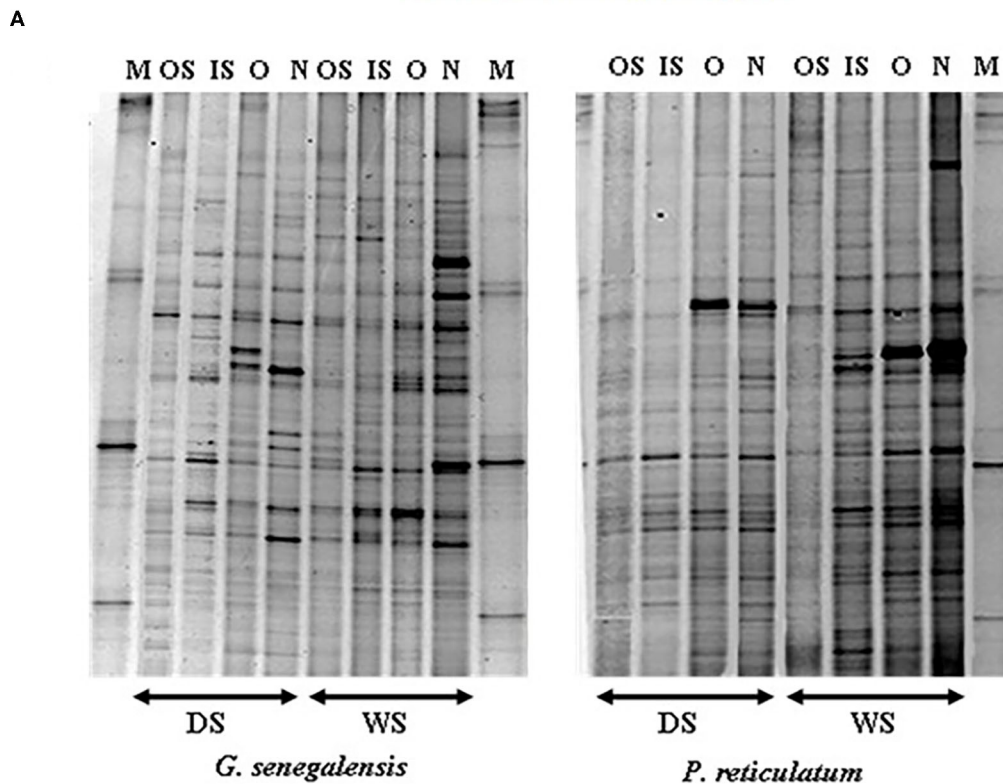
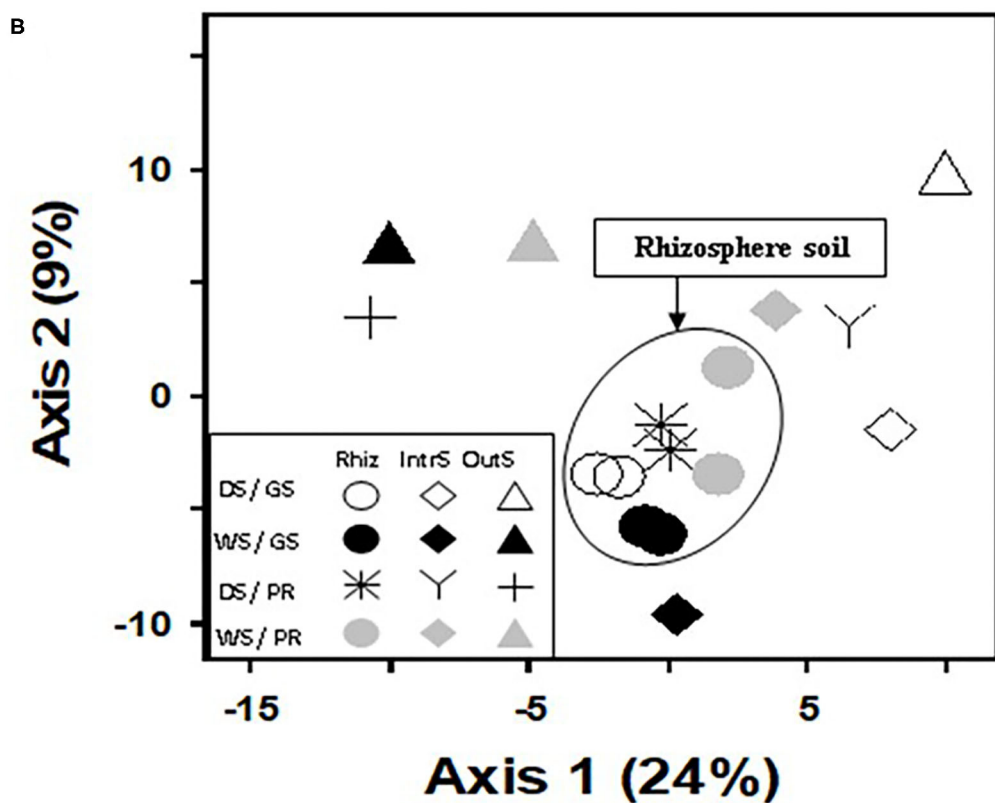


**FIGURE 3 |** Stress indicators (19:0cy/18:1ω7c or SAT/MONO ratios) and FUN/BACT PLFA ratio extracted from soil associated with **(A)** *G. senegalensis*, and **(B)** *P. reticulatum*. Soil samples (RhizS, shrub rhizosphere soil; IntrS, inter-root soil; and OutS, outside the influence of shrub soil) are from the dry season (DS) and wet season (WS). Bars are standard deviations. The lower case letters represent mean separation between sampling locations and upper case letters represent mean separation between seasons for the same soil location.

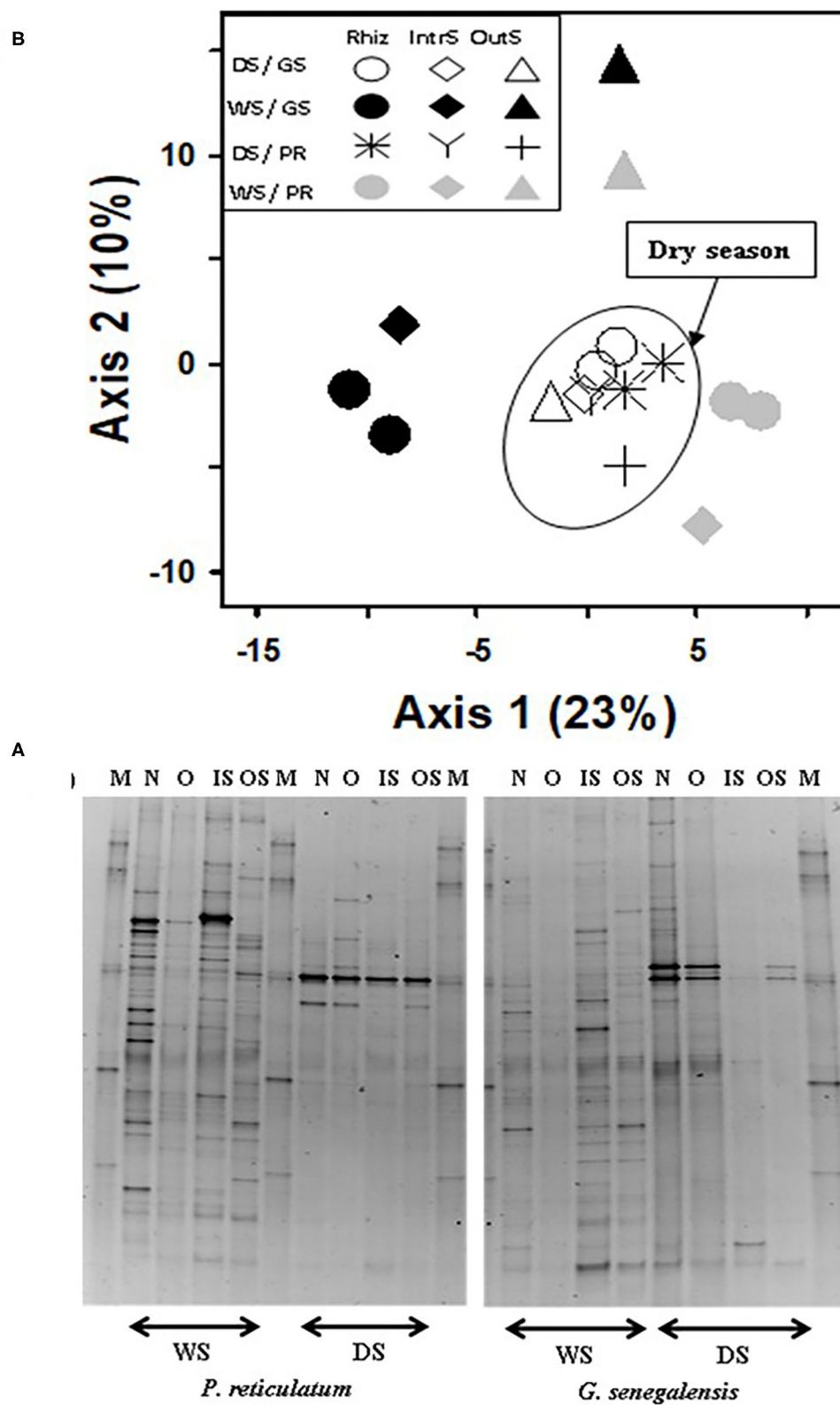
of bacteria and fungi compared to inter-root soil (Curl and Truelove, 1986; Norton and Firestone, 1991; Maloney et al., 1997; Semenov et al., 1999; Steer and Harris, 2000; Marschner

et al., 2002; Butler et al., 2003). Furthermore, it is consistent with Debenport et al. (2015), who showed greater diversity in soil beneath canopies of *P. reticulatum* and *G. senegalensis* than





**FIGURE 4 | (A)** Bacterial DGGE profile of 16S rDNA PCR product extracted from soil, and **(B)** PCA ordination analysis of DGGE banding profiles. Samples are from the wet (WS) and dry season (DS) in two sites with *G. senegalensis* (GS) and *P. reticulatum* (PR) shrubs. M, marker; OS, outside the influence of shrub soil; IS, inter-root soil; O, old roots; and N, new roots. In DGGE gel image M, marker; OS, outside the influence of shrub soil; IS, inter-root soil; O, old roots; and N, new roots. In PCA plot RhizS, shrub rhizosphere soil; IntrS, inter-root soil; and OutS, outside the influence of shrub soil.



**FIGURE 5 | (A)** Fungal DGGE profile of 28S rDNA PCR product extracted from soil and **(B)** PCA ordination analysis of DGGE banding profiles. Samples are from the wet (WS) and dry season (DS) in two sites with *G. senegalensis* (GS) and *P. reticulatum* (PR) shrubs. In DGGE gel image M, marker; OS, outside the influence of shrub soil; IS, inter-root soil; O, old roots; and N, new roots. In PCA plot RhizS, shrub rhizosphere soil; IntrS, inter-root soil; and OutS, outside the influence of shrub soil.

**TABLE 2 |** Diversity indexes based on PLFA profiles.

		Soil Sampling Location		
		Outside shrub	Inter-root	Rhizosphere
<b><i>G. senegalensis</i></b>				
Shannon index (H')	Dry	2.30 bA <sup>†</sup>	2.57 bB	3.97 aA
	Wet	2.66 bA	4.11 aA	3.96 aA
Evenness (E)	Dry	0.93 bA	1.03 bB	1.55 aA
	Wet	1.07 bA	1.60 aA	1.54 aA
Dominance (D)	Dry	0.119 aA	0.110 abA	0.099 bB
	Wet	0.102 abA	0.098 bB	0.109 aA
<b><i>P. reticulatum</i></b>				
Shannon index (H')	Dry	2.32 bA	2.59 bB	3.92 aA
	Wet	2.31 bA	3.88 aA	3.99 aA
Evenness (E)	Dry	0.93 bA	1.04 bB	1.53 aA
	Wet	0.93 bA	1.51 aA	1.56 aA
Dominance (D)	Dry	0.103 aA	0.107 aB	0.093 bB
	Wet	0.123 cA	1.551 aA	1.265 bA

<sup>†</sup>The lower case letters represent means separation between sampling locations and upper case letters represent mean separation between seasons for the same soil location; where values followed by the same letter are not significantly different at  $P < 0.05$ .

soil outside the influence of these shrubs using phylogenetic biomarkers. However, this study was only done on samples collected in the rainy season. Our current study shows that this diversity and increased microbial biomass in rhizosphere soil of these two shrub species is maintained in the dry season (~6 months after rains had stopped).

Summing PLFAs into the functional groups (Gr+, Gr-, fungi, and actinomycetes) indicated that across sampling locations, the pattern of response was quite similar for all these functional groups (in dry season RhizS >> IntrS ≥ OutS; in wet season RhizS similar to IntrS > OutS). This reflects the fact that PLFAs represent broad microbial functional groups. Nonetheless, diversity analysis of PLFAs and DGGE analysis for bacteria do suggest the RhizS is maintaining a more diverse community in both seasons and; on the other extreme, OutS has the lowest diversity. Based on PLFA profiling, the *G. senegalensis* site showed a major shift in fungal biomass between seasons in the IntrS compared to the OutS that was the lowest compared to all other samplings. The soil at the *G. senegalensis* site is very sandy and has low organic matter (as is typical of where *G. senegalensis* dominates in the northern/drier region of Senegal) (Dossa et al., 2010).

The most notable effects of the RhizS based on PLFA profiling was for the fungal and Gr+ bacteria which were significantly greater than OutS in both seasons and shrub species. This is in accordance with the study by Priha et al. (2001) who found that fungal markers 18:2ω6,9c and branched fatty acids, which have commonly been found in Gr+ bacteria (O'Leary and Wilkinson, 1988) were dominant in birch rhizosphere (*Betula pendula* Roth). They attributed this difference to a higher dominance of *Bacillus* species in birch rhizosphere. Butler et al. (2003) also showed a greater amount of the PLFA marker i15:0, which is generally attributed to Gr+ bacteria, in the rhizospheric than soil outside

the shrub canopy. The same authors found that 18:2ω6,9c was the most highly enriched when <sup>13</sup>C-labeled glucose or acetate was added to soils. Analysis of rhizosphere DNA indicated that Gram+ bacteria can be more dominant in the rhizosphere (Smalla et al., 2001).

When water is widely available in the rainy season, this RhizS microbial response in the rhizosphere is largely due to elevated organic inputs that are released and available around roots. Roots produce soluble organic compounds such as carbohydrates, proteins, ad amino acids, sloughed off root cells, and mucilage. This complex mixture of organic compounds provides a source of reduced carbon, nitrogen, and other nutrients to support larger and more diverse microbial communities. This is supported by the higher inorganic N level we found beneath the canopy of both shrubs and by Diedhiou-Sall et al. (2013) who, at the same site as our study, found 45 and 33% greater total C, and 10 and 11% greater total N soil beneath than outside the canopies of *P. reticulatum* and *G. senegalensis*, respectively.

However, in the dry season it would be expected that microbial biomass and diversity would be diminished, even with the organic inputs in the RhizS, because of a lack of water. This dry season response did happen for the soil outside the influence of both shrub species and the IntrS location. However, microbial biomass and the diversity based on PLFA markers in the RhizS were maintained in the dry season and significantly higher than IntS. In contrast, there was no difference in the rainy season between IntS and RhizS for these measures, but outside the influence of the shrub, these microbial properties were lower at that time. This would suggest that there is significant diffusion of C and nutrient-rich exudates from the shrub roots and/or that long-term root turnover beneath the shrubs has built up labile organic resources in the IntrS. So, in the summer when water is not limiting, it has very similar microbial properties to the RhizS.

Soil moisture varied widely in the IntrS and particularly for the OutS locations because of the extended dry season from November to June when there is virtually no rainfall. This is likely the major reason for the large changes in microbial communities we noted between seasons for these soil locations based on PLFA analysis. In contrast, PLFA profiling of the RhizoS microbial communities was much more similar and stable between seasons. This can be attributed to hydraulic lift by shrubs that release water year around (Kizito et al., 2012; Bogie et al., 2018). Bogie et al. (2018) confirmed, using isotopically labeled water, that hydraulically lifted water by *G. senegalensis* is transferred to adjacent millet plants.

The soil moisture data in our study supports the role of hydraulic lift by these shrubs in providing water to microorganisms during the dry season. Indeed, this is reinforced with RhizS having significantly higher levels than the other sampling locations at both sites in the dry season (**Table 1**). Extrapolating from Bogie et al. (2018) on the same soils, the Kuer Matar soil has a wilting point of 1.87%, whereas the soil moisture in the current study in the dry season was 2.64% for the RhizS soil which is estimated to be 47% of field capacity. Although a similar relationship of elevated RhizS at 2.41% soil moisture for the Nioro site it was only slightly higher than wilting point of 2.33% [based on estimate using data from Bogie et al. (2018)]. None-the-less, this was over 5-fold higher than soil outside the influence of *P. reticulatum* during the dry season.

Another effect is microclimate shading of the shrubs (Young, 1995; Stark and Firestone, 1996; Eviner, 2001; Myers et al., 2001). Indeed, Kizito (2006) showed that shrubs buffer diurnal temperature by a 5°C difference. This could affect the microorganisms directly by the lower temperature and by reducing moisture losses below the canopy that is present in the dry season. However, this effect would only occur in the extended dry season for our study site because, following farming practices, the shrubs were coppiced in late spring in preparation for the cropping season.

Litter inputs provide organic inputs and nutrient sources for the near-surface soil for both the RhizS and IntrS microorganisms (Ben-David et al., 2011). Since this is a cropped field, there is some shallow tillage which presumably could incorporate litter to a depth of 5–10 cm. This would be an additional factor for the lower levels of microbial properties outside the influence of the shrubs, that receives limited or only crop residue.

Diversity analysis followed the conclusions reported above for microbial biomass and PLFA profiling of the microbial community. The Shannon ( $H'$ ) and Evenness ( $E$ ) indices were higher for RhizS than IntrS and OutS during the dry season, but in the wet season,  $H'$  and  $E$  indices of RhizS and IntrS were similar—again showing that rhizosphere soil and soil between roots but below the canopy become similar from a diversity perspective when there is adequate water. The higher  $H'$  index of RhizS in the dry season is perhaps due to hydraulic lift by shrubs (Kizito et al., 2012), whereas IntrS moisture fluctuates between dry and wet season causing lower diversity in dry season. Over the year, soil beneath the canopy receives litter

inputs and has root turnover—thus, when there is adequate water in the wet season, IntrS supports greater diversity of microbial community.

The OutS showed a higher Simpson's dominance than RhizS and IntrS during the wet season due to low soil moisture and nutrient availability outside the canopy in the dry season. In the wet season, an increase in dominance was noticed in both RhizS and IntrS compared to the dry season, whereas the Simpson index in OutS remained unaffected between seasons. Enhancement of relative abundance of several populations in RhizS and IntrS in the wet season could be due to the influence of the shrubs on soil microorganisms under optimal moisture and nutrient conditions.

Enzyme activities provide information on the potential of the soil to carry out discrete reactions, which for our study were ones involved in degradation of organic inputs and nutrient mineralization. Of the sampling locations, RhizS consistently had the highest levels of activity, and joint plots of enzyme activities showed a strong correlation to the rhizosphere community composition. Thus, this would suggest that the greater diversity that increases from the OutS, to the IntrS, to the RhizS results in a soil community that has more potential to decompose litter and root inputs and to release nutrients. This follows Dossa et al. (2009) who found that soil from beneath these same shrub species had greater nitrogen and phosphorus mineralization potential.

Correlation analysis of PLFA biomarker functional groups showed high  $r$ -values among these groups and with PLFA<sub>tot</sub> (**Supplementary Table 1**). This would suggest that although all groups were different on an abundance basis this difference was similar or consistent across groups as evidenced by the high correlations.

During the dry season, the lack of water probably limited fungal growth and activity, more so than bacteria. This may explain the lack of difference between the OutS and the inter-root soil for chitinase activity, which is largely associated with the presence of fungi (Sinsabaugh et al., 1993; Sinsabaugh and Moorhead, 1994; Bandick and Dick, 1999). Urease activity was closely related to PLFA biomass which may be due to the fact that ammonium (the product of urease activity) is tightly linked to microbial assimilation (Sylvia et al., 2005). Since organic C and total P is higher in the soil beneath these shrubs (Dossa et al., 2009), increases in acid phosphatase and  $\beta$ -glucosidase activities in the rhizosphere would be expected, as the microbial community would stimulate these enzymes in response to elevated levels of litter substrates and root exudates into the RhizS over OutS that would lack these organic inputs. This is supported by inorganic N levels that were highest in the RhizS soil, which results from the activity of hydrolytic enzymes involved in releasing N from organic matter. Overall, there was a good correlation between enzyme activities and PLFA<sub>tot</sub> ( $r = 0.70$ ,  $p < 0.01$ ). Fernandes (2006) also found significant correlation between enzyme activities and PLFA analysis. These results and that of the joint plots indicate that RhizS of these shrubs has greater potential to decompose organic matter and mineralize nutrients than the IntrS and OutS.



## PLFA vs. DGGE

The PLFA profiling showed a consistent seasonal and sampling location effect on microbial community structure in both univariate microbial PLFA functional group data and based on diversity/clustering using multivariate PCA analysis. A somewhat similar effect for bacterial DGGE diversity analysis was found but not for fungal DGGE PCA analysis across both shrub species.

These divergent effects between PLFA and DGGE are because they measure different parameters. The PLFA method is a direct and quantitative chemical extraction of a cellular biomarker of living cells and is representative of major microbial groups and the overall community. Conversely, the PCR-DGGE analysis depends on amplifying DNA which, for one, may not have come from living material. Furthermore, amplification can be biased, while detecting only 1–2% of the microbial population representing the dominant species present in an environmental sample (MacNaughton et al., 1999). Thus, the DGGE analysis is likely representing a sub-population of what is represented by PLFA biomarkers. Smalla et al. (2001) reported a seasonal difference in the composition of the bacteria community using DGGE analysis. But in their study, they compared the microbial communities over many years. In the present study, the dry season was contrasted with the wet season in a 6-month period.

For the DGGE fungal community, the seasonal effect was more evident in separating the communities than the rhizospheric effect. Buyer et al. (2002), using PLFA methods, also reported the weak separation of the fungal community primarily based on the rhizosphere influence, but they found a clear rhizospheric effect for the bacterial community.

## Microbial Stress Indicators

In the present study, the stress indicators 19:0cy/18:1 $\omega$ 7c and SAT/MONO were higher in the IntrS than the RhizS during both dry and wet seasons, with greater differences noticed during the dry season, where higher levels means greater stress (Willers et al., 2015). The lower stress for the RhizoS location in dry season can be attributed to greater water availability (Table 1) that can only be due to hydraulic lift by the shrubs. In all seasons the RhizoS would be under lower nutrient stress due to the release of root exudates and root tissue turnover (Zelles et al., 1992; Kieft et al., 1994; Zelles et al., 1995; Bossio and Scow, 1998).

The FUN/BACT ratio was highest in RhizS during both seasons, with less difference, but still significantly different between IntrS and RhizS during the wet season. This ratio has been suggested as an indicator of soil microbial community stability (Bardgett and McAlister, 1999; Zelles, 1999). In the present study, water is available in the RhizS that not only directly supports the microbial community but also enables decomposition of litter to proceed in the dry season, providing C and nutrient resources for microorganisms. This could explain fewer differences in FUN/BACT ratio between IntrS and RhizS in the wet season than in the dry season.

## Perspectives

The two shrub species were on two different soil types. Therefore, it was not possible to compare shrub species. The degree to which rhizospheric communities are controlled by plant species, rather than soil type, is not clear, as there are studies in which

plant species growing in the same soil had similar rhizospheric microbial communities, indicating that the influence of the soil may be greater than that of the plant (Buyer et al., 1999; Latour et al., 1999; Blackwood and Paul, 2003). Overall, results of DGGE analysis should be analyzed carefully, because PCR-DGGE can detect only part of the dominant species present in an environmental sample (MacNaughton et al., 1999). The sensitivity of analysis is chiefly limited by the resolving power of the DGGE gel and the imaging system used (Jongman et al., 1995; Fromin et al., 2002). We found PLFA profiling and enzyme activities were more sensitive than DGGE in reflecting the microbial differences across the various soil sampling locations.

The presence of shrubs resulted in more active and diverse communities compared to soil outside the influence of the shrubs. Moreover, during the dry season, shrubs maintained a wetter soil environment, which can be attributed to hydraulic redistribution of water from the wet subsoil to the drier surface soil and was likely important in stimulating the microbial communities. The RhizS had higher enzyme activities in soil in the dry season that was quite similar to the wet season data. PLFA microbial profiling and enzyme activities were significantly inter-correlated and clearly showed strong rhizospheric and seasonal effects.

The results of the present study have implications for plant-microbial interactions relative to biogeochemical processes and organic matter decomposition. *A priori* it would be expected that in these semi-arid agro-ecosystems, any biologically-driven soil processes would largely stop during the dry season. The results of this study suggest otherwise: indeed, that diversity and activity of microbial communities are maintained in the shrub rhizosphere soil during the dry season, presumably due to hydraulic distribution. This is important because these shrubs co-exist with crops throughout the Sahel and therefore, during the dry season, shrub rhizospheres should be able to promote organic matter decomposition and nutrient mineralization and allow for release of plant available N, P, and S. The lack of rain during the dry season would allow for the accumulation of inorganic nutrients that are plant available, because there is no rainfall to cause leaching of nutrients beneath the rooting zone. This follows what farmers report: that if a crop can be established and maintained with the first rains, they get their highest yields on millet, because they can presumably take advantage of nutrients that have accumulated over the dry season. In contrast, if they have to replant when the early rains do not sustain crop growth of the first planting, yields are lower because the accumulated nutrients were leached out of the rooting zone during the early rains (personal communication, Ibrahim; Diedhiou, 2020). Indeed, the elevated levels of inorganic N in both seasons at the RhizS site would support the importance of shrubs combined with hydraulic lift in driving biological nutrient mineralization that would result in the accumulation of nutrients over the dry season that would subsequently be available to crops in the early growing season.

Clearly, our results show that shrubs promote microbial communities and their activity, but further studies are required to determine if these shrub rhizospheres also harbor beneficial microorganisms that could assist crops during the growing season and whether shrub rhizospheres influence the crop

rhizosphere microbial communities that grow within the influence of these shrubs. If shrubs harbor microorganisms that can fix N, solubilize P, produce plant hormones, and/or suppress diseases, this would be very helpful for subsistence farmers to reduce or eliminate external, purchased inputs for crop production. It offers the opportunity to develop shrub-crop systems that are optimized to take advantage of local biological interactions to promote food productivity and reduce risk in this semi-arid environment.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Alleviation of Submergence Stress in Rice Seedlings by Plant Growth-Promoting Rhizobacteria With ACC Deaminase Activity

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Submergence stress slows seed germination, imposes fatalities, and delays seedling establishment in rice. Seeds of submergence susceptible rice variety IR 42 were inoculated with four 1-aminocyclopropane-1-carboxylic acid (ACC) utilizing isolates viz., *Bacillus* sp. (AR-ACC1), *Microbacterium* sp. (AR-ACC2), *Methylophaga* sp. (AR-ACC3), and *Paenibacillus* sp. (ANR-ACC3) and subjected to submergence stress under controlled conditions for 7 days. Seeds treated with *Microbacterium* sp. AR-ACC2, *Paenibacillus* sp. ANR-ACC3, and *Methylophaga* sp. AR-ACC3 significantly enhanced the germination percentage (GP), seedling vigor index (SVI), and other growth parameters like root and shoot length and total chlorophyll contents, when compared with nonbacterized seeds submerged similarly. However, the values were statistically at par when control seeds were treated with 1- $\alpha$ -(2-aminoethoxyvinyl) glycine hydrochloride (AVG), a known inhibitor of ethylene production. Results suggest that stress ethylene production was significantly reduced by around 85% in seedlings treated with *Microbacterium* sp. AR-ACC2 as compared with untreated control seeds under submergence. *Paenibacillus* sp. ANR-ACC3 and *Methylophaga* sp. AR-ACC3 were the next effective strains. Ethylene synthesis in seedlings was statistically at par with seeds treated with AVG suggesting ACC deaminase can effectively reduce ethylene levels in plants subjected to submergence. *Bacillus* sp. (AR-ACC1) was neither able to significantly promote seedling growth parameters nor inhibit ethylene production as compared with control seeds. Results suggest that flooded soil planted to rice harbor microorganisms with plant growth-promoting properties that can be used effectively to alleviate submergence stresses in susceptible rice varieties under field conditions.

**Keywords:** ACC deaminase, ethylene production, growth promoting rhizobacteria, rice, seed germination, seedling growth, submergence stress

## INTRODUCTION

Soil flooding is one of the most important abiotic constraints for rice yields, with complete submergence of plants being particularly serious for rice farmers in the rainfed lowlands of humid and semihumid tropics of Asia (Jackson and Ismail, 2015). Indeed, submergence affects more than 5 million ha of rainfed lowlands in India resulting into drastic reduction in rice growth and yield

(Sandhu et al., 2019). Heavy rainfall and poor drainage causing accumulation of water in the fields shortly after sowing, leads to poor crop establishment and causes hindrance to widespread acceptance of direct-seeded rice practices. This is due to the inability of most rice varieties to germinate and reach water surface under complete submergence. Submergence is also an acute problem at the early growth stages which causes serious damages of plants by uprooting of the seedlings particularly at coastal lowland where traditional semidwarf varieties are cultivated (Kato et al., 2020). Plants tolerant to submergence stress exhibit morphological adaptations like shoot elongation, formation of aerenchyma and adventitious roots, and metabolic adaptations like activation of fermentation process, induction of anaerobic proteins (ANPs), and hormonal regulation (Magneschi and Perata, 2009; Jackson and Ismail, 2015).

Ethylene is an important plant growth hormone mediating growth and development of plant only at optimal concentration. Both indole acetic acid (IAA) and ethylene have ability to promote plant growth in a coordinate fashion. IAA-induced ethylene at lower concentration promotes initiation of root but at higher concentration, inhibiting root growth (Ali and Kim, 2018). Complete submergence induces ethylene biosynthesis and ethylene accumulation in most plant species (Khan et al., 2020). In contrast to other stresses, gas diffusion is inhibited under submergence because the diffusion coefficient of ethylene in water is 10,000 times lower than in air. The excess ethylene entrapped in the plant tissue and its effect under submergence is crop species specific. Rice is the most studied crop for submergence-tolerant species because it is an important crop in humid tropic and areas affected by high rainfall (Jackson, 2008; Khan et al., 2020).

During submergence, the increase in ethylene production is due to an increase in the activity of both 1-aminocyclopropane-1-carboxylic acid (ACC) synthase in the submerged roots and ACC oxidase in the shoots. Induction of ACC synthase enhances ACC level in root where as its oxidation to ethylene is blocked due to anoxic condition. This accumulated and unmetabolized ACC transport to the stem by the transpiration stream and convert to ethylene by ACC oxidase enzyme in the presence of oxygen. This boost in ACC oxidase activity in stem initiates the adventitious root formation during submergence (Fukao and Bailey-Serres, 2008).

The growth inhibitory effect of the submergence stress on crop plants can be alleviated by lowering the stress ethylene level. Beside several harmful chemical ethylene inhibitors, plant growth-promoting rhizobacteria (PGPR) having ACC deaminase enzyme alleviate stress ethylene levels by cleaving ACC to  $\alpha$ -ketobutyrate and ammonia (Oleńska et al., 2020). It has been the subject of much research due to its biochemical properties, action, substrate specificity, and even its genetic regulation and mode of heredity. Lowering of stress ethylene level facilitates the crop to substantially tolerate different environmental stresses, all of which induce the plant to increase its endogenous level of ethylene (Ali and Kim, 2018).

Grichko and Glick (2001a) studied the effect of inoculation with ACC deaminase producing PGPR on tomato subjected to flooding. Seeds of wild-type tomato plants were inoculated

either with *Pseudomonas putida* UW4, *Enterobacter cloacae* CAL2, *P. putida* (ATCC17399/pRKACC), or *P. putida* (ATCC17399/pRK415); the first three of these bacterial strains were carrying and expressing the gene for ACC deaminase. The inoculation of ACC deaminase containing PGPR resulted in statistically significant differences in overall plant growth, leaf chlorophyll content, and substantially decreased ethylene production in leaf petiole tissue, thereby ameliorating some of the damages to plants caused by flooding. Thus, the “protective effect” of ACC deaminase-containing plant growth-promoting bacteria on flooded tomato plants results from these bacteria acting as a sink for ACC, thereby lowering the level of ethylene that can be formed in the shoots.

Rice is the second most widely consumed cereal in the world next to wheat and more often than not is subjected to submergence stress during its growing period (Oladosu et al., 2020). In the present study, we examined the efficiency of ACC deaminase containing PGPR, isolated from rice field soils, in alleviating the inhibitory effect of submergence stress in germinating rice seeds and on seedling growth. Various parameters of rice growth at the seedling stage were thoroughly evaluated to understand their possible growth-promoting activities on rice. We also assessed whether inoculation with select PGPR would modulate the ethylene level in plant to enhance its growth.

## MATERIALS AND METHODS

### Bacterial Strains and Their Plant Growth-Promoting Traits

*Bacillus* sp. AR-ACC1 (HM063033), *Microbacterium* sp. AR-ACC2 (HM063034), *Methylophaga* sp. AR-ACC3 (HQ222610), and *Paenibacillus* sp. ANR-ACC3 (HM063032) were isolated from soil samples of Ganjam district of Orissa (19° 11' 04.7" to 20° 06' 48.7" N and 84° 48' 06.3" to 85° 12' 49.5" E) (Bal et al., 2013). These bacterial strains were isolated based upon their ability to utilize ACC, present in sterile DF salt minimal medium, as the sole nitrogen source (Dworkin and Foster, 1958). All the four strains were screened positive for IAA and ammonia production and only two strains (*Microbacterium* sp. AR-ACC2 and *Paenibacillus* sp. ANR-ACC3) produced siderophore but none of them were phosphate solubilizer and HCN producer (Bal et al., 2013). All the four isolates were believed to be plant growth-promoting rhizobacteria based on their ability to promote the elongation of rice roots under gnotobiotic conditions (Bal et al., 2013). The isolates were grown on either solid or liquid tryptic soy broth (TSB) medium at 30°C.

### Alleviation of Submergence Stress in Rice by PGPR Inoculation

#### Plant Materials and Experimental Set-Up

The seeds of two rice cultivars contrasting in their tolerance to submergence stress namely, *cv.* IR42 which is a susceptible cultivar and *cv.* Panikekua, resistant to submergence stress, were collected from the Germplasm collection center of Central Rice Research Institute, Cuttack. Seeds were surface sterilized by

dipping in 95% ethanol and in 0.2% HgCl<sub>2</sub> solution for 3 min followed by rinsing five times with sterile distilled water (Glick, 2020). Fifty seeds of each cultivar were placed on the field soil (150 g) surface in tall beakers (1,000 ml) and submerged with sterile distilled water to a depth of 10 cm. The beakers were maintained in the laboratory (28 ± 2°C) in diffuse light. Germination was measured daily up to 7 days.

## PGPR Inoculation to Rice Under Submergence Stress

Bacterial suspensions of four isolates were prepared by growing the strains in 250 ml conical flask containing DF salt minimal medium at 28°C for 24 h in an orbital shaking incubator at 100 rpm. The cultures were centrifuged at 8,000×g, and the bacterial cell pellets of the strains were suspended in 0.5 ml sterile 0.03 M MgSO<sub>4</sub> and the absorbance was adjusted to OD = 1 at 600 nm (Penrose and Glick, 2003). Surface sterilized rice seeds (cv. IR42) were incubated for 1 h at room temperature with the appropriate treatment: sterile 0.03 M MgSO<sub>4</sub> (negative controls), 10<sup>-4</sup>M AVG (Sigma, India) in 0.03 M MgSO<sub>4</sub> (positive control), and fresh bacterial suspensions in sterile 0.03 M MgSO<sub>4</sub> (Penrose and Glick, 2001).

The experiment was carried out in sterile 1,000 ml tall glass beaker for 7 days. After incubation with each treatment, 25 seeds were planted by sterilized forceps on the soil (150 g) surface in tall beakers and submerged with sterile distilled water to a depth of 10 cm inside laminar airflow. The beakers were placed in a growth chamber in completely randomized design with five replications for each treatment. Maximum and minimum temperatures were maintained at 28 and 20°C, respectively with a cycle of 12 h dark/light (Sapsirisopa et al., 2009). The number of seeds that sprouted and germinated was counted daily up to 7 days. After final count, germination percentage (GP) and seedling vigor index (SVI) were calculated using the equations described by Long et al. (2008). Plant growth parameters like root length (RL), shoot length (SL), root fresh weight (RFW), shoot fresh weight (SFW), root dry weight (RDW), and shoot dry weight (SDW) of 10 randomly selected seedlings from each replication were measured at the time of harvest. Chlorophyll concentrations were determined by spectrophotometry in 80% acetone extracts following the equation described by Porra (2002).

## Estimation of Ethylene Level in Plant Tissue

For ethylene estimation in rice seedlings by gas chromatography (GC), five seedlings each from different treatments were kept in tightly sealed vials in the dark for 1 h at 30°C. Headspace gas (1 ml) was drawn by airtight syringe (2 ml) and injected into GC (Model-Ceres 800 plus, Thermo-Scientific) packed with a Porapak-Q column (183 cm length and 0.3 cm internal diameter, 80/100 mesh) and equipped with flame ionization detector (FID). The GC was adjusted to 100, 300, and 150°C for oven, injection, and detection temperature, respectively. The carrier gas was N<sub>2</sub> at a flow rate of 30 ml min<sup>-1</sup>, and the combustion gas was H<sub>2</sub> at a flow rate of 30 ml min<sup>-1</sup> with air at the flow rate of 300 ml min<sup>-1</sup>. The amount of ethylene emission was expressed as nmol

of ethylene gfw (fresh weight)<sup>-1</sup> h<sup>-1</sup> by comparing the standard curve of pure ethylene (9.12 ppm in nitrogen, Matheson Tri-Gas) (Fišerová et al., 2008; Siddikee et al., 2011).

## Statistical Analysis

Data on various character sets were subjected to statistical analysis by using a statistical package (IRRISTAT version 3.1: International Rice Research Institute, Los Banos, Philippines). The mean difference comparison between the treatments was analyzed by analysis of variance wherever necessary and subsequently by Duncan's multiple range test (DMRT) at *P* < 0.05.

## RESULTS

### Screening Submergence Tolerance of Rice Cultivars

The submergence susceptible rice cultivar, IR42, and tolerant cultivar Panikiekua had the same germination efficiency (100%) after 3 days of growth under normal conditions, but GP of both the cultivar decreased under submergence, with a substantially greater reduction in the susceptible cultivar (54%) (Table 1). Moreover, the germinated seeds of the susceptible variety became brown in color and died after 6 days of submergence (Figure 1). Hence, for this study, susceptible variety, IR42 was selected to screen the effect of ACC deaminase producing PGPR strains on rice seedling growth under submergence.

### Effect of PGPR Strains on Seed Germination Under Submergence Stress

All the four ACC utilizing isolates identified as *Bacillus* sp., *Microbacterium* sp., *Methylophaga* sp., and *Paenibacillus* sp. respectively, enhanced the overall plant growth under normal condition (Bal et al., 2013) and were subsequently investigated to quantify the effect of these rhizobacteria in ameliorating the damage caused by submergence stress.

The effects of the bacterial strains on seed germination, seedling vigor, and ethylene synthesis are summarized in Table 2. Seeds treated with *Microbacterium* sp. AR-ACC2 and *Paenibacillus* sp. ANR-ACC3 enhanced the GP by 48.15 and 40.74% over the negative control. These effects were, however, statistically at par with that of the positive control of amendment with AVG (Sigma, India), a known inhibitor of ethylene production (44.4% more than negative control). Inoculation with *Microbacterium* sp. AR-ACC2 also increased seedling vigor when compared with the negative control. It was followed by the vigor of seeds treated with *Paenibacillus* sp. ANR-ACC3 and *Methylophaga* sp. AR-ACC3 and was statistically at par with the treatment by AVG (Table 2). However, the isolate *Bacillus* sp. AR-ACC1 was not able to enhance the seedling vigor under submergence.

The effect of inoculation of ACC deaminase producing PGPR on growth of flooded rice plants was assessed after 7 days of submergence (Figure 1). Seven days of continuous submergence resulted in a decrease in all the growth parameters studied (Table 3). However, inoculation with the ACC deaminase containing PGPR strains noticeably stimulated the growth of



**TABLE 1** | Germination percentage of different rice cultivars during 7 days submergence.

Rice cultivars	Germination percentage (%)													
	Days of submergence <sup>a</sup>													
	Day 1		Day 2		Day 3		Day 4		Day 5		Day 6		Day 7	
	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F
IR-42	45.4 ± 2.3 <sup>b</sup>	0	87.2 ± 2.9	5.0 ± 0.9	100 ± 0.0	25.4 ± 3.0	100 ± 0.0	54.4 ± 4.7	100 ± 0.0	54.4 ± 4.7	100 ± 0.0	54.4 ± 4.7	100 ± 0.0	54.4 ± 4.7
Panikekoa	42.4 ± 1.9	0	90.2 ± 2.4	24.4 ± 1.6	100 ± 0.0	66.4 ± 1.0	100 ± 0.0	79.4 ± 1.4	100 ± 0.0	85.4 ± 0.9	100 ± 0.0	85.6 ± 0.8	100 ± 0.0	85.6 ± 0.8

<sup>a</sup>NF, non-flooded; F, Flooded submerged.<sup>b</sup>Mean of five replicates ± SEM (standard error of mean).**FIGURE 1** | Root and shoot growth of 7-day-old rice (cv. IR-42) seedlings exposed to submergence stress under gnotobiotic conditions. **(A)** 0.03 M MgSO<sub>4</sub> (negative control); **(B)** *Bacillus* sp. (AR-ACC1); **(C)** *Methylophaga* sp. (AR-ACC3); **(D)** *Paenibacillus* sp. (ANR-ACC3); **(E)** *Microbacterium* sp. (AR-ACC2); **(F)** AVG (positive control).

the plants both under normal and submerged conditions. Under submerged condition, seedlings treated with all bacterial strains except *Bacillus* sp. AR-ACC1 significantly ( $P \leq 0.05$ ) enhanced all the plant growth parameters studied, as compared with the negative control.

Seedlings treated with *Microbacterium* sp. AR-ACC2 showed highest root elongation to 3.16 cm. The bacterium also caused maximum shoot elongation (3.94 cm) followed by *Methylophaga* sp. AR-ACC3 and *Paenibacillus* sp. ANR-ACC3. Data regarding SFW and RFW showed that *Microbacterium* sp. AR-ACC2 caused maximum promotion to 14.94 and 5.33 mg, respectively.

Strain *Methylophaga* sp. AR-ACC3 and *Paenibacillus* sp. ANR-ACC3 were the next effective strain to enhance fresh weights. In the case of SDW and RDW, seedlings treated with the three strains *Microbacterium* sp. AR-ACC2, *Methylophaga* sp. AR-ACC3, and *Paenibacillus* sp. ANR-ACC3 showed maximum promotion. These were significantly ( $P \leq 0.05$ ) higher than the negative control but statistically at par with each other. The ratio of SFW and SL of submerged plants was in the following comparative order of *Microbacterium* sp. AR-ACC2 > *Paenibacillus* sp. ANR-ACC3 > *Methylophaga* sp. AR-ACC3 > AVG > *Bacillus* sp. AR-ACC1 > negative control. The ratios



**TABLE 2 |** Effect of select PGPR inoculation on Germination percentage (GP), Seedling Vigor Index (SVI), chlorophyll a and b contents and ethylene production by rice (cv IR-42) seedlings under submergence stress for 7 days.

Treatments	GP (%)		Seedling vigor index (SVI)		Chl a (mg.gm fresh wt)		Chl b (mg.gm fresh wt <sup>-1</sup> )		Chl a+b (mg.gm fresh wt <sup>-1</sup> )		Chl a/b		C <sub>2</sub> H <sub>4</sub> (nmol C <sub>2</sub> H <sub>4</sub> gfw <sup>-1</sup> hr <sup>-1</sup> )	
	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F
MgSO <sub>4</sub>	100 <sup>a</sup>	54 <sup>c</sup>	1858 <sup>d</sup>	83 <sup>c</sup>	2.14 <sup>d</sup>	0.13 <sup>c</sup>	0.61 <sup>c</sup>	0.04 <sup>c</sup>	2.75 <sup>c</sup>	0.17 <sup>c</sup>	3.51 <sup>a</sup>	3.55 <sup>a</sup>	0.18 <sup>a</sup>	13.02 <sup>d</sup>
AVG	98 <sup>a</sup>	78 <sup>a</sup>	1828 <sup>d</sup>	247 <sup>b</sup>	2.05 <sup>e</sup>	0.19 <sup>c</sup>	0.57 <sup>d</sup>	0.05 <sup>c</sup>	2.62 <sup>d</sup>	0.24 <sup>c</sup>	3.59 <sup>a</sup>	3.51 <sup>a</sup>	0.07 <sup>a</sup>	1.72 <sup>a</sup>
AR-ACC1	98 <sup>a</sup>	50 <sup>c</sup>	2190 <sup>c</sup>	75 <sup>c</sup>	2.17 <sup>d</sup>	0.13 <sup>c</sup>	0.61 <sup>c</sup>	0.04 <sup>c</sup>	2.79 <sup>c</sup>	0.17 <sup>c</sup>	3.54 <sup>a</sup>	3.47 <sup>a</sup>	0.06 <sup>a</sup>	10.32 <sup>c</sup>
AR-ACC2	100 <sup>a</sup>	80 <sup>a</sup>	2486 <sup>a</sup>	568 <sup>a</sup>	2.71 <sup>a</sup>	0.41 <sup>a</sup>	0.76 <sup>a</sup>	0.12 <sup>a</sup>	3.46 <sup>a</sup>	0.53 <sup>a</sup>	3.59 <sup>a</sup>	3.52 <sup>a</sup>	0.06 <sup>a</sup>	1.98 <sup>a</sup>
AR-ACC3	100 <sup>a</sup>	74 <sup>b</sup>	2319 <sup>b</sup>	293 <sup>b</sup>	2.22 <sup>c</sup>	0.30 <sup>b</sup>	0.62 <sup>c</sup>	0.09 <sup>b</sup>	2.84 <sup>c</sup>	0.38 <sup>b</sup>	3.61 <sup>a</sup>	3.49 <sup>a</sup>	0.06 <sup>a</sup>	3.35 <sup>b</sup>
ANR-ACC3	100 <sup>a</sup>	76 <sup>a</sup>	2316 <sup>b</sup>	298 <sup>b</sup>	2.38 <sup>b</sup>	0.31 <sup>b</sup>	0.67 <sup>b</sup>	0.09 <sup>b</sup>	3.06 <sup>b</sup>	0.40 <sup>b</sup>	3.54 <sup>a</sup>	3.43 <sup>a</sup>	0.07 <sup>a</sup>	3.14 <sup>b</sup>
LSD (5%)	5.973		78.52		0.073		0.024		0.094		0.166		1.08	

Mean of five replicate observations.

In a column, means followed by a common letter are not significantly different ( $p < 0.05$ ) by Duncan's multiple range test.

of SDW and SFW of rice seedlings treated with *Microbacterium* sp. AR-ACC2, *Methylophaga* sp. AR-ACC3, and *Paenibacillus* sp. ANR-ACC3 were statistically at par (Table 3).

Under submergence stress, the chlorophyll concentration of rice seedlings significantly decreased compared with the normal plants (Table 2). However, significant increase in total chlorophyll and both Chl *a* and Chl *b* contents were observed in the submerged plants that were treated with the rhizobacteria excepting *Bacillus* sp. AR-ACC1 when compared with flooded nonbacterized plants. Treatment with *Microbacterium* sp. AR-ACC2 caused maximum increase in the concentration of total chlorophyll (0.53 mg) and also both Chl *a* (0.41 mg) and Chl *b* (0.12 mg), followed by *Methylophaga* sp. AR-ACC3 and *Paenibacillus* sp. ANR-ACC3 as the next effective strains. However, seedlings treated with *Bacillus* sp. AR-ACC1 did not show any significant enhancement of chlorophyll content under submergence.

## Effect of PGPR on Ethylene Production

The submergence stress resulted in a significant increase in the production of ethylene as compared with the nonflooded plants (Table 2). Highest amount of ethylene production was observed in the negative control plants as compared with plants from the positive control (treated with AVG) under submergence. Ethylene synthesis was reduced by around 85% in seedlings treated with *Microbacterium* sp. AR-ACC2 and was statistically at par with the positive control plants. *Paenibacillus* sp. ANR-ACC3, *Methylophaga* sp. AR-ACC3, and *Bacillus* sp. AR-ACC1 were the next effective strains to reduce ethylene level by around 76, 74, and 21%, respectively (Table 2).

## DISCUSSION

The underlying postulate that was tested in this study was to lower the stress ethylene level by the oxidation of ACC with the help of ACC deaminase enzyme and subsequent colonization of roots by PGPR strains having multiple plant growth-promoting traits to stimulate the plant growth under submergence stress. The four bacterial strains used in this investigation belonged to *Bacillus* sp., *Microbacterium* sp., *Methylophaga* sp., and *Paenibacillus* sp. that were tested as PGPR in earlier studies under normal conditions (Bal et al., 2013). To study their role in the alleviation of submergence stress in rice seedlings, the performance of the four ACC deaminase-producing PGPR strains was monitored in plant growth chamber under submergence stress.

Submergence stress slows seed germination, imposes mortality, and delays seedling establishment in direct-seeded rice (Kato et al., 2020). The primary experiment for selection of rice cultivar to study the inhibitory effect of submergence stress on rice seedling showed that the GP was decreased by around 50% in the case of the susceptible variety, IR-42 whereas the GP decreased by only 15% in the case of the tolerant variety (Table 2) as reported earlier (Das et al., 2004). Hence, the susceptible cultivar, IR-42 was selected for submergence stress study.

**TABLE 3 |** Effect of select PGPR on different growth parameters of submergence susceptible rice (cv. IR-42) seedlings exposed to submergence stress for 7 days.

Treatments	Length (cm)		Shoot (SL)		Root (RL)		Fresh weight (mg)		Dry weight (mg)		Shoot (SDW)		SFW/SL (mg.cm <sup>-1</sup> )		SDW/SFW	
	Shoot (SL)		Root (RL)		Fresh weight (mg)		Dry weight (mg)		Shoot (SDW)		SFW/SL (mg.cm <sup>-1</sup> )		SDW/SFW			
	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F	NF	F
MgSO <sub>4</sub>	9.02 <sup>d</sup>	0.48 <sup>d</sup>	9.56 <sup>d</sup>	1.06 <sup>d</sup>	22.20 <sup>d</sup>	1.00 <sup>d</sup>	37.86 <sup>c</sup>	1.54 <sup>d</sup>	2.14 <sup>e</sup>	0.15 <sup>d</sup>	3.61 <sup>d</sup>	0.14 <sup>b</sup>	3.96 <sup>b</sup>	1.46 <sup>d</sup>	0.10 <sup>a</sup>	0.09 <sup>b</sup>
AVG	8.86 <sup>d</sup>	1.00 <sup>c</sup>	9.79 <sup>d</sup>	2.16 <sup>b</sup>	22.60 <sup>d</sup>	2.34 <sup>c</sup>	38.40 <sup>c</sup>	4.84 <sup>c</sup>	2.44 <sup>d</sup>	0.23 <sup>b</sup>	4.38 <sup>c</sup>	0.50 <sup>b</sup>	3.92 <sup>b</sup>	2.26 <sup>c</sup>	0.11 <sup>a</sup>	0.10 <sup>b</sup>
AR-ACC1	10.10 <sup>c</sup>	0.50 <sup>d</sup>	12.25 <sup>c</sup>	1.00 <sup>d</sup>	25.60 <sup>c</sup>	0.95 <sup>d</sup>	52.14 <sup>b</sup>	1.75 <sup>d</sup>	3.20 <sup>c</sup>	0.19 <sup>c</sup>	5.30 <sup>b</sup>	0.21 <sup>b</sup>	4.26 <sup>a</sup>	1.79 <sup>d</sup>	0.10 <sup>a</sup>	0.12 <sup>a</sup>
AR-ACC2	11.16 <sup>a</sup>	3.16 <sup>a</sup>	13.71 <sup>a</sup>	3.94 <sup>a</sup>	26.78 <sup>a</sup>	5.33 <sup>a</sup>	57.42 <sup>a</sup>	14.94 <sup>a</sup>	4.00 <sup>a</sup>	0.49 <sup>a</sup>	6.07 <sup>a</sup>	1.68 <sup>a</sup>	4.21 <sup>a</sup>	3.81 <sup>a</sup>	0.11 <sup>a</sup>	0.11 <sup>a</sup>
AR-ACC3	10.48 <sup>b</sup>	1.92 <sup>b</sup>	12.71 <sup>b</sup>	2.50 <sup>b</sup>	25.64 <sup>c</sup>	4.01 <sup>b</sup>	52.60 <sup>b</sup>	8.44 <sup>b</sup>	3.44 <sup>b</sup>	0.40 <sup>a</sup>	5.40 <sup>b</sup>	1.34 <sup>a</sup>	4.15 <sup>a</sup>	3.40 <sup>b</sup>	0.10 <sup>a</sup>	0.11 <sup>a</sup>
ANR-ACC3	10.32 <sup>b</sup>	2.06 <sup>b</sup>	12.85 <sup>b</sup>	1.86 <sup>c</sup>	27.11 <sup>b</sup>	4.28 <sup>b</sup>	56.49 <sup>a</sup>	6.80 <sup>b</sup>	3.54 <sup>b</sup>	0.42 <sup>a</sup>	5.86 <sup>a</sup>	1.39 <sup>a</sup>	4.40 <sup>a</sup>	3.68 <sup>a</sup>	0.10 <sup>a</sup>	0.11 <sup>a</sup>
LSD (5%)	0.254		0.578		0.647		2.509		0.19		0.36		0.352		0.014	

Mean of five replicate observations.

In a column, means followed by a common letter are not significantly different ( $p < 0.05$ ) by Duncan's multiple range test.

RL, root length; SL, shoot length; RFW, root fresh weight; SFW, shoot fresh weight; RDW, root dry weight; SDW, shoot dry weight; NF, non-flooded; F, flooded.

The inhibitory effect of submergence stress on plant growth is directed by accelerated synthesis of ethylene (Kumar et al., 2020). Hypoxia during submergence causes an increase in the synthesis of ACC in roots due to both induction of ACC synthase genes and arrest of ACC oxidation (Houben and Van de Poel, 2019). During flooding, the concentration of ACC in roots increases and roots release high amount of ACC to the soil. The enzyme-substrate relationship demonstrates that ACC deaminase does not have a particularly high affinity for ACC (Gamalero and Glick, 2015). Moreover, ACC levels in plants are typically in micromolar range; therefore, in most plant tissues, the ACC concentration will be dramatically below the  $K_m$  of ACC deaminase for this substrate. Hence, based on the Michaelis–Menten rate equation, an increase in the ACC concentration due to flooding caused parallel increase in the rate of ACC cleavage (Olanrewaju et al., 2017). In this way, the conversion of ACC to  $\alpha$ -ketobutyrate and ammonia is favored over its oxidation to ethylene despite the fact that ACC oxidase binds ACC with a much higher affinity than does ACC deaminase (Glick et al., 1998).

The results obtained in the present investigation agree with the abovementioned predictions. A simple correlation analysis between *in vitro* ACC deaminase production and ethylene synthesis reduction by the isolates indicated a positive correlation ( $r = 0.92$ ,  $n = 4$ ), suggesting a direct impact of ACC deaminase activity on ethylene content under submergence. Similar to an earlier report by Grichko and Glick (2001b) on flooded tomato plants, this study revealed that inoculation with all the four ACC deaminase-containing PGPR strains caused a significant reduction in ethylene production compared with the negative control plants under submergence (Table 2). The ethylene level in plant tissue had significant negative correlation with GP ( $r = -0.96$ ,  $P = 0.05$ ) and seedling vigor ( $r = -0.78$ ,  $P = 0.05$ ) which suggested that ethylene content has direct negative impact on germination rate. Inoculation with strain *Microbacterium* sp. AR-ACC2 caused maximum reduction (around 85%) in ethylene synthesis which was statistically at par with the plants treated with ethylene inhibitor AVG (Table 2).

Waterlogging accelerates the synthesis of stress ethylene and higher concentrations of ethylene have inhibitory effects on root growth that may lead to significant reduction in plant height, plant fresh and dry weights, and chlorophyll content (Loreti et al., 2016). Hence, it is imperative to regulate the ethylene production in the close vicinity of plant roots for normal growth and development of the plants (Kumar et al., 2020). Earlier studies (Grichko and Glick, 2001a; Ali and Kim, 2018) on inoculation of tomato and *Ocimum sanctum* plants with ACC deaminase producing PGPR showed substantial tolerance to flooding stress implying that bacterial ACC deaminase lowered the effects of stress-induced ethylene. Present study on rice plant treated with ACC deaminase containing PGPR strains also revealed that rice seedlings exhibited alleviation of stress ethylene production and significantly increased tolerance to submergence stress than the negative control plants (Figure 1). Except *Bacillus* sp. AR-ACC1 other three strains exhibited significant ( $P \leq 0.05$ ) growth-promoting activities in rice seedlings under gnotobiotic conditions, including increased rate of germination, root and shoot length, fresh and dry weight of

root and shoot, and total chlorophyll content. Inoculation with *Microbacterium* sp. AR-ACC2 enhanced the RL maximum up to fivefold over the negative control plants. It was the most promising strains to enhance other plant growth parameters: SL (~3-fold), RFW (~4-fold), SFW (~9-fold), RDW (~2-fold), SDW (~11-fold), SFW/SL (~2-fold), and SDW/SFW (~0.2-fold) (Table 3). Inoculation with ACC deaminase-containing bacteria promotes root growth of developing seedlings of various crops (Glick, 2020). The differences in plant growth promotion among the isolates are also attributed to their individual rhizospheric competencies and hydrolyzing the ACC synthesized in roots. The elongation of root system in submerged plant might be due to the alleviation of ethylene inhibitory effect due to the ACC deaminase producing PGPR treatment.

Regulation of stress ethylene level is not the only trait of PGPR strains to enhance plant growth but other growth-promoting mechanisms also contribute to growth promotion. IAA produced by most of the PGPR strains play an imperative role as a direct mechanism of plant growth enhancement (Kochar and Srivastava, 2012). It is likely that IAA and ACC deaminase stimulated root growth in a coordinated fashion (Nascimento et al., 2018). The complex cross-talk between IAA and ethylene in plant growth promotion by PGPR suggested that ACC deaminase producing PGPR might decrease the extent of IAA signal transduction inhibition by ethylene (Glick et al., 1998; Nascimento et al., 2018). Use of PGPR strains having multiple plant growth-promoting traits is expected to help increase crop productivity on a sustainable basis. All the bacteria used in the present study enhancing seed germination and seedling growth had the ability to produce IAA and ammonia (Bal et al., 2013) which might have helped the plants to withstand the submergence stress. *Microbacterium* sp. and *Paenibacillus* sp. were also siderophore producers.

Enhanced ethylene synthesis in submerged plants could promote chlorophyll degradation and leaf senescence that may reduce photosynthetic carbon fixation during and after submergence resulting in the depletion of carbohydrate reserves with a consequent increase in plant mortality (Adak et al., 2011). Leaf chlorophyll content decreased in submerged plants as compared with plants without submergence stress. However, treatment with ACC deaminase containing strains (except *Bacillus* sp. AR-ACC1) significantly increased the concentration of total chlorophyll and both Chl *a* and *b* in submerged plants in comparison with negative control plant (Table 2). *Microbacterium* sp. AR-ACC2 enhanced the total chlorophyll content by twofold over negative control plants. The increased chlorophyll content and expanded root architecture resulting from inoculation of PGPR strains would likely have improved

photosynthetic capacity and higher nutrient uptake efficiency, respectively which in turn would have favored higher ratio of SFW to SL and SDW to SFW and provide some protection against submergence stress (Table 3) (Biswas et al., 2000; Grichko and Glick, 2001b). Thus, the microbial treatments provided to the plants were found beneficial under submerged condition as it reduced the inhibitory effect of stress ethylene and consequently enhanced the plant growth parameters of inoculated plants to withstand the stress.

Tolerance to anaerobic conditions during germination, often referred to as anaerobic germination is a complex genetically controlled process (Yang et al., 2019). Accordingly, breeding direct seeded rice varieties with anaerobic germination has been difficult. Most rice varieties fail to germinate and thrive under anaerobic conditions in waterlogged fields, leading to poor seedling establishment. ACC deaminase can significantly decrease ACC levels in plants, especially plants subjected to flooding stress, thereby decreasing the amount of stress ethylene and the subsequent damage to the plant that might occur as a consequence of that stress ethylene. This can be achieved either through the interaction of ACC deaminase-containing plant growth-promoting bacteria with plant roots or by the development of transgenic plants expressing this enzyme (Grichko and Glick, 2001b). Our study shows that flooded soil planted to rice harbor several microorganisms with plant growth-promoting properties that can be used effectively to alleviate such stresses due to submergence under field conditions.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

HB carried out the research under the supervision of TA and also HB wrote the manuscript. TA reviewed and edited the manuscript. Both authors approved the submitted version.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Evaluation of Apple Root-Associated Endophytic *Streptomyces pulveraceus* Strain ES16 by an OSMAC-Assisted Metabolomics Approach

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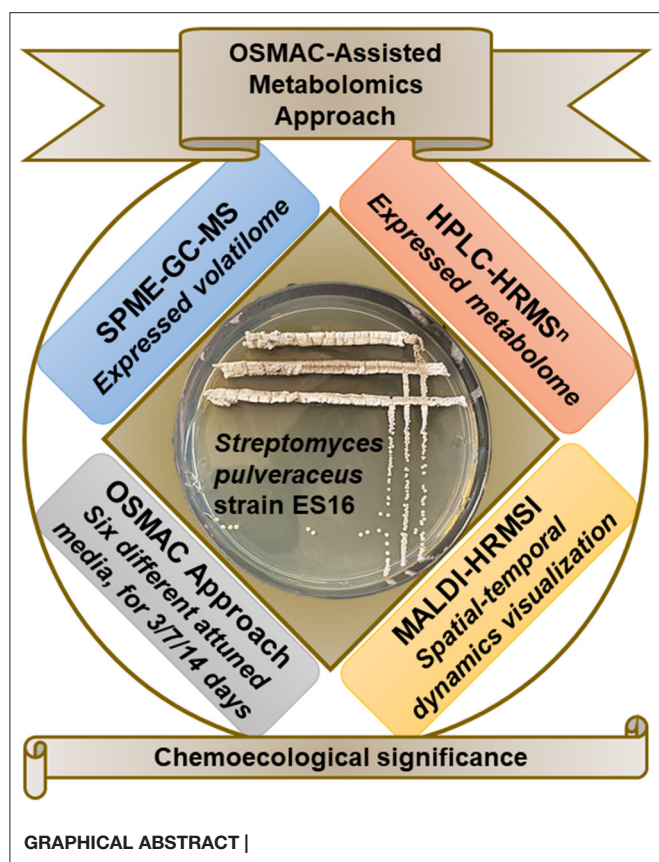
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The One Strain Many Compounds approach (OSMAC) is a powerful and comprehensive method that enables the chemo-diversity evaluation of microorganisms. This is achieved by variations of physicochemical cultivation parameters and by providing biotic and abiotic triggers to mimic microorganisms' natural environment in the lab. This approach can reactivate the silent biosynthetic routes of specific metabolites typically not biosynthesized under standard laboratory conditions. In the present study, we combined the OSMAC approach with static headspace solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS), high-performance liquid chromatography-high-resolution tandem mass spectrometry (HPLC-HRMS<sup>n</sup>), and matrix-assisted laser desorption/ionization high-resolution mass spectrometry imaging (MALDI-HRMSI) to evaluate the chemoeological significance of an apple root-associated endophytic *Streptomyces pulveraceus* strain ES16. We employed the OSMAC approach by cultivating the endophyte in six different media conditions and performed temporal studies over 14 days. Analysis of the volatilome revealed that only under stressful conditions associated with sporulation, endophytic *S. pulveraceus* ES16 produces geosmin, a volatile semiochemical known to attract the soil arthropods Collembola (springtails) specifically. Subsequently, targeted metabolic profiling revealed polycyclic tetramate macrolactams (PTMs) production by the endophyte under stress, which are bioactive against various pathogens. Additionally, the endophyte produced the iron-chelating siderophore, mirubactin, under the same conditions. The structures of the compounds were evaluated using HRMS<sup>n</sup> and by comparison with literature data. Finally, MALDI-HRMSI revealed the produced compounds' spatial-temporal distribution over 14 days. The compounds were profusely secreted into the medium after production. Our results indicate that endophytic *S. pulveraceus* ES16 can release the signal molecule geosmin, chemical defense compounds such as the PTMs, as well as the siderophore mirubactin into the host plant apoplast or the soil for ecologically meaningful purposes, which are discussed.

**Keywords:** apple replant disease, geosmin, HPLC-HRMS, MALDI-HRMSI, mirubactin, OSMAC, SPME-GC-MS, *Streptomyces pulveraceus*



## INTRODUCTION

Plant-associated mutualistic microorganisms such as endophytic microorganisms, commonly known as endophytes, which colonize plants' internal tissues, frequently contribute to host metabolic function and protect plants against pests and diseases by producing biocontrol traits such as bioactive secondary metabolites (Carrión et al., 2019; White et al., 2019; Morales-Cedeño et al., 2020; Newman and Cragg, 2020). For example, endophytes are known to produce natural products that possess

a plethora of biological activities ranging from antibacterial, antifungal, cytotoxic, anticancer, insecticidal to communication molecules (Kusari et al., 2017; Ancheeva et al., 2020; Joo et al., 2020). These biocontrol traits might be amenable to utilization by genetic, epigenetic, and metabolic fine-tuning of endophytes that can then be used as biologics (or biological control agents) to protect host plants in the field conditions without the negative environmental impacts of conventional pesticides. However, to date, biologics or endophytic microbial biocontrol agents have mostly failed to work in field conditions. This is frequently attributed to insufficient expression of biocontrol traits *in planta* or the introduced organism's failure to be competitive in the host environment. Whole-genome sequencing data shows that only a minor portion of endophytes' biosynthetic potential is expressed under standard *in vitro* culture conditions (Khare et al., 2018). Current evidence indicates that the synthesis of these "cryptic" compounds is induced *in planta* in the presence of other interacting micro- and macro-organisms through transcriptional and epigenetic regulatory mechanisms (Mhlongo et al., 2018; Cheng et al., 2019; Jones et al., 2019; Babalola et al., 2020). Chemical interactions among endophytes or between endophytes and interacting partners such as associated endophytes, host plant, invading pathogens, insects, and soil arthropods lead to sustained production of physiologically relevant metabolites, and interspecies crosstalk is often necessary for triggering the production of natural products that are not produced without a suitable trigger (Mhlongo et al., 2018; Cheng et al., 2019; Jones et al., 2019; Babalola et al., 2020). Only suitable interactions lead to the continuous and sustained production of one or more metabolites in an ecological niche.

Hence, it is necessary to understand the chemical-ecological interactions of endophytes on a case-by-case basis to fully exploit the potential of natural product biosynthesis and sustainable production for agriculture and industrial production of desired pharmaceutically relevant compounds, as well as to elucidate the intricate mesh of endophytic microbial chemoecological networks with coexisting organisms. A comprehensive and robust methodological approach employed to achieve this is called the One Strain Many Compounds (OSMAC) approach (Bode et al., 2002; Maghembe et al., 2020). The OSMAC approach enables establishing a comprehensive metabolic profile for each microorganism, revealing the compounds produced by the organism in its natural ecological niche (Romano et al., 2018; Pan et al., 2019; Maghembe et al., 2020). *In vitro*, culture-based OSMAC approach includes modulation of the physiochemical culture parameters, the addition of ecological niche-mimetic biotic and abiotic stress factors and triggers, and bioprocess optimization in a bid to selectively express cryptic gene clusters that are only expressed under particular conditions in the original natural habitat of the microorganism under study (Pan et al., 2019; Maghembe et al., 2020).

Numerous studies have substantiated the biosynthetic capacity of *Streptomyces* species, many of which exhibit an endophytic lifestyle, synthesize various bioactive metabolites, and communication molecules that are not only important from a biochemical and molecular standpoint but also from an ecological perspective (van der Meij et al., 2017; Spasic et al.,

**Abbreviations:** ARD, Apple Replant Disease; CID, Collision Induced Dissociation; COSY, Correlation Spectroscopy; ESI, Electrospray Ionization; DHB, 2,5-Dihydroxybenzoic Acid; EI, Electron Ionization; fhOrn,  $\delta$ -N-formyl- $\delta$ -N-hydroxyornithine; GC, Gas Chromatography; GYM, Glucose Yeast Malt; HESI, Heated Electrospray Ionization; HPLC, High-Performance Liquid Chromatography; HR, High-Resolution; HRMS, High-Resolution Mass Spectrometry; HSAF, Heat Stable Antifungal Factor; MALDI, Matrix-Assisted Laser Desorption/Ionization; min, Minute; LB, Lysogeny Broth (Medium); LC, Liquid Chromatography; LOD, Limit of Detection; LTQ, Linear Trap Quadrupole; MeOH, Methanol; MS, Mass Spectrometry; MS<sup>n</sup>, Tandem Mass Spectrometry, *m/z*, Mass-to-Charge Ratio; NA, Nutrient Agar; NCBI, National Center for Biotechnology Information; NIST, National Institute of Standards and Technology; NMR, Nuclear Magnetic Resonance; NOESY, Nuclear Overhauser Effect Spectroscopy; OSMAC, One Strain Many Compounds; PDA, Potato Dextrose Agar; ppm, Parts Per Million; PTM, Polycyclic Tetramate Macrolactam; Rpm, Rotations Per Minute; RT, Retention Time; SM, *Streptomyces* Medium; SPME, Solid Phase Microextraction; TIC, Total Ion Current; UV, Ultra-Violet; VOC, Volatile Organic Compound.

2018; Law et al., 2020; Quinn et al., 2020). Over the past few decades, *Streptomyces* have received a significant amount of attention due to their vast production of secondary metabolites and their contribution to modern medicine, agriculture, and veterinary practice, among others. These species also play a significant role in ecology by processing and breaking down enzymes, as well as plants and fungi cell walls. For example, *Streptomyces reticuli* can degrade complex carbohydrates and cellulose (Wibberg et al., 2016), whereas *Streptomyces* sp. TH-11 has shown chitinolytic activities (Hoang et al., 2010). Bafilomycins produced by an endophytic *Streptomyces* species demonstrate antifungal, antitumor, and ionophore properties (Yu et al., 2011). In another study, endophytic *Streptomyces* spp. have successfully reduced the disease severity of *Fusarium* spp. on wheat (Colombo et al., 2019). Apart from inhibiting phytopathogens, they also promote plant growth through phytohormones and facilitate the uptake of nutrients by producing compounds such as siderophores (Terra et al., 2020).

The replant disease of apple orchards is a major recurring problem in different regions of the world, the etiology of which is agreed to be a combination of various biotic factors, including multiple pathogenic fungal complexes and is influenced by abiotic factors (Mazzola and Manici, 2012; Winkelmann et al., 2019). Apple Replant Disease (ARD) typically presents a stressful environment for apple plants, endophytes, and associated microbiome (Mazzola and Manici, 2012; Yim et al., 2015; Radl et al., 2019; Winkelmann et al., 2019). The plant roots show signs of browning and blackening, tip necrosis, and reduced root hairs to exemplify this (Grunewaldt-Stöcker et al., 2019). Above the soil, stunted growth and reduced plant biomass are observed, as shown in several studies (Yim et al., 2013, 2015; Weiß et al., 2017). There is a decrease in the fruit yield, and the fruit size and taste are negatively influenced. Building upon an earlier study (Nicola et al., 2018), we recently found an increased abundance of actinobacteria (particularly *Streptomyces* species) in roots grown in ARD soils compared to healthy roots grown in non-ARD soils using molecular barcoding (Mahnkopp-Dirks et al., 2020). Interestingly, we found that the increased abundance of *Streptomyces* in apple roots grown in ARD soils negatively correlated with increased shoot length and fresh mass. Furthermore, gene sequencing revealed that *Streptomyces* taxa are selectively enriched in the rhizosphere in ARD soils in split-root experiments (Balbín-Suárez et al., 2020). However, whether these observations are causal or correlated remains unclear. Further, the involvement and the precise role of yet-unexplored “ecological links” in the form of interacting micro- or macro-organisms such as associated endophytes, rhizosphere microbes, or soil arthropods, among others, is not fully known. Notably, the chemical basis of these observations, particularly the role of natural products such as signal or communication molecules and compounds with niche-relevant biological activities that mediate the underlying chemical ecology, has not been studied.

Therefore, we investigated a cultivable endophytic *Streptomyces pulveraceus* strain ES16 that we isolated from apple plant roots (*Malus domestica*) within the Central Experiment 1 setup (CE1; Ellerhoop, Spring 2018) of the BMBF BonaRes

program ORDIAmur (Mahnkopp et al., 2018). In particular, we wanted to answer the following three questions about the endophytic *S. pulveraceus* strain ES16:

1. What are the secreted volatile semiochemicals or signal molecules?
2. What are the bioactive, chemical defense compounds produced?
3. What are the spatial-temporal dynamics of the produced compounds?

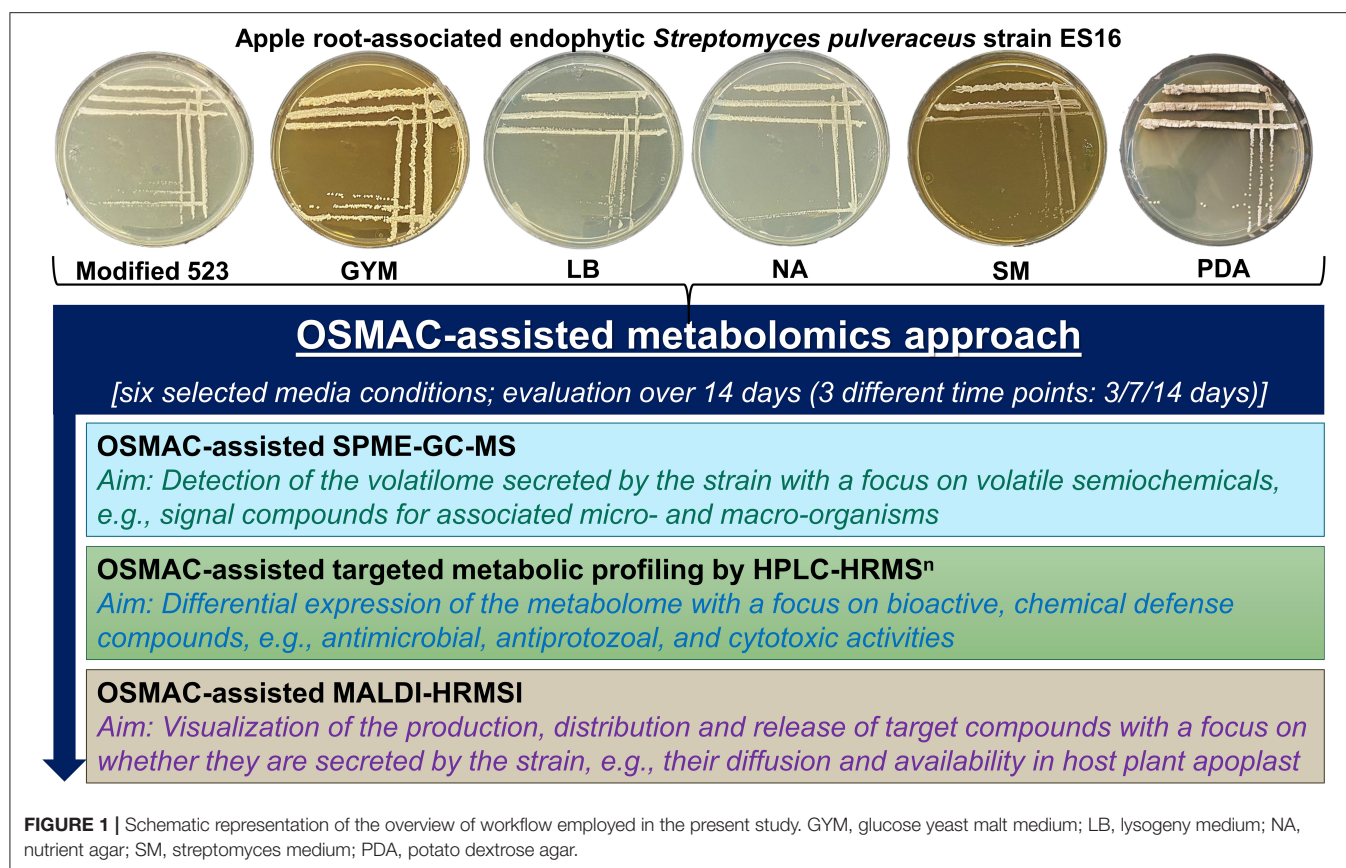
To answer the above questions, we employed an OSMAC approach by cultivating endophytic *S. pulveraceus* ES16 in six different media compositions ranging from nutrient-rich to minimal conditions, including media to stress the organism under submerged conditions (broth) as well as on agar (Figure 1). Under the influence of different OSMAC conditions, we investigated the volatilome produced by the strain by static headspace solid-phase microextraction-gas chromatography-mass spectrometry (SPME-GC-MS) focusing on volatile semiochemicals or signal compounds. After that, we established the selectively expressed metabolic profiles under the different OSMAC conditions, typically focusing on bioactive compounds such as antimicrobial, antiprotozoal, and cytotoxic activities using high-performance liquid chromatography-high resolution tandem mass spectrometry (HPLC-HRMS<sup>n</sup>). Finally, we employed matrix-assisted laser desorption/ionization high-resolution mass spectrometry imaging (MALDI-HRMSI) to visualize the production, distribution, and release of target compounds in high spatial resolution and temporally for 14 days. We discuss the results obtained concerning the possible chemoecological significance of apple root-associated endophytic *S. pulveraceus* strain ES16.

## MATERIALS AND METHODS

### Isolation, Identification, and Establishment of an Axenic Culture of Endophytic *Streptomyces pulveraceus* ES16

Endophytic *Streptomyces pulveraceus* strain ES16 (“Ellerhoop Spring Isolate No. 16”) was isolated from surface-disinfected apple roots (*Malus domestica*) grown in grass control (ARD-unaffected) soil within the Central Experiment setup 1 (CE1; Ellerhoop, Spring 2018) of the BMBF BonaRes ORDIAmur program (Mahnkopp et al., 2018). For isolation, 1 cm pieces of surface-disinfected fine roots ( $\varnothing < 2$  mm) were placed in Petri dishes containing 523 medium (Viss et al., 1991) and stored for ~7 days at room temperature. After this, emerging colonies were picked and incubated for 1–7 days in liquid 523 medium until growth was visible. 1 mL of suspension was used for DNA extraction based on Quambusch et al. (2014). The 16S rRNA gene was amplified using the primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (GGYTACCTTGTTACGACTT) (Weisburg et al., 1991). 16S rDNA fragments were sequenced with the Sanger method (Sanger et al., 1977) by Microsynth Seqlab (Göttingen, Germany), the obtained sequence (Supplementary Table 1) was blasted





(Blastn) (Zhang et al., 2000) against the NCBI database (<https://www.ncbi.nlm.nih.gov/>), and submitted (accession no. MW580619).

## OSMAC-Assisted Cultivation and Fermentation

We optimized our OSMAC approach established for endophytic bacteria (Eckelmann et al., 2018) to cultivate endophytic *Streptomyces pulveraceus* strain ES16. Thus, the endophyte was cultivated in six different attuned media conditions, ranging from nutrient-rich to minimal conditions, for selecting different phenotypes (e.g., sporulation under stress) and metabolic expression patterns for this particular strain, both under submerged conditions (broth) as well as on agar. These included the enriched media *viz.* modified 523 medium and streptomyces medium (SM), the moderately nutrient-rich lysogeny medium (LB) and basal nutrient medium (agar, NA; broth, NB), as well as glucose yeast malt medium (GYM) and potato dextrose medium (agar, PDA; broth, PDB) that served as minimal media for this strain by selectively limiting phosphate and/or nitrogen, thereby inducing moderate to high-stress and sporulation. The components of the media and related references are detailed in **Supplementary Table 2**. The cultures were incubated at 28°C (Memmert Incubator, Schwabach, Germany), and the bacterial growth, morphology, and OSMAC-relevant phenotypic characteristics were monitored and documented regularly over

14 days. For submerged cultivation and fermentation, 500 mL broth of each of the six media was prepared in 1,000 mL Erlenmeyer flasks and autoclaved at 121°C for 15 min (Autoclave VB-55, Systec, Wetzten, Germany). One loop of the bacteria was added to the broth under sterile conditions, and the flask was sealed. The inoculated broths were placed in a shaker incubator (Lab-Rotation Incubator Multitron 2, INFORS HT, Einsbach, Germany) and shaken at 125 rpm at 28°C for 3, 7, and 14 days, respectively. As negative controls, 100 mL of all six sterile, uninoculated media broths were incubated simultaneously. All setups and experiments were performed in biological triplicates.

## Extraction of Agar Plates

The 3-, 7- and 14-day old bacterial agar plates and negative control plates were crushed to small pieces using a spatula. Each plate was covered with 20 mL HPLC-grade MeOH (J. T. Baker, Deventer, The Netherlands) and was mixed well. The mixture was transferred to a beaker and was extracted in an ultrasonic bath (Sonorex Longlife, Bandelin, Berlin, Germany). After 15 min, the mixture was filtered. This step was repeated thrice (*i.e.*, extraction with 3 × 20 mL fresh MeOH). The filtrate was concentrated to dryness under reduced pressure in a rotary evaporator (Laborota 4001 Efficient, Heidolph, Schwabach, Germany) and resuspended in 4 mL HPLC-grade MeOH (J. T. Baker, Deventer, The Netherlands) for further analyses.



## Extraction of Fermented Cultures (Broths)

On the 3rd, 7th, and 14th day of cultivation and under sterile conditions, 100 mL of each inoculated broth was transferred to a 250 mL Erlenmeyer flask. Each flask was placed in an ice bath and ultrasonicated thrice for 15 min in the Sonoplus ultrasonic device (Bandelin, Berlin, Germany) equipped with ultrasonic lance UW 3200 (Bandelin, Berlin, Germany). The flasks' content was transferred to Eppendorf centrifuge tubes and was centrifuged at 10,000 rpm for 10 min to separate the biomass (Centrifuge Allegra™ IR, Beckman Coulter GmbH, Krefeld, Germany). The supernatant was decanted, frozen, and was freeze-dried overnight in a Vaco 5 freeze dryer (ZIRBUS technology GmbH, Bad Grund, Germany). The residue was extracted thrice with 20 mL HPLC-grade MeOH (J. T. Baker, Deventer, The Netherlands) in an ultrasonic bath (Sonorex Longlife, Bandelin, Berlin, Germany) for 15 min, and filtered. The filtrate was concentrated to dryness under reduced pressure in a rotary evaporator (Laborota 4001 Efficient, Heidolph, Schwabach, Germany), and was resuspended in 4 mL HPLC-grade MeOH (J. T. Baker, Deventer, The Netherlands). The uninoculated negative control blanks were extracted following the same procedure.

## Static Headspace Solid-Phase Microextraction-Gas Chromatography-Mass Spectrometry (SPME-GC-MS)

Static headspace SPME-GC-MS was performed according to the published procedure (Yalage Don et al., 2020), suitably modified. Agar plates seeded with *S. pulveraceus* ES16 were incubated for 5 days at 28°C, and ca. one gram (small pieces) of each bacterial agar plate was cut under sterile conditions and transferred to a headspace vial. The vial was then incubated for another 2 days so that the vial atmosphere was enriched with the volatilome secreted by the bacterium cultivated in a specific agar medium. After that, the vial septum was punctured with a solid-phase microextraction (SPME) needle coating, and the fiber (PDME, 75 µm) was exposed to absorb the bacterial volatilome. After 15 min, the fiber was retracted, and the SPME was inserted directly into the GC-MS, where the bacterial volatilome was thermally desorbed. The analysis of volatile organic compounds (VOCs) was performed with a Trace GC Ultra system (Thermo Fisher Scientific, Waltham, USA), coupled to an ISQ mass spectrometer (Thermo Fisher Scientific, Waltham, USA). The separation of the compounds was achieved via an Optima 5 MS (30 m, 0.25 mm ID, 1 µm, Macherey-Nagel, Düren, Germany) GC column. The initial oven temperature was set to 60°C for the first 2 min and was ramped to 175°C at a rate of 20°C/min. The temperature was again increased to 260°C with the second ramp of 4°C/min and held for 2 min resulting in a total run time of 30 min. The mass spectrometer equipped with an electron impact source (IE) was operated at 70 eV with an ion source temperature of 200°C and a scan range of  $m/z$  50–550.

## High-Performance Liquid Chromatography-High Resolution Tandem Mass Spectrometry (HPLC-HRMS<sup>n</sup>)

100 µL of each extract was transferred to an HPLC vial and was concentrated to dryness in a concentrator (Savant SDP1010 SpeedVac, Thermo Fisher Scientific, Waltham, USA). 100 µL of a 2:1 H<sub>2</sub>O and MeOH mixture was added to the vial, and the residue was reconstituted by vortexing (Vortex mixer VF2, IKA-Werke GmbH, Staufen, Germany). The sample was centrifuged at 6,600 rpm for 5 min (Mini Centrifuge, MCF-2360, LMS CO., LTD., Tokyo, Japan), and the supernatant was transferred to an inlet and measured. The measurements were either carried out with an Agilent 1200 system HPLC (Waldbronn, Germany) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, USA) or a Nexera X2 HPLC (Shimadzu Scientific Instruments, Maryland, USA) coupled with an LTQ-Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, USA). A Nucleoshell C18 reverse-phase column (2.7 µm, 150 × 4.6 mm, Macherey-Nagel, Düren, Germany) was used for chromatographic separation at 30°C with H<sub>2</sub>O (+ 0.1% HCOOH) (A) and MeOH (+ 0.1% HCOOH) (B) gradient (flow rate 300 µL min<sup>-1</sup>). The gradient program was as follows: 95% A isocratic for 2 min, linear gradient to 100% B over 26 min, 100% B isocratic for 6 min, the system returned within 0.5 min to initial conditions of 95% A and was equilibrated for 2.5 min. The LTQ-Orbitrap XL and the LTQ-Orbitrap were equipped with a HESI ion source with 5 kV voltage at 350°C. The ion source was operated with He as collision gas, and N<sub>2</sub> as sheath- (40 arbitrary units), and auxiliary gas (8 arbitrary units). The spectrometers were operated in positive modes with a mass range of  $m/z$  100–1,600 with a nominal mass resolving power of 60,000 at  $m/z$  400 with a scan rate of 1 Hz, with the internal lock masses N-butylbenzenesulfonamide ([M+H]<sup>+</sup>  $m/z$  214.0896) and dibutyl phthalate ([M+H]<sup>+</sup>  $m/z$  279.1591). The analyses were performed using Xcalibur software v. 2.2 SP1.48 (Thermo Scientific, Bremen, Germany). The acquired masses were sorted by intensity ( $I > 1.00E3$ ). A maximum mass tolerance of 2 ppm was accepted. For structure elucidation, tandem HRMS experiments were performed with the LTQ-Orbitrap mass spectrometer with collision-induced dissociation (CID) energies of 15, 25, and 35 eV. Background subtraction was performed as required using Xcalibur software v. 2.2 SP1.48 (Thermo Scientific, Bremen, Germany). Scifinder, Knapsack, and PubChem were used as reference databases.

## Matrix-Assisted Laser Desorption/Ionization-High-Resolution Mass Spectrometry Imaging (MALDI-HRMSI)

MALDI-HRMSI experiments were performed using our previously established procedure (Eckelmann et al., 2018), suitably modified. Areas of interest of the respective agar plates with the seeded or cultivated microorganisms were cut to square pieces with a razor blade, transferred, fixed onto a 4 × 1 cm microscopical glass slide, and dried for 24 h. A photographic image was taken for each sample using a specialized digital

microscope (VHX-5000, Keyence Deutschland GmbH, Neu-Isenburg, Germany) to evaluate the measured area and record the optical image. A SMALDI Prep spray device (TransMIT GmbH, Giessen, Germany) was utilized for matrix spraying. The samples were sprayed uniformly with matrix MBT (2-mercaptobenzothiazole; 5 mg/mL) prepared in H<sub>2</sub>O:acetone 1:4 (v/v). The spray application was performed using the Matrix Sprayer Control [v. 1.9.2890] (TransMIT GmbH, Giessen, Germany) employing the following parameters: matrix flow 16 µL/min; gas flow 3 L/min; 27 min under the Area Mode. The measurements were carried out at a resolution of 30 µm with an AP-SMALDI5 ionization source (TransMIT GmbH, Giessen, Germany) coupled to a Thermo Q-Exactive mass spectrometer (Thermo Scientific Inc., Bremen, Germany). Data processing and mapping of mass pixels corresponding to the target compounds were done using the software package Mirion V3.36.4.13 (TransMIT GmbH, Giessen, Germany). Ion images were generated with a bin width of ±5.0 ppm. Mass pixels are shown as false colors.

## RESULTS

### OSMAC-Based Microbiological and Phenotypic Observations

Endophytic *S. pulveraceus* ES16 colonies' growth on modified 523 agar started within the first 24 h of incubation. After 3 days, the colonies were visible to the naked eye and continued to increase in size until the 7th day of incubation. However, the colonies' growth stopped after the 1st week, as the colonies did not continue to grow in diameter until 14 days. The size of each colony did not exceed 1 mm and therefore could be characterized as punctiform. On this medium, *S. pulveraceus* ES16 formed circular colonies with a minor convex elevation. They showed a smooth texture with an opaque light beige/white color during the first few incubation days. The color altered slightly after a week to a light pink color. There were no visible changes in the medium's color, i.e., no visible microbial exudates were observed. No sporulation was observed (**Figure 2A**). This bacterium demonstrated a soft colony consistency when touched with the loop on the 3rd day of incubation, which gradually hardened over the 14-day experimental period.

However, the endophyte demonstrated markedly different morphology on GYM agar than that on modified 523 agar (**Figure 2B**). The colonies started forming after just 12 h of incubation and were completely visible at 48 h. The size of the colonies had a relatively considerable increase with time. On day 3, a single colony's diameter was measured around 1 mm, and it increased to 4 mm and 6 mm on days 7 and 14, respectively. Apart from growth in size, a drastic change in the bacterium's color and shape was observed with incubation time. The initial colony shape of *S. pulveraceus* ES16 on GYM agar was an irregular convex form with a wrinkled surface. The colonies were firstly small and had a light beige color. On day 7, it was noticeable that some parts of the colonies had started to sporulate while other parts had a darker brown color and no longer had an elevated convex colony character. After 14 days of incubation, the

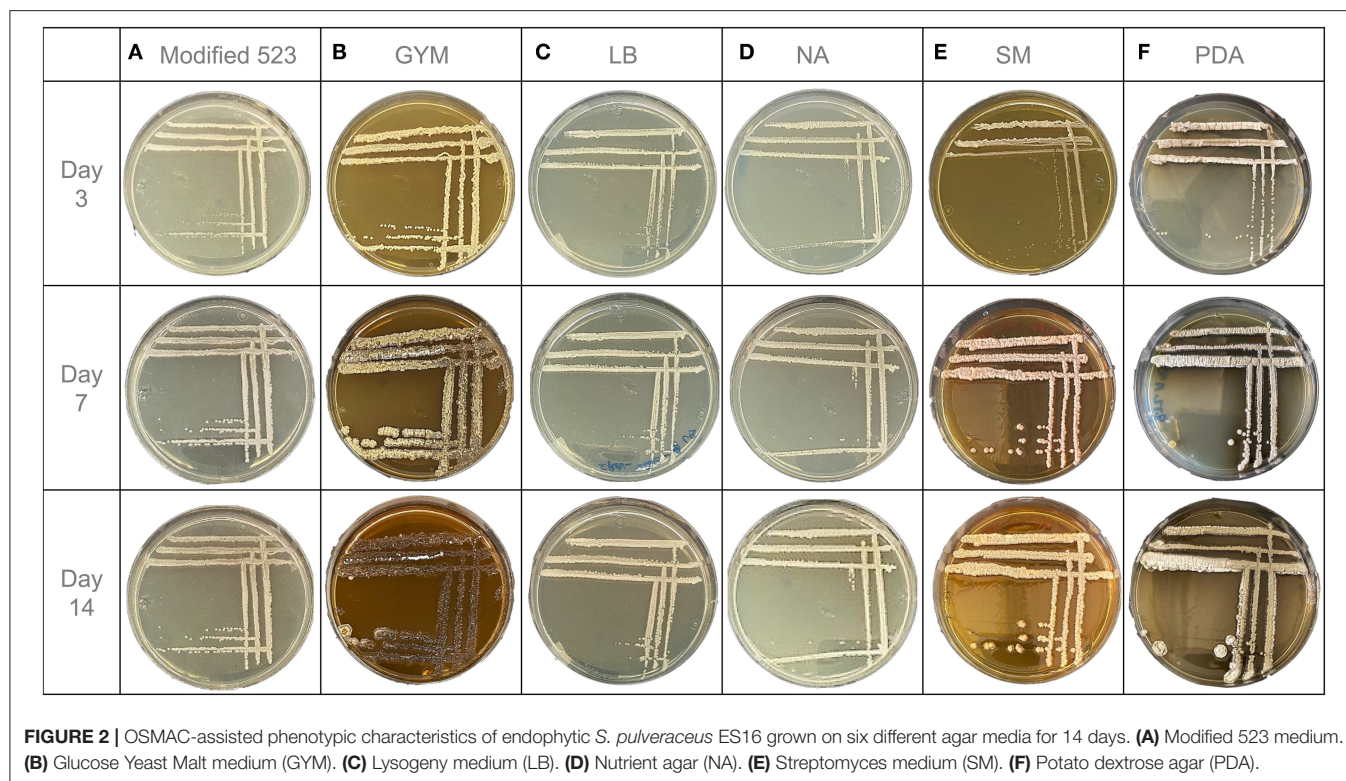
bacterium's color altered almost entirely to dark brown, which indicated that the bacteria could be in the stationary or decline phase. On day 7, a potting soil odor was detected as the plate was opened. The change in the medium color around the colonies to a darker shade over time was indicative of visual exudates secreted by the endophyte. On day 3, the bacterial colony consistency was soft and smooth, whereas it was brittle on day 7. On day 14, the bacterium had developed a hard and dry texture.

The growth of *S. pulveraceus* ES16 on LB agar was typically similar to that on modified 523 agar (**Figure 2C**). The colonies' formation started 24–36 h after the incubation, and after 3 days, the colonies were visible to the naked eye. The single colonies showed a minimal increase in size from day 3 to day 7, and the overall diameter of a single colony did not exceed 1 mm. The endophytic colonies were light beige/white with a smooth texture. The colonies were circular and raised. The color of the bacteria remained the same for the first 7 days. After 14 days of incubation, the color altered to light yellow. There were no visible changes in the agar's texture and shade, and no sporulation was observed. When touched with the inoculation loop, *S. pulveraceus* ES16 showed a viscid consistency throughout the 14 days of observation.

In the case of NA, the colonies of *S. pulveraceus* ES16 were not visible on agar until 48 h after incubation. Like that of modified 523 and LB agar, the colonies were relatively small (1 mm) and fully grown by day 7. They maintained the same light beige/white color throughout the 14-day incubation time. The colonies had a circular shape with a smooth texture. No alterations in the color of the medium, and no sporulation were observed (**Figure 2D**).

The colonies of *S. pulveraceus* were visible within the first 6 h after cultivation on SM agar. On day 3 of incubation, tiny punctiform colonies (<1 mm) could be observed (**Figure 2E**). From day 3 till day 7, a drastic increase in colony size was observed. The colony color was initially opaque white/light beige, similar to the other tested media. On day 7, the observed color had transformed into pink/beige. The endophyte formed irregular curled colonies with a pulvinate elevation and demonstrated a rough, brittle surface. No sporulation was observed.

Entirely different morphology of *S. pulveraceus* ES16 was observed on PDA (**Figure 2F**). The first colonies appeared after 24 h. The colony size increased with time while remaining ca. 1 mm on day 3, 3 mm on day 7, and 5 mm on day 14. At 48 h of incubation, the colonies had a similar morphology to that of modified 523 agar, NA, and LB agar with light beige color and circular shapes. However, by day 3, the bacterium began to sporulate vigorously, revealing a high-stress environment. The colony color changed rapidly to light gray and darkened after 7 and 14 days, respectively. By day 7, extensive sporulation of the colonies was noted. Despite sporulation, the colonies remained circular and convex in shape. Strikingly, from around day 7 of the incubation, the bacterial plate emitted a potting soil scent, which intensified by day 14. Another significant observation on the PDA plates was the pigmentation of the medium. Already by day 3, the color of the medium changed from yellow to dark brown, which intensified during the 14 days of incubation time, revealing secretion of visible exudates. *S. pulveraceus* ES16 had



a texture similar to that of a fungal mycelium on PDA. Once in contact with the loop, it was viscid and soft.

### Analysis of the Expressed Volatilome Using SPME-GC-MS With a Focus on Volatile Signaling Compounds

SPME-GC-MS was employed to evaluate the production of volatile organic compounds (VOCs) (Tholl et al., 2006; Iijima, 2014; Yalage Don et al., 2020) by endophytic *S. pulveraceus* ES16 in different media conditions. Notably, the strong earthy odor released by the endophyte, when growing on PDA plates, coupled with extensive sporulation, provided a scientific handle to focus on stress-induced compounds and semiochemicals (signal molecules). Extensive analyses of the samples compared to the NIST EI-MS spectral library revealed the endophytic production of the volatile sesquiterpene, geosmin (trans-1,10-dimethyl-trans-9-decalol; compound **1**; **Figure 3**) specifically on PDA but not in other media conditions. The fragmentation pattern of the geosmin (**Figure 4**) matched with the reference NIST EI-MS spectral library, confirming its identity. Geosmin (**1**) production was only observed when the endophyte was subjected to stressful conditions, concomitant with extensive sporulation and a strong earthy smell. We also searched for other known signal molecules that might be produced along with geosmin, including 2-methylisoborneol (2-MIB), germacerene D, germacradienol (Becher et al., 2020), among others. However, these compounds were not detected (<LOD) while analyzing the expressed volatilome of endophytic *S. pulveraceus* ES16.

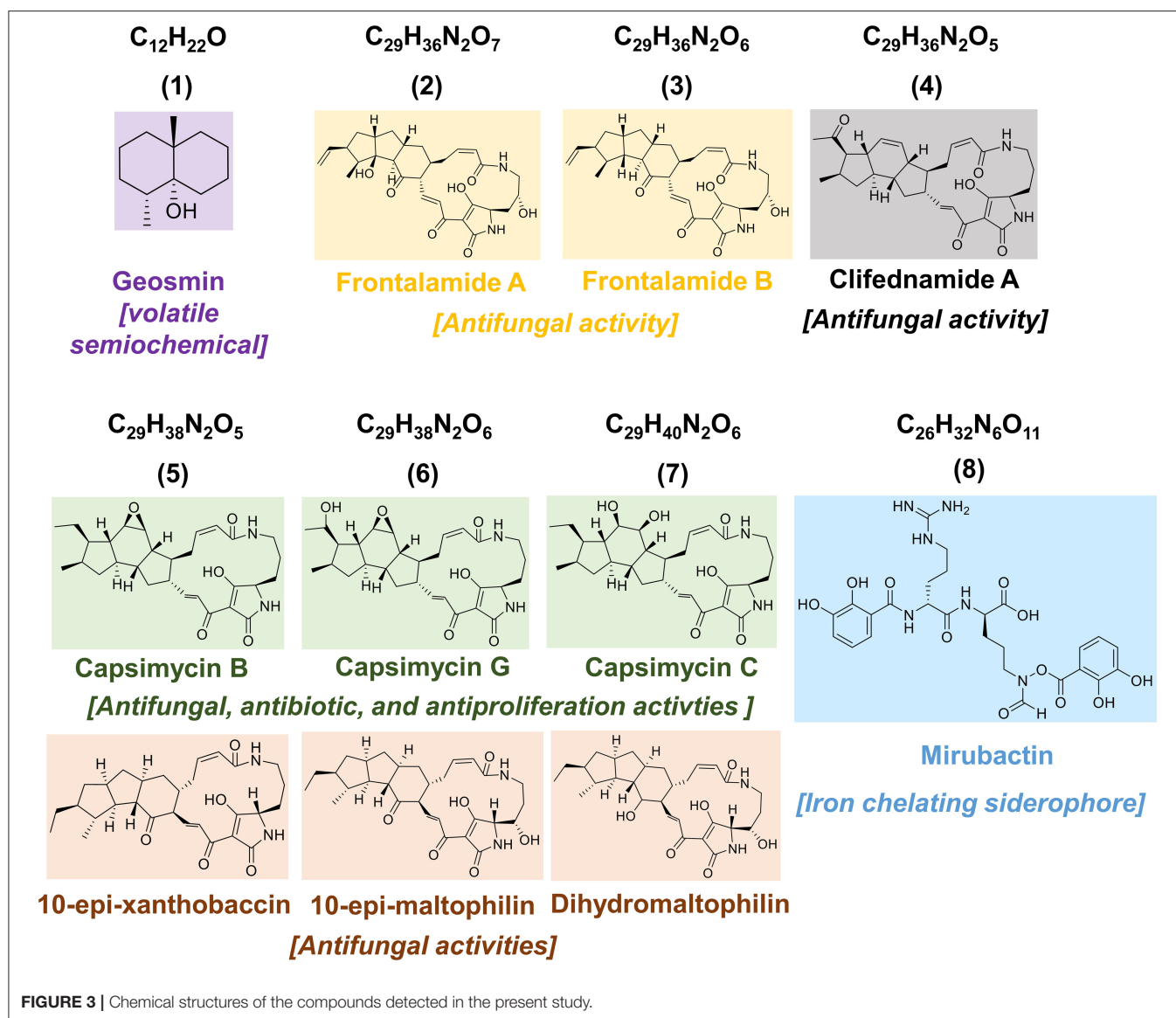
### HPLC-HRMS<sup>n</sup> Based Selective Metabolic Profiling and Structural Dereplication of Target Bioactive Compounds

Guided by the OSMAC approach, the chemo-diversity of endophytic *S. pulveraceus* ES16 cultivated in different media conditions was investigated using HPLC-HRMS. The extracts of plates and broths were analyzed in parallel to examine the compounds produced by the bacterium both under submerged cultivation conditions and agar (solid media). Moreover, HPLC-HRMS based temporal analyses were performed after cultivation for 3-, 7- and 14 days, respectively, to unravel the production pattern of the compounds and set up the basis of further MALDI-HRMSI experiments. Furthermore, a bioactivity-guided selection of the produced compounds was carried out to target the “hits,” focusing on antibacterial, antifungal, antiprotozoal, and relevant plant beneficial compounds, and the targeted metabolic profile of the endophyte was established. Finally, the target compounds were identified by extensive HRMS<sup>n</sup> experiments and by comparison with the literature. Endophytic *S. pulveraceus* ES16 demonstrated the production of two classes of compounds with relevant bioactivities: polycyclic tetramate macrolactams (PTMs) and siderophores.

### Polycyclic Tetramate Macrolactams (PTMs)

While examining the secondary metabolites of endophytic *S. pulveraceus* ES16 using the OSMAC approach, a family of polycyclic tetramate macrolactams (PTMs) was detected (**Figure 3**, compounds 2–7). Interestingly, the PTMs were only produced in two specific media conditions that induced stress in





the organism, exemplified by sporulation and visible exudation into the media, namely GYM agar, and PDA. The production of these compounds (2–7) was first detected by the 7th day of incubation, which increased till day-14 of observation. Overall, six different compounds/masses belonging to this family were detected. Apart from  $m/z$  525.2601 (2), which was only detected on GYM agar, all five other compounds (3–7) were produced in both media conditions.

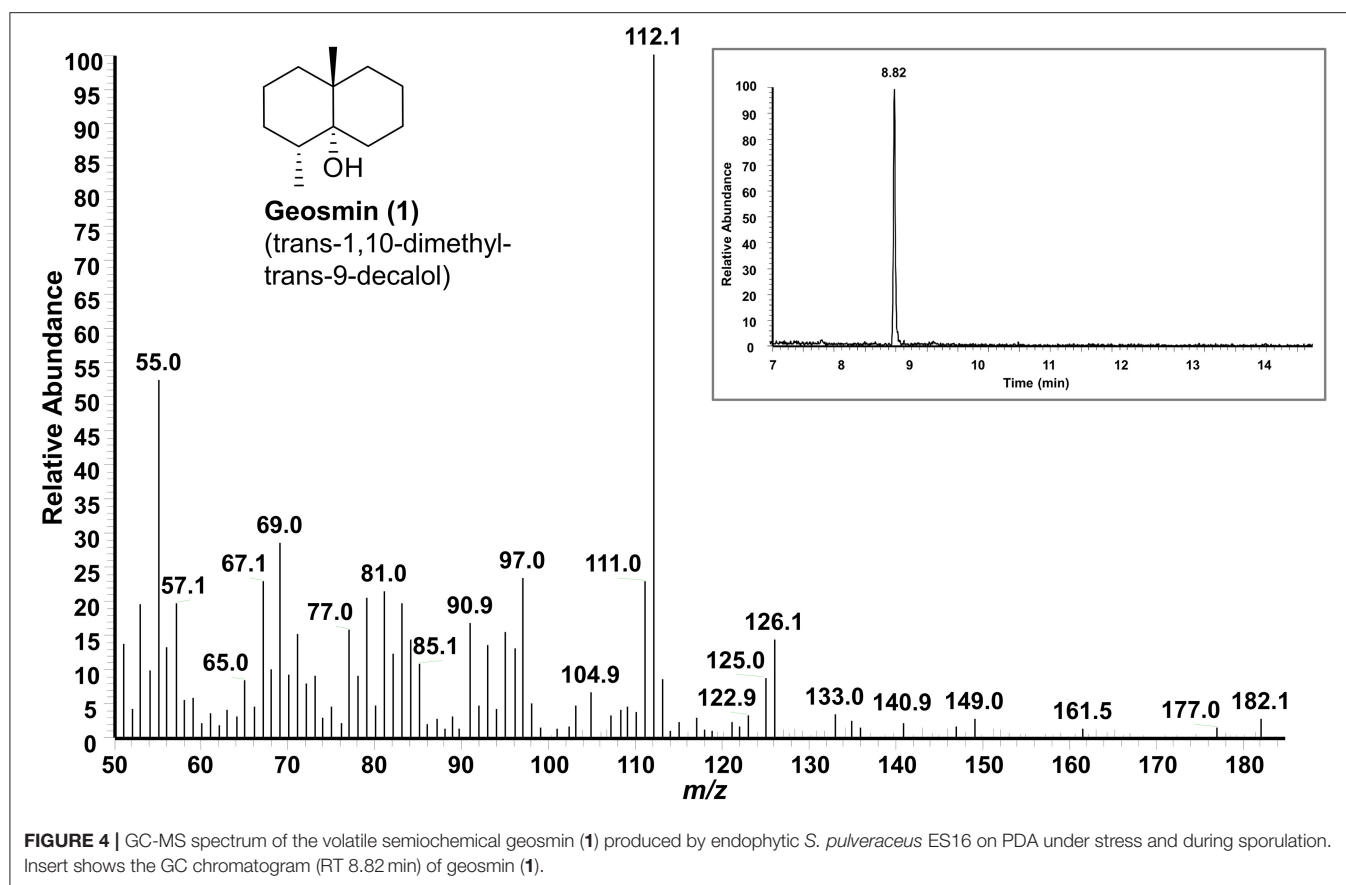
Although various PTMs are known in the literature, their analysis was restricted to NMR techniques such as NOESY and COSY. However, as expected, in our study, endophytic *S. pulveraceus* ES16 produced the PTMs (2–7) in low, physiologically-relevant amounts, which did not allow the isolation and purification of PTMs (2–7) using preparative HPLC. Therefore, the structures of compounds 2–7 were dereplicated using HRMS<sup>n</sup>. Due to similarities in the structures of the compounds, we performed HRMS<sup>2</sup> and HRMS<sup>3</sup> to evaluate their structures based on typical fragmentation patterns and by

comparison with the literature (HRMS<sup>2</sup> could not be done for compound 2 due to low production). The HRMS<sup>n</sup> spectra of compounds 2–7 are presented in **Supplementary Figures 1–10**. Yu et al. (2017) and Qi et al. (2018) have reported the MS<sup>n</sup> fragmentation of some PTMs, which enabled us to compare the fragmentation patterns of compounds 2–7 and assign their plausible structures.

### Mirubactin

In addition to the bioactive PTMs (2–7), endophytic *S. pulveraceus* ES16 also produces an unusual siderophore called mirubactin (compound 8, **Figure 3**). This siderophore contains an O-acyl hydroxamic acid ester group with a high affinity to the valuable ferric ions in iron-depleted environments. It also consists of one D-arginine and one d-hfOrn unit in addition to two DHB units. Our OSMAC approach revealed that this compound's production critically depends on the medium on which the bacterium is cultivated. Mirubactin (8) was detected





only on GYM agar and on PDA ( $[M+H]^+$ ,  $m/z$  605.2192,  $\pm 2$  ppm, RT 15.02 min). Interestingly, the endophyte produces this siderophore 10-fold more pronounced on PDA than on GYM agar (PDA<sub>intensity</sub> 4.2E5; GYM<sub>intensity</sub> 1.9E4) concomitant with the strain's higher stress and extensive sporulation on PDA.

HRMS<sup>n</sup> experiments confirmed the structure of mirubactin (8) (Figure 5). The HRMS<sup>2</sup> measurement yielded the primary fragment ion with an  $m/z$  of 469.2042 after one DHB unit's cleavage. Consecutive HRMS<sup>3</sup> of the fragment  $m/z$  469.2042 yielded in three additional fragments:  $m/z$  333.1877 is the result of the loss of the two dehydrated DHB groups from each side, while  $m/z$  293.1242 arises from the cleavage of one DHB moiety together with the fhOrn; the fragment  $m/z$  311.1349 follows the same fragmentation pattern as  $m/z$  293.1242 after a rearrangement (Giessen et al., 2012). Strikingly, the mirubactin-iron complex ( $C_{26}H_{30}FeN_6O_{11}$ ,  $[M+H]^+$ ,  $m/z$  658.1316,  $\pm 2$  ppm) was also produced by the endophyte on both GYM agar and PDA.

### Spatial-Temporal Dynamics of the Endophytic Metabolites Visualized by MALDI-HRMSI

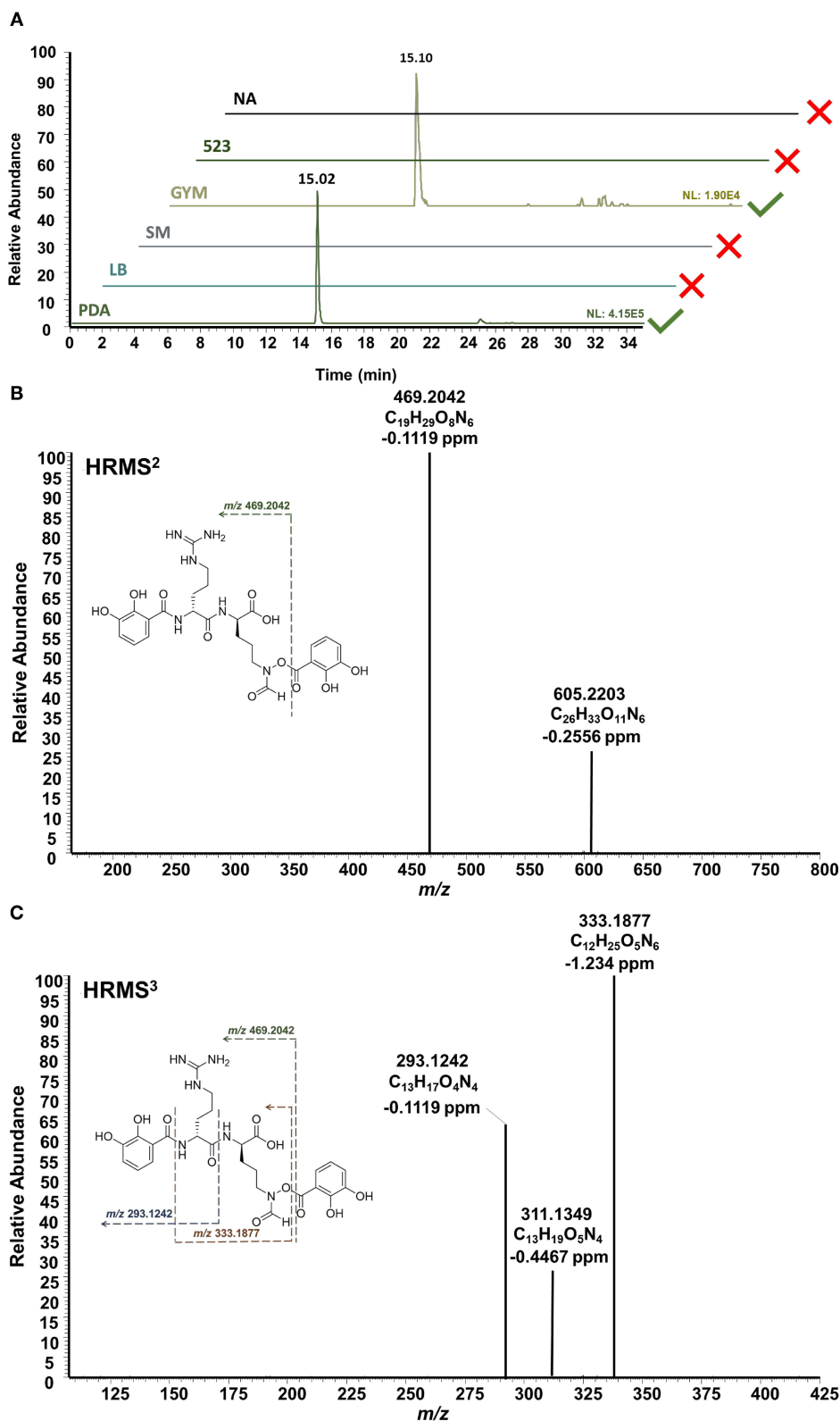
In order to visualize the production, distribution, and secretion of the above compounds produced by endophytic *S. pulveraceus* ES16 in high spatial resolution, we performed MALDI-HRMSI

to monitor the target compounds over 14 days. Figure 6 depicts the ion intensity images of the PTMs 3–7 on day 14 ( $[M+H]^+$  and  $[M+K]^+$  adducts). Compound 2 was below the limit of detection ( $<LOD$ ) and could not be mapped. MALDI-HRMSI revealed that compounds 5–7 were secreted more profusely compared to the other PTMs. These results are consistent with the data obtained from inoculated agar extractions and HPLC-HRMS analyses. The spatial-temporal distribution of the produced PTMs showed that the compounds were readily secreted into the medium after production. The catecholate siderophore, mirubactin (8), produced on PDA and GYM agar, was also subjected to visualization by MALDI-HRMSI (Supplementary Figure 11). Similar to the PTMs, the endophyte also secretes mirubactin (8) into the media over 14 days.

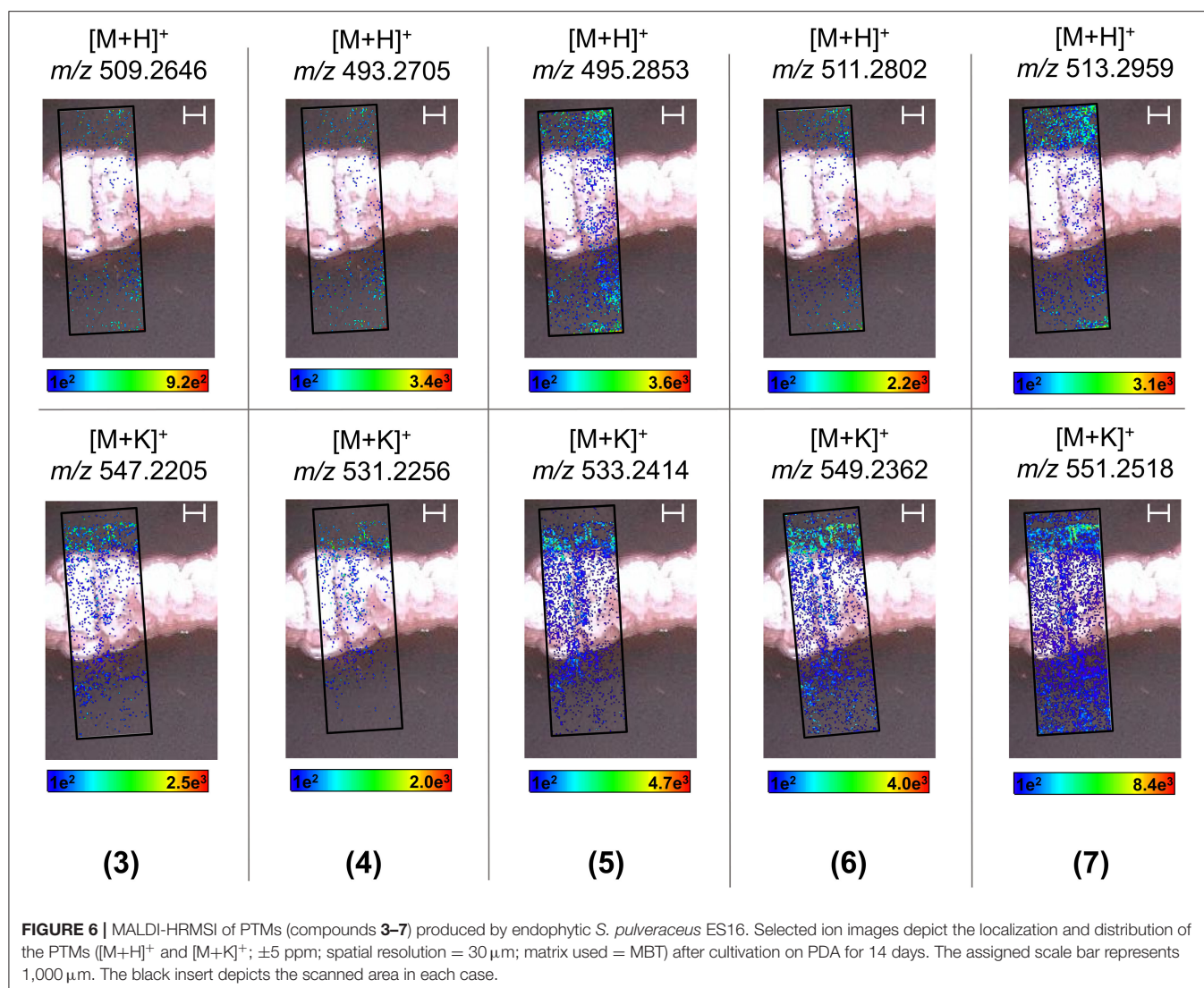
## DISCUSSION

### Phenotypic Differentiation of Endophytic *S. pulveraceus* ES16 in Six Different Cultivation Conditions (OSMAC Approach) Directing the Targeted Evaluation of Its Volatilome and Metabolome

The stressful and dysbiotic environment characteristically associated with ARD, not only for apple plants but also for associated microbiota, is well-established (Mazzola and Manici,



**FIGURE 5 |** HPLC-HRMS<sup>n</sup> analysis of mirubactin (**8**). **(A)** Extracted ion chromatograms ( $[M+H]^+$ ,  $m/z$  605.2192,  $\pm 2$  ppm) shows the selective production of mirubactin (**8**) only on GYM agar and on PDA. **(B)** HRMS<sup>2</sup> of mirubactin (**8**). **(C)** HRMS<sup>3</sup> of mirubactin (**8**). The proposed mass spectral fragmentation pathway is annotated on the compound structure.



2012; Yim et al., 2013, 2015; Weiß et al., 2017; Grunewaldt-Stöcker et al., 2019; Radl et al., 2019; Winkelmann et al., 2019; Balbín-Suárez et al., 2020). For example, an increased abundance of environmental stress sensing genes, particularly in the rhizosphere microbiome of ARD soils compared to ARD-unaffected soils, was recently unraveled by a metagenomics approach (Radl et al., 2019). Against this background, in the present study, we aimed to study apple root-associated endophytic *S. pulveraceus* ES16 by employing a chemical OSMAC approach (Romano et al., 2018; Pan et al., 2019; Maghembe et al., 2020) to induce high stress and sporulation in the organism. Earlier studies have substantiated that *Streptomyces* species are stressed and forced to sporulate by regulation of the pleiotropic transcriptional factors such as *GlnR*, *MtrA*, and *PhoP*, which can be achieved by manipulating the nature of carbon and nitrogen sources, their quantity (specifically the carbon/nitrogen ratio as well as their depletion), and phosphate availability in the medium (Karandikar et al., 1996; Martín and Liras, 2020). In particular, by limiting the nitrogen availability,

*Streptomyces* species can be starved of essential amino acids leading to a stringent response that restricts transcription of numerous genes involved in vegetative growth (i.e., induces sporulation) and stimulates the expression of stress response genes (Bibb, 2005; Tiffert et al., 2008, 2011; Dalebroux and Swanson, 2012; Martín and Liras, 2020). Additionally, phosphate starvation influences the two-component phosphate control of the metabolism system, *PhoR-PhoP* (Hutchings et al., 2004; Martín et al., 2012), slows down primary metabolism and triggers the production of secondary metabolites (Sola-Landa et al., 2003, 2005; Martínez-Castro et al., 2018). Therefore, we successfully employed PDA limited in both nitrogen and phosphate (**Supplementary Table 2**) as a high stress-inducing and sporulating medium for endophytic *S. pulveraceus* ES16. Earlier studies have also demonstrated how PDA can induce sporulation and production of antimicrobial compounds by different marine *Streptomyces* species (Vijayakumar et al., 2012; English et al., 2017). Furthermore, we used the GYM medium following the recommendation of Shirling and Gottlieb (1966)

in the International Streptomyces Project (ISP), which is limited in phosphate but not in nitrogen and has been used earlier to induce sporulation in some *Streptomyces* strains (Arasu et al., 2009). Endophytic *S. pulveraceus* ES16 sporulated mildly on GYM agar. We also used a modified 523 medium that does not limit nitrogen or phosphate and provides the disaccharide saccharose as the carbon source. Along with the basal medium NA that does not induce sporulation and nutrient-rich LB and SM media (favorable growing conditions), this medium is used for optimal cultivation of *Streptomyces* species (Atlas, 2010; Vijayakumar et al., 2012; English et al., 2017). Taken together, we evaluated the phenotypic differentiation of endophytic *S. pulveraceus* ES16 under stress compared to unstressed as well as optimal growth conditions, which laid a scientific handle to study the concomitant effects on its secreted volatilome and expressed metabolome.

### Endophytic *S. pulveraceus* ES16 Produces the Volatile Semiochemical, Geosmin, Only Under Stressful Conditions and During Sporulation

Several reports have documented the production of geosmin (1) by a plethora of microorganisms inhabiting a variety of ecological niches, including filamentous fungi, actinomycetes, cyanobacteria, and proteobacteria, to name a few (Yamada et al., 2015; Vurukonda et al., 2018; Churro et al., 2020; Melo et al., 2020). Becher et al. (2020) recently unraveled this compound's central role in *Streptomyces* species' lifecycle since it plays a specific role as the volatile signal molecule attracting the soil arthropods Collembola (springtails) to the producer strains. Several studies have demonstrated that Collembola illustrates biocontrol potential by feeding on pathogenic fungi. Curl (1979) examined the biocontrol potential of Collembolan species for the first time by observing their interactions with pathogenic fungi. He suggested that the Collembola species *Proisotoma minuta* and *Thalassaphorura encarpata* decreased pathogenic fungal growth rate such as *Rhizoctonia solani* and *Fusarium oxysporum*, which are associated with root disease. Shiraishi et al. (2003) showed the suppression of the damping-off disease in cabbage and Chinese cabbage by the Collembola *Folsomia hidakana*. Recently, Innocenti and Sabatini (2018) reported that Collembola prefers feeding on pathogenic fungi such as *Fusarium culmorum* rather than biocontrol fungi such as *Trichoderma harzianum*. In the present study, the phenotypic observations of endophytic *S. pulveraceus* ES16 under specific OSMAC parameters revealed an earthy soil odor release during this bacterium's prolific sporulation on PDA plates that induced stress to the organism. One important reason why we employed the six different defined media conditions to cultivate the organism was to study its secreted volatilome and metabolome expressed under the influence of stressful media conditions compared to nutrient-rich and optimum conditions (Romano et al., 2018; Pan et al., 2019; Maghembe et al., 2020). OSMAC-guided SPME-GC-MS analyses further revealed that the organism produces the volatile signal compound geosmin (compound 1, Figure 3;  $\gamma\epsilon\omega$ -“geo”: earth,  $\sigma\mu\eta$ -“osmi”: smell) only under stressful conditions and during

sporulation, and it is responsible for this scent. The endophyte did not emit this signal compound in the other tested media conditions. As expected, endophytic *S. pulveraceus* ES16 mildly sporulated on GYM agar. However, we could not detect the compound's production, possibly because its production is only associated with high stress and extensive sporulation or produced in meager amounts (<LOD). Moreover, the volatile terpenoid 2-methylisoborneol (2-MIB), which is also responsible for the earthy smell similar to geosmin (1) and found to be secreted by some *Streptomyces* species (Rabe et al., 2013; Yamada et al., 2015), was also not detected (<LOD) on PDA or GYM agar.

### Production of Bioactive PTMs and the Siderophore Mirubactin Is Also Associated With Similar Stressful Media Conditions

PTMs are natural products containing one tetramic acid and a polycyclic system fused to a macrolactam. Their polycyclic system is categorized into three main groups: the PTMs containing a 5/5, those with a 5/6/5, or PTMs with a 5/5/6 ring system in their structure (Luo et al., 2013; Liu et al., 2019). PTMs are well-known for possessing various biological activities such as antibacterial, antifungal, antiprotozoal, and antiviral (Luo et al., 2013; Liu et al., 2019; Jiang et al., 2020). For example, capsimycin B was discovered from *Streptomyces* sp. Tü 6239 and showed antibiotic activities against Gram-positive bacteria (Bertasso et al., 2003). In another study, *Lysobacter enzymogenes* and *Streptomyces* spp. both produced dihydromaltophilin (also known as HSAF) (Li et al., 2008). HSAF is an antifungal agent capable of harming fungal biosynthetic pathways and is therefore suitable against phytopathogens. Recently, marine sponge-associated *Streptomyces zhaozhouensis* strain MCCB267 was shown to produce clifednamide A that demonstrated remarkable antiproliferative activities by inducing apoptosis in lung carcinoma cells (Dhaneesha et al., 2019). A detailed examination of endophytic *S. pulveraceus* ES16 metabolome guided by the OSMAC approach revealed the production of several bioactive PTMs (compounds 2–7, Figure 3), a series of antibiotic agents. MALDI-HRMSI experiments further revealed that the produced PTMs are part of the endophytic *S. pulveraceus* ES16 strain's secretome and are released into the media. Accordingly, the compounds can be considered to be released by the endophyte into the host plant, leading to an impact on the plant itself. Further experiments are required to elucidate the positive (e.g., warding off pathogens or associated endophytes) or negative (e.g., phytotoxic) effects of the endophytic PTMs (2–7) on its host plant.

Siderophores play an essential role in host-pathogen interactions (Kramer et al., 2020). For instance, van Loon et al. (2008) showed that the production of siderophores by endophytes contributes to protecting the host plant through induced systemic resistance (ISR), a mechanism in the plant activated by an infection. Furthermore, siderophores are considered potential biocontrol agents; endophytic siderophore production reduces the amount of ferric ions available for uptake by pathogens, contributing to the plant defense (Ghosh et al., 2020; Kramer et al., 2020). Endophytic *S. pulveraceus* ES16



can produce the rare iron-chelating siderophore mirubactin (compound **8**, **Figure 3**), which it also secretes out as revealed by MALDI-HRMSI. The isolation, structure elucidation, and biosynthesis of this compound have been reported by Giessen et al. (2012). This siderophore's production has only been reported from *Actinosynnema mirum* (Giessen et al., 2012), and gene sequencing has been employed to predict the possibility of its production by *Streptomyces* species (Jackson et al., 2018; Almeida et al., 2019). To the best of our knowledge, we report for the first time the *in vitro* production of mirubactin (**8**) by a *Streptomyces* species using targeted metabolic profiling.

## Insights Gained From the Chemical Evaluation of Endophytic *S. pulveraceus* ES16

In the present study, we employed a targeted metabolomics approach on endophytic *S. pulveraceus* ES16 comprising of three different methodologies, namely SPME-GC-MS to evaluate the secreted volatilome, HPLC-HRMS<sup>n</sup> to evaluate the expressed metabolome, and MALDI-HRMSI to visualize the spatial dynamics of target compounds, all guided by the OSMAC approach. We used the cultivable apple root-associated endophytic *S. pulveraceus* strain ES16 as a representative working model to investigate its chemoecological relevance.

Firstly, the results of our volatilome analyses led us to propose that *S. pulveraceus* ES16 succumbs to high stress during the onset of ARD (concomitant to its host plant), starts sporulating, and synthesizes and secretes geosmin (**1**). The precise function of this semiochemical inside apple plants, whether roots further secrete it into the soil along with plant exudates, and its function in ARD soil remains to be tested in future experiments. Given the recently-established ecological function of geosmin (**1**) for *Streptomyces* (Becher et al., 2020), it can be hypothesized that if the compound is released into the soil, it can signal selected soil arthropod species. This further opens up a conceivable chemoecological “link” between the endophyte, its host plant, and associated organisms in soil that can receive and respond to the endophytic signaling cues. Secondly, it is plausible that production and secretion of the bioactive PTMs (**2–7**) by endophytic *S. pulveraceus* ES16 can provide a competitive advantage to the bacterium in the host plant apoplast because it can ward off a plethora of pathogens or associated endophytes, thereby enabling better colonization and increased abundance of the endophyte in the host. However, some of the endophytic PTMs possess cytotoxic activities in addition to having antimicrobial efficacies (Luo et al., 2013; Liu et al., 2019; Jiang et al., 2020). Whether these compounds also negatively affect the host plant by exerting phytotoxic effects should be studied in the future. Thirdly, mirubactin (**8**) was also secreted by the endophyte, as evidenced by the MALDI-HRMSI, similar to the PTMs (**2–7**). Therefore, it can be anticipated that this siderophore is also released into the plant apoplast by endophytic *S. pulveraceus* ES16 and can add to the PTMs-mediated “warfare” against various microorganisms residing in the immediate environment

by chelating ferric ions available for uptake by associated or invading microorganisms (Ghosh et al., 2020; Kramer et al., 2020).

Future studies, for instance, by combining phytotoxicity assays, plant inoculation experiments, and MALDI-HRMSI, can precisely unravel the production, release, and effect of the compounds *in planta*. Noteworthy, apple roots in ARD soil are often highly disturbed so that endophytic *S. pulveraceus* ES16, as well as the chemicals produced by the organism, can also be assumed to enter the rhizosphere soil, whose precise function in the soil remains to be explored. Furthermore, our results provide a basis for employing a similar approach to other *Streptomyces* strains and species or other apple root-associated organisms to comprehend the chemical basis of their ecological functions and interactions. It would also be useful to investigate the phenotypic and metabolic effect of ARD biomarkers such as phytoalexins (Reim et al., 2020; Rohr et al., 2020) and apple root exudates or compounds therein (e.g., phlorizin) on apple root-associated microorganisms and vice versa. Further investigations on the etiology of ARD as well as designing effective and sustainable biocontrol remedies must take into account both the molecular and the chemical networks between associated ecological partners in order to gain a holistic view.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

RA performed the OSMAC experiments, phenotypic characterizations, SPME-GC-MS, HPLC-HRMS<sup>n</sup>, MALDI-HRMSI, and analyzed and interpreted the data. SZ enabled realization and optimization of mass spectrometry and MALDI-HRMSI experiments, and interpreted the data. FM-D undertook the central experiments, isolated, identified, and established the axenic culture of the *Streptomyces* strain. TW conceptualized the central experimental designs, supervised FM-D, and coordinated the BonaRes ORDIAmur project consortia. SK conceived and led this research, designed the experiments, supervised RA, interpreted the data, and wrote the manuscript. RA and SK prepared the figures and tables. All co-authors contributed to the manuscript and reviewed and approved the final version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.643225/full#supplementary-material>

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# Temporal Soil Bacterial Community Responses to Cropping Systems and Crop Identity in Dryland Agroecosystems of the Northern Great Plains

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Industrialized agriculture results in simplified landscapes where many of the regulatory ecosystem functions driven by soil biological and physicochemical characteristics have been hampered or replaced with intensive, synthetic inputs. To restore long-term agricultural sustainability and soil health, soil should function as both a resource and a complex ecosystem. In this study, we examined how cropping systems impact soil bacterial community diversity and composition, important indicators of soil ecosystem health. Soils from a representative cropping system in the semi-arid Northern Great Plains were collected in June and August of 2017 from the final phase of a 5-year crop rotation managed either with chemical inputs and no-tillage, as a USDA-certified organic tillage system, or as a USDA-certified organic sheep grazing system with reduced tillage intensity. DNA was extracted and sequenced for bacteria community analysis via 16S rRNA gene sequencing. Bacterial richness and diversity decreased in all farming systems from June to August and was lowest in the chemical no-tillage system, while evenness increased over the sampling period. Crop species identity did not affect bacterial richness, diversity, or evenness. Conventional no-till, organic tilled, and organic grazed management systems resulted in dissimilar microbial communities. Overall, cropping systems and seasonal changes had a greater effect on microbial community structure and diversity than crop identity. Future research should assess how the rhizobiome responds to the specific phases of a crop rotation, as differences in bulk soil microbial communities by crop identity were not detectable.

**Keywords:** 16S rRNA gene, chemical no-tillage, yellow sweetclover [*Melilotus officinalis* (L.)], Illumina MiSeq®, organic grazed, organic tilled, winter wheat (*Triticum aestivum* L.), safflower (*Carthamus tinctorius* L.)

## INTRODUCTION

Modern industrial agriculture results in overly simplified landscapes where soil mediated regulatory ecosystem functions have been hampered or replaced with intensive off-farm inputs aimed principally at securing high yields. The overreliance on synthetic chemical and mechanical inputs, however, comes with a high potential for soil degradation and soil loss (Landis, 2017; Vanwalleghem et al., 2017). Worldwide, agricultural erosion has diminished soil capacity to function as a resource

and a living ecosystem on 40% of all agricultural land, underscoring the need to restore and maintain soil health (Doran and Zeiss, 2000). In particular, tillage is used in many conventional and organic agricultural systems to cultivate fields, remove crop and weed residues, and incorporate manure inputs. Intensive conventional tillage is known to facilitate soil erosion and a loss of nutrients (Jat et al., 2019; Schneekloth et al., 2020), particularly in dry regions (Clay et al., 2014). Moreover, this practice can dramatically reduce bacterial (de Quadros et al., 2012; García-Orenes et al., 2016; Ishaq et al., 2020a) and fungal (Drijber et al., 2000; Castillo et al., 2006) diversity in soil, potentially impairing the functionality of these systems. Alternatives to conventional tillage are being sought for organic systems which maintain crop productivity but sustain soil or biodiversity loss.

The soil microbiome—the array of bacteria, fungi, archaea, protozoa, viruses, and their collective genomes which interact with each other and with macroorganisms, is an essential component of the soil ecosystem underpinning numerous ecosystem functions (Lal, 2016). Soil microorganisms influence soil physical structure, drive nutrient cycling through decomposition of organic matter and mineralization of nitrogen, and suppress disease in plants (Miller and Jastrow, 2000; Brussaard et al., 2007; Martínez-García et al., 2018). Soil bacteria can produce hormones that directly influence plant growth or modify the production of plant growth hormones (Patten and Glick, 2002; Mohite, 2013), and mutualistic soil biota aid in plant water and nutrient uptake, increasing aboveground productivity (Bender and van der Heijden, 2015; van der Heijden et al., 2015). Understanding how these interactions between aboveground and belowground communities condition productivity is essential to enhance the sustainability of farming, especially as intense agricultural management is known to reduce micro- and macrobiological diversity with associated negative consequences to ecosystem functions.

Organic farming is one approach to improve agricultural sustainability through increasing reliance on ecological processes rather than synthetic inputs. Compared to chemically managed systems, organic systems place a greater emphasis on soil regeneration and aboveground biodiversity. Previous literature has examined the differential impact of organic and conventional management systems on soil microbiota, which can lead to distinct belowground communities (Li et al., 2012; Hartmann et al., 2015; Harkes et al., 2019). For example, soil microbiota is reported to increase in phylogenetic richness and community variability in organic systems (Lupatini et al., 2017), but this may be linked to the greater bioavailability of nutrient inputs in a more neutral soil pH (Lauber et al., 2009; Zhalina et al., 2015). Nutrient input, in particular, may be the driving force of these observations (Hartmann et al., 2015; Zhang et al., 2017; Semenov et al., 2020). There is also a knowledge gap of how soil microbial communities vary among contrasting organic systems.

In the semiarid regions of the Northern Great Plains, fallow periods where fields are left bare for a growing season to aid in the preservation of soil moisture do not contribute as many benefits to the soil as more diverse cropping systems (Rosenzweig et al., 2018). Also, the highly simplified landscapes that dominate

conventional agriculture of the region are characterized by low plant diversity and a high dependence on synthetic inputs (Adhikari et al., 2019) which negatively impact soil biota and result in less diverse belowground communities (Chaudhry et al., 2012; Ishaq et al., 2017). In contrast, organic cropping systems are characterized by more complex crop rotations and diverse associated biodiversity, including weed communities, than those observed in conventional wheat-summer fallow rotations across the region (Adhikari and Menalled, 2018).

Diversified crop rotations that include for-profit or for-health (i.e., green manure or bioremediation) plant species reduce or replace fallow periods, benefiting soil ecosystems and increasing soil biodiversity (Maarastawi et al., 2018; Peralta et al., 2018). Carbon and nitrogen from microbial biomass can increase by roughly 20 and 27%, respectively, in diversified crop rotations compared to a simple monoculture (McDaniel et al., 2014). Functional and metabolic diversity in heterotrophic soil bacterial communities also increase under more diverse crop rotations (D'Acunto et al., 2018). It is unclear whether the soil physicochemical changes, increases in crop detritus, host-specific promotion, or inclusion of various functional groups associated with diversifying crop rotations have the greatest impact on soil microbial communities (Venter et al., 2016). While there is not a specific sequence of crops that will achieve these benefits, cover crop mixtures are often included in these rotations toward the same end.

Cover crops can recruit specific microorganisms via plant inputs (Ishaq et al., 2017), and while there is not a comprehensive understanding of what microbial communities are recruited by specific plant species, there is some evidence that certain plants act more selectively than others (Massensini et al., 2015; Trognitz et al., 2016; Aguilera et al., 2017). It is also known that cover crops can alter soil microbial community dynamics because microbiota with fast growth rates and the capacity to utilize temporally limited nutrients can take advantage of organic carbon from cover crop roots, root turnover, and crop residue (Wortman et al., 2013) and may favor bacterial communities while those that become low quality residue favor fungi (Frasier et al., 2016). These inputs of biomass and root exudates shape soil microbial activity and diversity, even after cover crops are terminated for the season (Wortman et al., 2013; Calderón et al., 2016).

In semi-arid agroecosystems, cover crops must be terminated early enough in the growing season to preserve soil moisture for the commercial crop. Cropping systems employ various methods of cover crop termination that have their own effects on soil microbial communities. For example, conservation, or reduced tillage lessens disruption to soil organic carbon pools and no tillage leaves cover crop residue on the soil surface. This can result in increased soil carbon, soil nitrogen, and microbial biomass at the surface of the soil (Helgason et al., 2010; Sapkota et al., 2012; Nivelle et al., 2016) but has been criticized for its heavy reliance on herbicides and the associated selection of herbicide-resistant weed biotypes (Menalled et al., 2016). In recent years, consumer demands and market opportunities have driven an expansion of organic agricultural systems (USDA ERS, 2020) which use tillage to terminate cover crops and incorporate residue into the soils.

However, tillage disturbs the soil ecosystem by accelerating soil organic matter oxidation and labile carbon cycling (McLauchlan, 2006), factors that have driven a growing interest to reduce soil disturbance practices in organic systems (Carr, 2017). The integration of crop and livestock practices has been explored as an approach to reduce tillage intensity in organic cropping systems (Miller et al., 2015; McKenzie et al., 2016) while increasing soil nitrogen, soil carbon, and microbial biomass (Ishaq et al., 2017). However, little is known about specific effects of grazing on soil microbial communities.

Semiarid, dryland agriculture makes up a significant portion of global crop production, and understanding how soil communities interact with crops under different farming practices is crucial for global agricultural security (Delgado-Baquerizo et al., 2017). In this study, we assessed soil bacterial community responses to contrasting cropping systems in semiarid regions of the Northern Great Plains. To do so, we took advantage of a field experiment that began in 2012 at the Montana State University Fort Ellis Research and Teaching Center in Bozeman, MT to test a 5-year crop rotation under three different management systems: chemical inputs with no-tillage, a USDA-certified organic system with tillage, and a USDA-certified organic system with targeted sheep grazing for cover crop and weed termination (Ishaq et al., 2020a). We hypothesized that (1) organically managed systems support higher soil bacterial diversity than chemically managed systems, (2) the two organic systems result in dissimilar soil bacterial communities, and (3) diversity is highest in the cover crop phases. We did this by comparing bacterial communities from winter wheat, safflower/sweet clover, and sweet clover under different management systems in fields where the crop rotation has been established for several years.

## METHODS

### Site Description

A field experiment to evaluate approaches to minimize soil disturbances in organic cropping systems was established in 2012 at the Montana State University Fort Ellis Research and Teaching Center in Bozeman, MT (45.653 N, -110.972 W). The underlying soil is a Blackmore silt loam (University of California, Davis, 2019) (fine-silty, mixed superactive, frigid Typic Argiustolls) with 0–4% slopes, composed of about 22% clay, 10% sand, and 68% silt down to 25 cm (Natural Resources Conservation Service Soil Survey Staff, 1999). Fort Ellis usually receives 465 mm of precipitation annually, with monthly mean air temperatures between -5.7 and 18.9°C (PRISM Climate Group, 2020). Prior to 2004, the study site was planted with perennial grasses [*Bromus inermis* L., *Thinopyrum intermedium* (Host) Barkworth and D.R. Dewey, and *Poa compressa* L.]. Between 2004 and 2009, the experimental site followed either continuous spring wheat (*Triticum aestivum* L.), spring wheat-fallow, or winter wheat-fallow crop rotation. From 2009 to 2012, the study site followed either a continuous alfalfa (*Medicago sativa* L.) or a 3-year crop rotation consisting of spring wheat in the first year followed by pea (*Pisum sativum* L.), and hay barley (*Hordeum vulgare* L.) in the second and third years, respectively.

In the spring of 2012, the entire experimental site was planted with glyphosate tolerant rapeseed (*Brassica napus* L.) and treated with herbicide. The rapeseed was tilled to a depth of 15 cm in July 2012 and planted in September 2012 following the experimental design described below. Additional site information can be found in Miller et al. (2015) and Barsotti et al. (2013).

### Study Design

This study followed a split-plot design with three replications where cropping systems were the main plot level and crop phases the split-plots (**Supplementary Figure 1**). The cropping systems included a chemical no-till system where synthetic off-farm inputs were utilized to manage weeds, pathogens, and soil nutrient levels (hereafter, chemical no-till). This system, often referred to as conventional in the context of industrial-age farming, was considered the basis of comparison for other treatments. The other cropping system treatments included a USDA-certified organic system with tillage used for cover crop termination and weed management (hereafter, organic tilled), and a USDA-certified organic system that employed reduced tillage and targeted sheep grazing (*Ovis aries* L.) for cover crop termination and weed management with the overall goal of reducing tillage intensity (hereafter, organic grazed) (Ishaq et al., 2020a). The organic tilled treatment represents current organic practices which are effective yet not ecologically sustainable, and the organic grazed treatment provided an experimental approach to reduce tillage intensity. Each cropping system was randomly assigned to a 75 × 90 m plot with three entire field replications and further divided into five 90 × 13 m split-plots separated by a 1 m fallow track, and randomly assigned to one phase of a 5-year crop rotation: Year 1, safflower (*Carthamus tinctorius* L.) under-sown with yellow sweet clover (*Melilotus officinalis* (L.) Lam); Year 2, yellow sweet clover; Year 3, winter wheat (*Triticum aestivum* L.); Year 4, lentils (*Lens culinaris* Medik); and Year 5, winter wheat. Austrian winter pea (*Pisum sativum* subsp. arvense.) was planted in fall 2012 for the first year (2013) of the experiment because the biennial nature of yellow sweet clover would have required a previous year of seeding. In 2017, sweet clover was terminated in early July (with allowance for regrowth) and winter wheat was harvested in the last week of July. Safflower, a late spring crop, was performing negligible soil chemistry by the time samples were collected in August and was harvested in September.

A no-till double-disk seeder was used on all cropping systems to minimize soil disturbance. Chemical inputs in the conventional system mimicked standard practices in the Northern Great Plains and included 2,4-D, bromoxynil, dicamba, fluroxypyr, glyphosate, MCPA, pinoxaden, and urea to manage weeds and nutrient availability (Adhikari and Menalled, 2020). Crops from both organic treatments were USDA certified by 2015, after completing the transition to organic that began in 2012. A chisel plow, tandem disk, and field cultivator were utilized in the organic tilled treatment as needed to terminate cover crops, prepare the seedbed, and incorporate cover crop residue into the soil (Ishaq et al., 2020a). Targeted sheep grazing at a stocking density of 50 sheep/ha for 30 days terminated cover crops and managed weeds in the organic grazed treatment

(Menalled et al., 2020). The agronomic management details are provided in more detail elsewhere [see Adhikari and Menalled (2020)].

## Soil Collection

Soil samples for microbial DNA extraction and sequencing were collected from the safflower/sweet clover, sweet clover, and year 3 winter wheat split-plots, from each of the three cropping systems, in each of the three cropping system replicates, in June and in August of 2017 (i.e., 3 systems  $\times$  3 replications  $\times$  3 rotations  $\times$  2 time points). Due to logistic problems, two safflower/clover split-plots (one chemical no-till and the other organic grazed) in the second field replication were not sampled in June, but all were sampled in mid-August following residue removal in the respective treatments for a total of 52 soil samples. Each split-plot was divided into quartiles to account for spatial variation in the soil environment and allow for more representative microbial community samples. One bulk soil sample from each of these quartiles was taken with a 2 cm diameter core sampler to a depth of 15 cm after clearing the ground of debris and crop residue. The four cores were manually homogenized to one sample per subplot and kept at  $-20^{\circ}\text{C}$  until analysis. Extremely dry soil conditions in August 2017 necessitated the use of a pickaxe to break the ground before sampling. The soil sampler was sterilized with 70% isopropyl alcohol and air dried between samples to prevent cross contamination of microbial communities.

## Laboratory Methods

Metagenomic soil DNA was extracted from 0.25 g of the 20–30 g homogenized samples using a Qiagen DNeasy PowerSoil kit (Qiagen, Hilden, Germany). Amplicon libraries of the V4 region of the bacterial 16S rRNA gene were prepared following procedures outlined by Kozich et al. (2013), using dual indexed primers (515F and 806R) to target soil bacteria active at the time of sampling. This method amplifies both bacteria and archaea, but the resulting libraries are primarily bacterial as the latter are not amplified well. Amplicons were normalized using SequelPrep plate normalization kit (Invitrogen) and the resulting product was pooled to equimolar concentrations. These pooled amplicons were cleaned with AmpureXP magnetic beads at a concentration of 0.8 (vol/vol) beads to pool ratio. Paired-end sequencing was performed at Michigan State University's RTSF Genomics Core using an Illumina MiSeq with a maximum read length of 250 base pairs on MiSeq v2 reagent cartridge (Illumina Inc.). Illumina Real Time Analysis v1.18.54 was used to perform base calling, and the resulting output was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1. Raw sequences and metadata are available from NCBI under BioProject Accession PRJNA672991.

## Bioinformatics

The DADA2 pipeline run in the software environment R 3.6.1 was used to filter paired end reads, remove chimeric sequences, and conduct taxonomic assessment (Callahan et al., 2016; R Development Core Team, 2020). Sequences were trimmed by 10 bases at the start and end positions during filtering, with no ambiguous bases allowed and a maximum expected error

of two. The error rates were learned on  $2 \times 10^6$  randomly selected dereplicated reads and then used to identify Amplicon Sequence Variants (SVs), which are analogous to individuals in that sequences have been grouped down to single-nucleotide polymorphism/single base differences by assessing overall error rate and probability of base error vs. polymorphism occurrence.

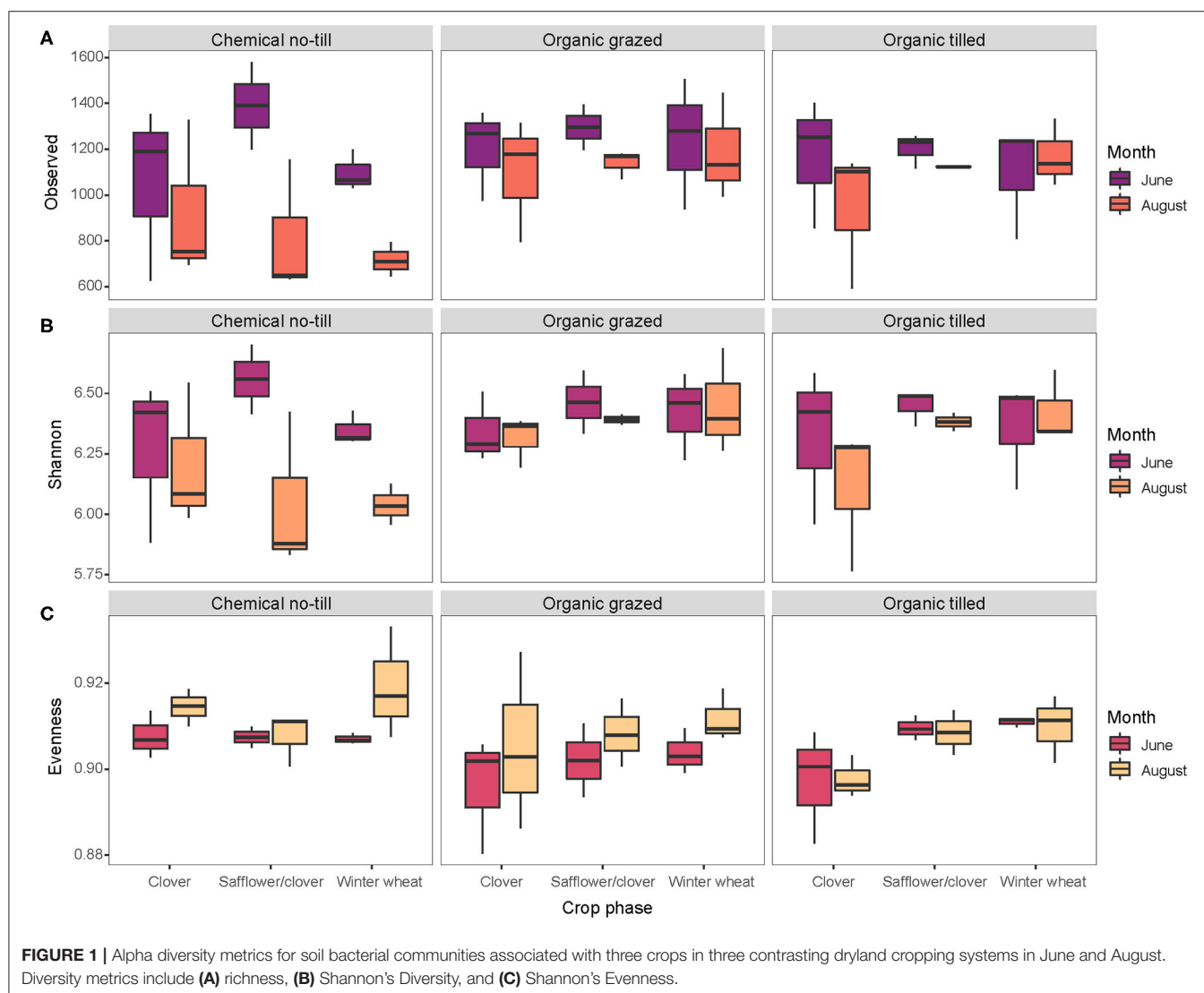
Then, using DADA2, two-parent chimeras (bimeras) were removed. The Silva NR version 138 database was used to assign taxonomy to sequence variants (Yilmaz et al., 2014). Data were first rarefied to the size of the smallest sample library (38,776 sequenced reads), which allowed all 52 samples to pass this quality control step. One sample with only 220 sequence variants made it through as a result. This low outlier was from an organic tilled safflower/clover plot sampled in August 2017. Data were rarefied to the next lowest number of reads per sample (65,165 sequenced reads). The outlier was subsequently removed along with 42 absolute sequence variants and the 6,932 sequenced reads unique to them.

## Statistical Procedures

All data analysis was performed in R 3.6.1–4.0.2 (R Development Core Team, 2020). The phyloseq and vegan packages were used for statistical analysis (McMurdie and Holmes, 2013; Okansen et al., 2019), while ggplot2 was used for data visualization (Wickham, 2016). Alpha or within-community diversity was assessed via observed sequence variants (bacterial community richness), Shannon's Diversity, and Shannon's Evenness. Linear regression with mixed effect models explored the relationships between treatments and alpha diversity. The model for bacterial richness and diversity included cropping system and sampling month as fixed effects and plot as a random effect, while the model for evenness used only month as a fixed effect and plot as a random effect. Crop phase (i.e., plant species identity) was tested as a predictor for richness, diversity, and evenness, but was not a significant predictor for any of them. Field replication was also considered as a random effect because of variation at the main plot level, but split-plot explained more variation in the model for bacterial richness, Shannon's Diversity, and Shannon's Evenness. Nesting split-plot in plot also did not increase the conditional  $R^2$  of these models enough to be considered an improvement. Soil data on percent carbon, percent nitrogen, C:N ratio, and pH were available along with crop and weed biomass but could not be used to model alpha diversity because all except C:N ratio and weed biomass (Supplementary Figures 3, 4) varied by cropping system, split-plot, or were highly correlated with one another ( $r = 0.90$ ). There were no interactions between treatments in any of the models for alpha diversity. Type III One-Way Analysis of Variance determined which variables significantly affected alpha diversity while Tukey's *post-hoc* comparisons evaluated differences in means among treatment levels.

Changes in relative bacterial abundance from June to August among cropping systems were represented with heatmaps that displayed the fifty most abundant sequence variants labeled by genera. Permutational random forest analysis was used with the rfrpermute package to determine the taxa which were significantly discriminatory between treatment states, highlighting the ones that were unique or a defining feature of the three cropping





systems. For each comparison, 500 trees were made with 100 permutations each. Relative abundance of important taxa was then visualized by sampling month within each cropping system. Unspecified genera in both the heatmaps and random forest analysis were identified at the family level.

Bray-Curtis dissimilarities (based on species' presence/absence and relative abundance) were calculated to assess between-community diversity and visualized using non-metric multidimensional scaling ordination. Mean dissimilarities of soil bacterial communities grouped by treatment were compared using analysis of variance and Tukey's *post-hoc* tests. A permutational analysis of variance (PERMANOVA) compared differences among bacterial communities using the adonis function with 9,999 permutations. Data were stratified by split-plot to account for repeated measures. Homogeneity of dispersions—an assumption for using a PERMANOVA—was evaluated using the betadisper function.

## RESULTS

### Alpha Diversity

There were 11,193 unique sequence variants in the 51 soil samples collected in 2017. Bacterial richness decreased in every crop across all three cropping systems between June and August but varied the most in the chemical no-till cropping system and the least in the organic systems (**Figure 1A**). Safflower/clover and winter wheat in the chemical no-till systems had bacterial communities with a wider range of Shannon's diversity compared to those under organic management (**Figure 1B**). Bacterial evenness changed the least in organic tilled winter wheat, organic tilled safflower/clover, and chemical no-till safflower/clover (**Figure 1C**).

All measures of alpha diversity changed with respect to sampling month (**Table 1**). Bacterial richness varied a function of cropping system and month ( $p = 0.049$ , equivocal, 0.008, respectively, **Table 1**). Richness was higher in the organic

**TABLE 1** | Statistics from Type III Analysis of Variance for mixed effect linear models of bacterial richness, Shannon's Diversity, and Shannon's Evenness.

	Numerator Df	Denominator Df	F statistic	p-value
<b>Richness</b>				
Cropping System	2	24.14	3.43	0.049
Month	1	25.70	8.41	0.008
<b>Diversity</b>				
Cropping System	2	23.38	2.62	0.094
Month	1	25.08	4.87	0.037
<b>Evenness</b>				
Month	1	24.61	7.57	0.011

**TABLE 2** | Pairwise comparisons from Tukey's *post-hoc* analysis of alpha diversity models.

	Contrast	Estimate	t ratio	p-value
<b>Richness</b>				
Cropping System	Organic grazed–Chemical no-till	212.7	2.59	0.041
	Organic tilled–Chemical no-till	131.5	1.60	0.266
	Organic grazed–Organic tilled	81.2	0.99	0.592
Month	June–August	171	2.89	0.008
<b>Diversity</b>				
Cropping System	Organic grazed–Chemical no-till	0.169	2.26	0.082
	Organic tilled–Chemical no-till	0.107	1.43	0.344
	Organic grazed–Organic tilled	0.062	0.83	0.690
Month	June–August	0.123	2.20	0.037
<b>Evenness</b>				
Month	June–August	–0.005	–2.74	0.011

grazed system than in the chemical no-till system, an estimated difference in means of 212 observed sequence variants when averaged across months ( $p = 0.041$ , **Table 2**). Bacterial richness was significantly higher in June than in August with an estimated difference in means of 171 observed sequence variants when averaged across cropping systems ( $p = 0.008$ , **Table 2**). Bacterial richness did not differ between the grazed and tilled organic systems. Cropping system as a predictor of Shannon's diversity was trending toward significance ( $p = 0.09$ , **Table 1**). The chemical no-till system was trending toward less diversity than the organic grazed system, with an estimated difference in mean Shannon's diversity measure of 0.169 when averaged across months ( $p = 0.08$ , **Table 2**). Soil bacterial diversity and evenness also changed significantly by month ( $p = 0.037$  and  $p = 0.011$ , respectively, **Table 1**). Shannon's diversity was higher in June than in August, with an estimated difference in means of 0.123 ( $p = 0.011$ , **Table 2**). Mean Shannon's evenness, however, increased by an estimate of 0.005 by the end of the summer ( $p = 0.011$ , **Table 2**).

## Overall Community Composition: Abundance

Taxa from the phyla Actinobacteria, Proteobacteria, Verrucomicrobia, and Firmicutes consistently had the highest relative abundance across all three cropping systems (**Supplementary Figure 2**), though there were some

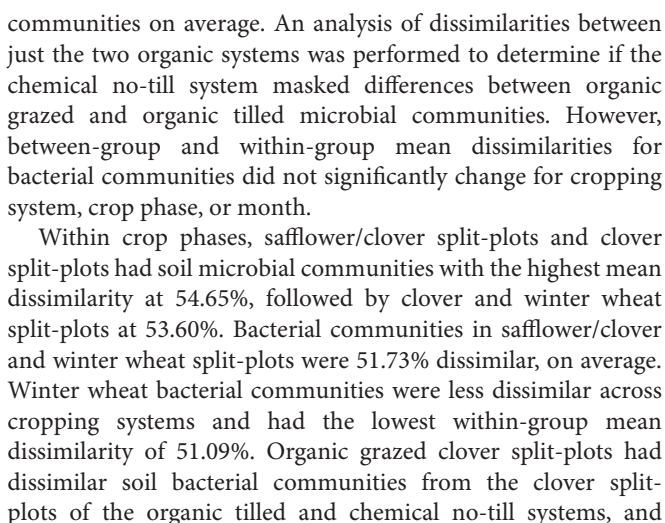
shifts in community composition. Sequence variants from *Bradyrhizobium* (Proteobacteria) and *Candidatus Udaeobacter* (Verrucomicrobia) were more abundant in June while those from *Candidatus Nitrososphaera* (Thaumarchaeota, Archaea) and *Blastococcus* (Actinobacteria) were more abundant in August (**Figure 2**). *Pseudarthrobacter* (Actinobacteria) remained highly abundant throughout the summer in almost every treatment plot (**Figure 2**). Some changes in bacterial abundance occurred within particular crop phases or cropping systems. For example, one sample in an organic tilled clover plot had a high abundance of *Candidatus Udaeobacter* in June but not in August, while organic tilled plots had higher abundance of Bacillaceae in August than in June. Other sequence variants were abundant throughout the summer, such as Nitrososphaeraceae, Xanthobacteraceae (Proteobacteria), *Sphingomonas* (Proteobacteria), and a number of taxa not identified at the genus level (**Figure 2**).

A random forest analysis identified the top 50 sequence variants, by calculated importance ( $p < 0.05$ ), that responded to cropping systems in each month (**Figure 3**). The relative abundances of these taxa faceted by month indicated which predictor taxa were important to different cropping systems in June and August (**Figure 3**). *Pseudarthrobacter* was the most abundant, followed by Nitrososphaeraceae and *Candidatus Udaeobacter*. Log relative abundance of *Pseudarthrobacter*, increased in all three cropping systems from June to August. Nitrososphaeraceae increased in the chemical no-till and organic tilled systems and decreased in the organic grazed system while *Candidatus Udaeobacter* did the opposite (**Figure 3**). The random forest analysis had an out-of-bag error estimate of 19.61% as a prediction error for bootstrapped samples that did not contain elements of the original dataset.

## Beta (Between-Community) Diversity

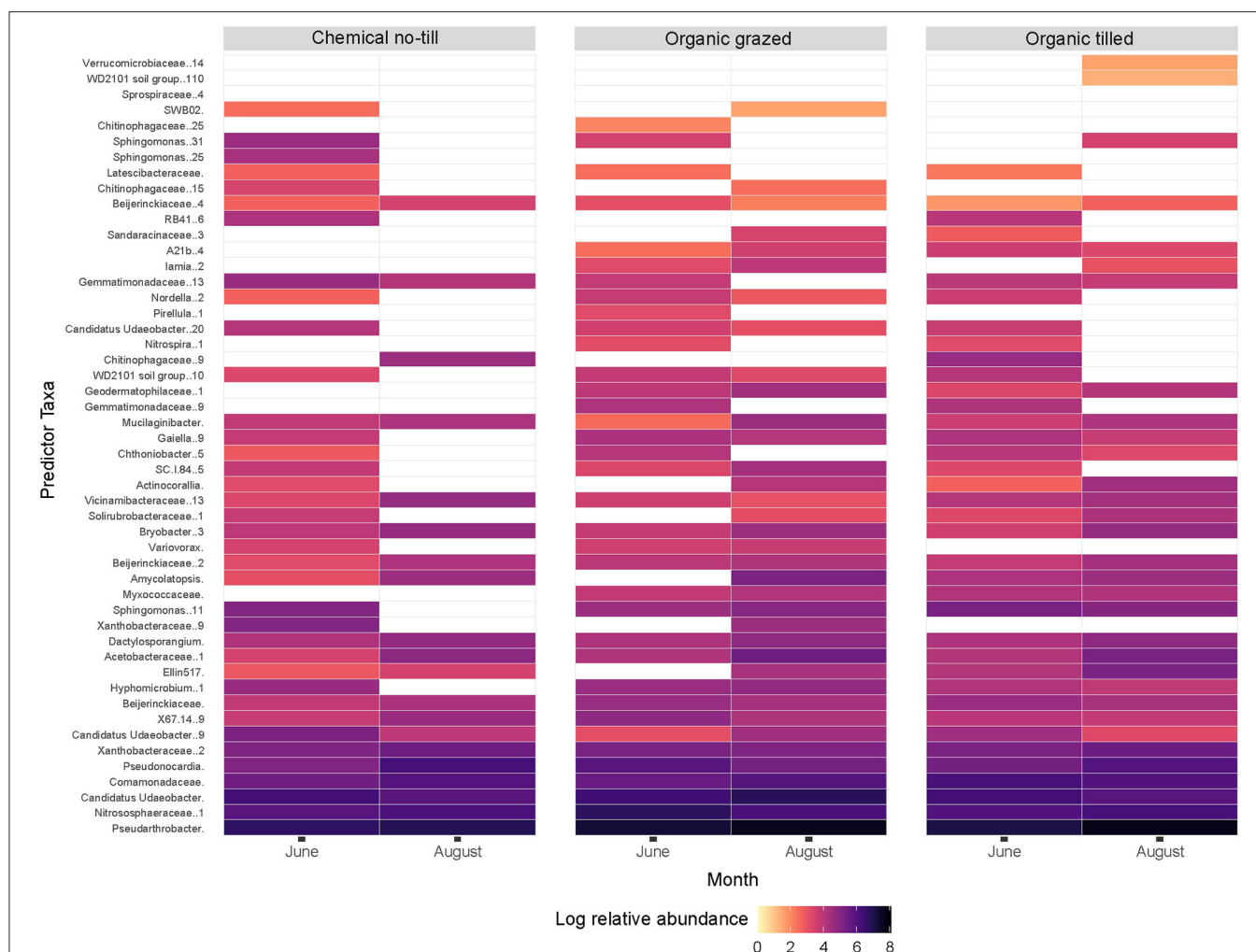
The PERMANOVA indicated that cropping system, crop, month, and the interaction between cropping systems and crop identity impacted bacterial communities (**Table 3**). Of the three treatments, cropping system explained the most variation among communities (10.1%) and the interaction between cropping system and crops explained second (8.3%). The organic grazed system had different soil bacterial communities from the organic tilled and chemical no-till systems (**Figure 4**). Organic grazed communities and chemical no-till communities had a mean dissimilarity of 55.06%, followed by 54.94% between organic grazed and organic tilled communities. Chemical no-till and organic tilled systems had less dissimilar communities in comparison with a mean dissimilarity of 52.26%. Dissimilarity decreased in the chemical no-till system, increased in the organic grazed system, and changed little in the organic tilled system as the summer progressed (**Figure 4**). Communities in organic grazed systems had the highest within-group mean dissimilarity of 53.25% on a weighted Bray-Curtis scale. Communities in the organic tilled and chemical no-till systems had within-group dissimilarities of 50.65 and 51.04%, respectively.

Soil microbial communities become more dissimilar throughout the summer, and this was driven more by cropping system than the crop phase (i.e., plant species identity) (**Figure 4**). June communities were 53.95% dissimilar from August



communities under organic tilled safflower/clover split plots were more dissimilar from the organic grazed and chemical no-till safflower/clover (**Figure 5**). Clover communities had the highest mean within-group dissimilarity in all cropping systems at 56.05%. Bray-Curtis dissimilarities did not differ among crop phases in the cropping systems, but there was an interaction between cropping system and month ( $p = 0.033$ ). However, there were no significant *post-hoc* comparisons to indicate which systems changed in beta diversity as a function of month.

The sustainable productivity of arable land is dependent on preventing or remediating soil degradation, and alternatives to common agricultural practices need to be thoroughly studied to allow researchers to make accurate recommendations for producers. With that goal in mind, this study was part of a



**FIGURE 3 |** Log relative abundance of the top 50 predictor sequence variants in all cropping systems by month represented by a random forest analysis. White spaces indicate taxa that are absent or at very low abundance. Sequence variants from unspecified genera are identified at the family level.

field experiment comparing the effects of cropping systems on crop production, soil bacterial communities, and other ecosystem dynamics. In this study, we confirmed that organically grazed systems support higher soil bacterial richness and Shannon's diversity than chemically managed systems but did not find evidence suggesting that soil bacterial diversity differed between organic systems or was higher in cover crops compared to winter wheat, when accounting for cropping systems and sampling month.

## Differences Between Cropping Systems

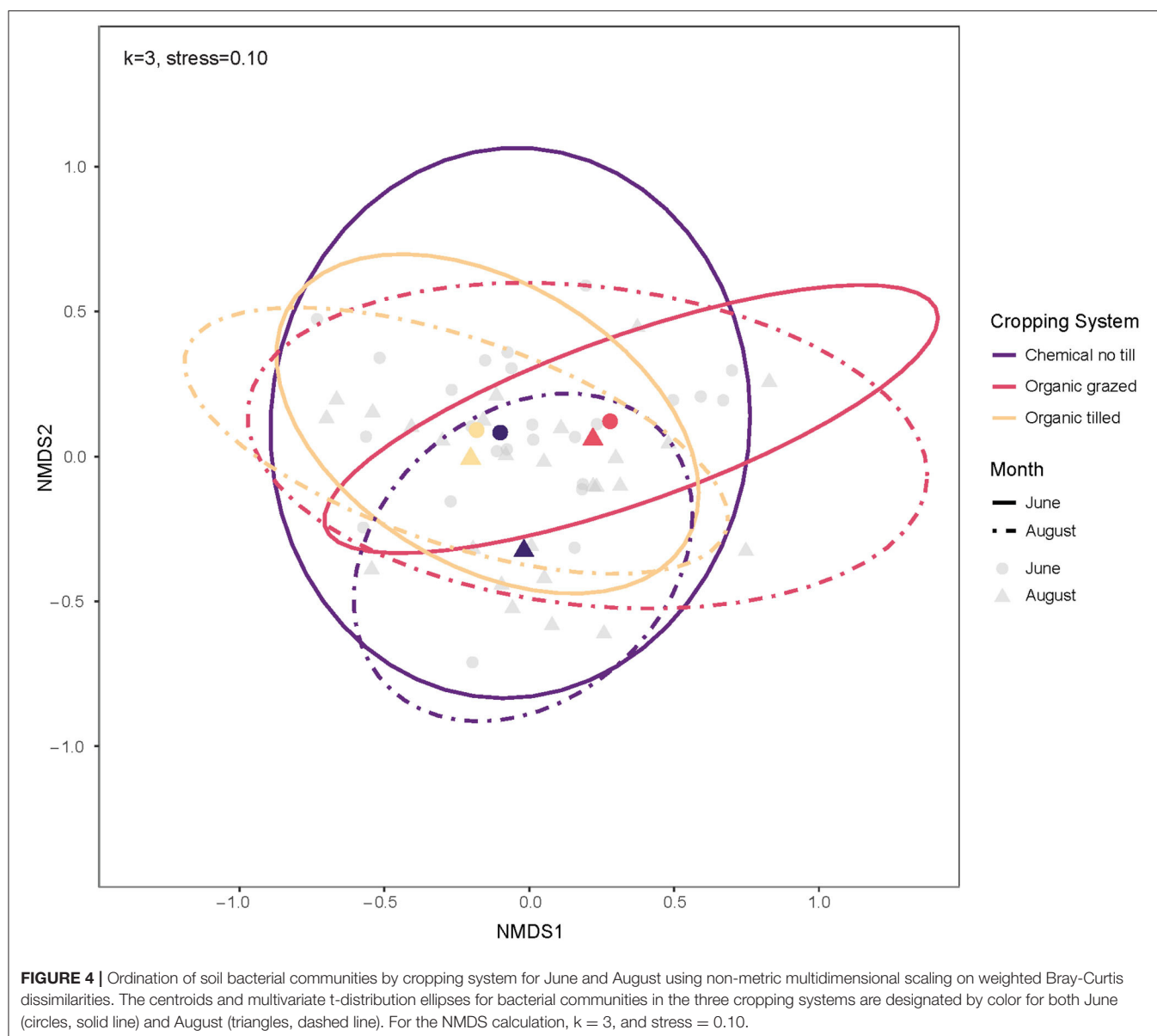
Management practices used by chemical and organic systems can result in divergent microbial communities, principally due to the effects of tillage, fungicides, fertilizers, herbicides, and divergent plant communities, factors which are known to affect macro- and microbial diversity (Hartmann et al., 2015; Smith et al., 2015; Nettles et al., 2016; Zuber and Villamil, 2016). In this study, the chemical no-till system resulted in the least diverse microbial communities, which were dissimilar to those

**TABLE 3 |** PERMANOVA of the effects of cropping system, crop, month, and the interaction between cropping system and crop on soil bacterial communities.

Variable	Df	F model	R <sup>2</sup>	p-value
Cropping system	2	2.85	0.101	0.0001
Crop	2	1.288	0.045	0.0001
Month	1	2.434	0.043	0.0001
Cropping system: Crop	4	1.176	0.083	0.0003
Residuals	41		0.727	
Total	50		1	

communities under grazed but not tilled organic management. Both organic systems in this study utilized different levels of tillage for weed management and cover crop termination. Specifically, the organic grazed system did not receive any tillage for the first 3 years of the rotation while soil at the organic tilled system was mechanically disturbed on a regular basis



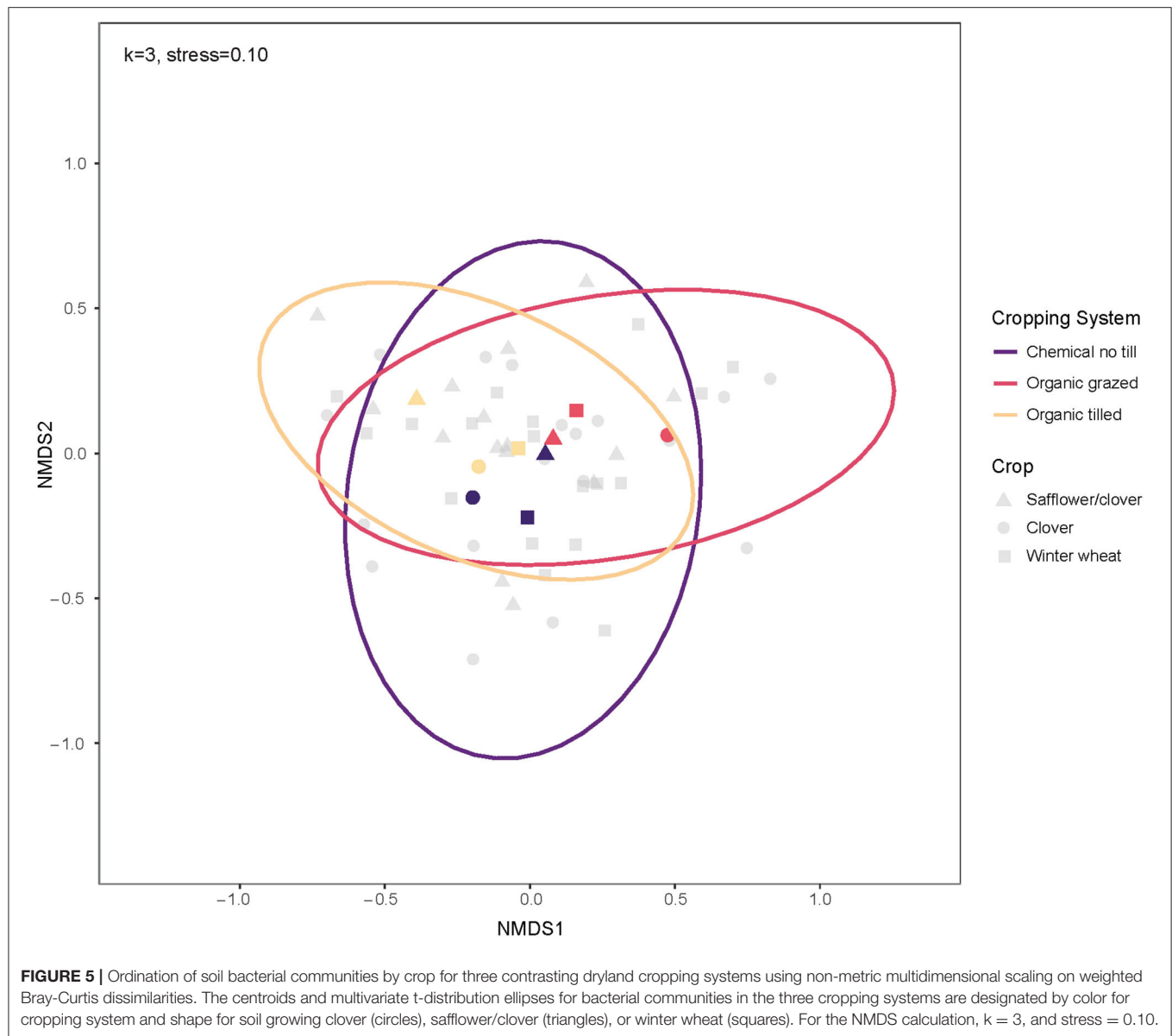


during this study. Tillage can act as a selective pressure leading to a difference in soil microbial community composition under contrasting soil disturbance regimes (Navarro-Noya et al., 2013). Breaking apart soil aggregates exposes previously protected soil organic matter to oxidizing conditions (Six et al., 2000; Van Groenigen et al., 2010) favorable to fast growing copiotrophic bacteria (Srouf et al., 2020). However, findings on the effects of tillage on soil microbial communities are not consistent. Studies have reported higher microbial richness and diversity in no-till systems compared to tilled systems while others find no difference between the two (Navarro-Noya et al., 2013).

### No Impact of Crop Rotation Phase

Soil microbial community alpha diversity was less affected by plant species in the crop rotation than by the overall cropping

system and seasonality, a result previously demonstrated in this field experiment (Ishaq et al., 2020a). The lack of a response to crop phase (i.e., plant species identity) indicates other factors were more important in determining microbial community composition. It is presumed that chemical- vs. organic-based inputs and soil disruptions from tillage (Ishaq et al., 2020a), as well as seasonal temperature and precipitation (Ishaq et al., 2020b), are stronger selective pressures of soil microbial communities than the effects that crop species have on quality of plant residue inputs (Schmatz et al., 2017) or microbial-recruitment by plants (Ishaq et al., 2017). A similar study of crop rotations and management strategies in the Central Great Plains found that conservation tillage and arable weed diversity affected soil microbial communities much more than cover crop diversity (Wortman et al., 2013).



This study identified that soil microbial communities were impacted by an interaction between cropping systems and crop identity. In particular, within-group community dissimilarity was higher in the organic grazed system, regardless of crop. Soil bacteria from clover subplots had the highest within-group dissimilarity in all cropping systems, indicating that local environmental factors created more variation in the membership of bacterial communities. This may be due to the effect of local soil conditions on the soil microbial assembly (Brown et al., 2020), or to genetic variation in plants and their relative interactions with soil microbiota (Pérez-Jaramillo et al., 2019; Brown et al., 2020).

Previous research on the impact of tillage and crop identity on soil microbial communities indicated that tillage selects

for faster growing taxa while cover crops select for moderate-growth taxa with more biochemical capacities (Schmidt et al., 2018). Moreover, the crop species used in rotations can alter soil microbial communities both taxonomically and functionally. While clover is known to recruit nitrogen-fixing bacteria in root nodules, clover and grass crops increase soil respiration from the community as a whole and presumably digestion of complex carbohydrates (Martínez-García et al., 2018). The effects of safflower planted with clover on soil microbiota has not been thoroughly evaluated, but previous studies linked safflower fertility to high soil bacterial abundance and low fungal abundance (Lu et al., 2013). This may be due to the phosphate-solubilizing bacteria recruited to the safflower rhizosphere (Zhang et al., 2019) taking the place of mutualistic soil fungi

that create bioavailable phosphorus, which form the basis of their nutritional symbiosis with plants in exchange for sugars.

In the present study, given the drought resistance of safflower, the diversity of two plant species in the safflower/clover year, and the bacterial recruitment capacity of clover, it was anticipated that the bacterial diversity in soil would differ among crop phases. It was expected that winter wheat would recruit the lowest bacterial diversity, clover the next highest, and safflower/clover would result in the highest bacterial diversity. Variation in bacterial diversity among crop phases was moderated by cropping system and did not differ based solely on crop. Safflower/clover did not have a higher bacterial richness, but it did exhibit less variability in richness between plots and appeared to lose fewer taxa between June and August. Climate data at the experimental site show a trend of decreasing precipitation and increasing temperature over the course of the growing season (Adhikari and Menalled, 2020), which can lead to a decrease in microbial community diversity (Naylor and Coleman-Derr, 2018).

## Differences Between Sampling Months

Low bacterial richness observed in August was presumably related to low soil moisture due to severely dry late summer conditions, an effect which has been observed previously (Fuchslueger et al., 2014; de Vries et al., 2018; Naylor and Coleman-Derr, 2018; Ishaq et al., 2020b). Soil bacteria are in closer contact with their surroundings and are limited by the availability of resources. In addition to the lack of moisture itself, microbial communities are altered by a reduction in plant-soil feedbacks which occur under drought conditions (Fuchslueger et al., 2014; de Vries et al., 2018). The large decrease in alpha diversity observed in the chemical no-till safflower/clover split-plots may have been compounded by crop senescence in these plots, heightening community vulnerability to dry soil conditions.

Dissimilarity in soil communities between June and August reflect a reduction in species richness and an increase in evenness associated with late summer dry soil conditions. In agreement with Ishaq et al. (2020b), we observed that soil bacterial communities become more disparate toward the end of the growing season when plant growth and moisture are no longer selecting bacterial growth and localized differences in fields may determine which bacteria can survive. If these spatially specific effects persist over time, it can lead to legacy effects on the microbial community over several growing seasons, affecting system resiliency (Seipel et al., 2019).

Overall, soil microbial communities in bulk soil respond to overarching management systems but not necessarily crop species. Conditions within the growing season such as precipitation and soil moisture have a more pronounced effect on community richness and composition than crop phase. Looking to future research, this study generated additional hypotheses and considerations. The rhizosphere needs to be sampled

rather than bulk soil to assess how microbiota respond to the specific phases of a crop rotation (i.e., plant species identity), as differences in bulk soil microbial communities among the crop rotation plots were not detectable. Additionally, studies need to incorporate multiple time points in the growing season and assess long-term changes in soil microbial communities caused by disturbances. This knowledge, in turn, will allow an improved understanding of how management systems and their associated ecological disturbances create circumstances from which it is more difficult for microbial communities, soil health, and plant productivity to recover.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: <https://www.ncbi.nlm.nih.gov/bioproject/?term=prjna672991>, PRJNA672991.

## AUTHOR CONTRIBUTIONS

TO performed data analysis, interpretation, and wrote the manuscript. JE performed laboratory protocols and facilitated sequencing. SI contributed to data analysis code development. JE, TS, FM, and SI performed high-level development of the experimental design and analysis, as well as review of the manuscript. FM was principal investigator for USDA funding and reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.624242/full#supplementary-material>

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# Plant Microbiota Beyond Farming Practices: A Review

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Plants have always grown and evolved surrounded by numerous microorganisms that inhabit their environment, later termed microbiota. To enhance food production, humankind has relied on various farming practices such as irrigation, tilling, fertilization, and pest and disease management. Over the past few years, studies have highlighted the impacts of such practices, not only in terms of plant health or yields but also on the microbial communities associated with plants, which have been investigated through microbiome studies. Because some microorganisms exert beneficial traits that improve plant growth and health, understanding how to modulate microbial communities will help in developing smart farming and favor plant growth-promoting (PGP) microorganisms. With tremendous cost cuts in NGS technologies, metagenomic approaches are now affordable and have been widely used to investigate crop-associated microbiomes. Being able to engineer microbial communities in ways that benefit crop health and growth will help decrease the number of chemical inputs required. Against this background, this review explores the impacts of agricultural practices on soil- and plant-associated microbiomes, focusing on plant growth-promoting microorganisms from a metagenomic perspective.

**Keywords:** farming practices, PGPR, metagenomics, microbiome engineering, microbiota, soil microbial ecology

## INTRODUCTION

For 10 millennia, humankind has continuously reshaped its environment for the purpose of food production. With the green revolution, farmers began to intentionally reshape their microbial niches through the massive use of chemical inputs such as pesticides. The intensification of farming practices dramatically unbalanced crop-associated microbial communities. The emergence in the 1980s of plant growth-promoting rhizobacteria (PGPR) was designed to take account of microbial communities and their beneficial traits for crops as a whole (Kloepper et al., 1980). Since then, numerous beneficial microorganisms have been identified and broadly characterized.

Most PGPR or biocontrol agents are associated with rhizosphere and root endosphere microbiota that may be considered derivatives of surrounding bulk soil microbiota. Understanding the fate of such microbiota is fundamental to developing smart farming practices, although a tremendous amount of work is required to determine how to achieve this. Rhizosphere microbial communities are modulated by various abiotic and biotic factors. There are numerous underlying mechanisms explaining the composition, structure, and fate of belowground microbiota, such as the rhizosphere effect mediated by bipartite interactions (Hartmann et al., 2008; Mendes et al., 2013). Some bacteria can be vertically transmitted from the seed to the next generation and thrive

in the early stages of root development (Hardoim et al., 2012; Liu et al., 2012). Aboveground microbial community assembly is also strongly host- and compartment-dependent, suggesting an important relationship between a plant and its epiphytic microbiota, which can originate from seed, soil, and air (Vorholt, 2012; Hardoim et al., 2015). Despite the tremendous progress that has been made in the study of crop-beneficial microbes, there has been no successful development of field-effective bio-based plant protection products. This highlights the need to consider the fate of such microorganisms in the agroecosystems to which they have been introduced and the biological functionalities effectively provided by a crop-associated microbiome.

A microbiome was initially defined as the genome of microbial communities inhabiting specific ecological niches and interacting through distinctive and specific functions (Whipps et al., 1988). Recently, Berg G. et al. (2020) expanded this definition, enlarging the concept of the microbiome to include all microbiota, including prokaryote and eukaryote microorganisms, their habitats, and their “theater of activity” mediated by microbial structures, metabolites, and nucleic elements.

Addressing the diversity, composition, and structure of a microbiome will provide deeper insights in the numerous microbial functionalities supporting plant health and growth (Lemanceau et al., 2017; Compant et al., 2019). Such metagenomic studies have only been achievable following the emergence of next-generation sequencing (NGS) technologies. NGS also contributes to the development of other omics technologies (transcriptomic and proteomic), enabling the description of biological functions carried out by microbiota. In the coming years, considerable progress in the understanding of microbiomes will be achieved through a combination of NGS technologies and descriptive approaches that equate more closely to biological facts.

One can expect to decrease the need for chemical inputs by modulating microbial communities in a way that benefits crop health and growth. Against this background, the present work reviews, from a metagenomic perspective, the impacts of cropping practices on soil and plant microbiomes with a focus on microorganisms promoting plant growth.

## IMPACT OF TILLAGE PRACTICES ON SOIL MICROBIOTA

For centuries, tilling has been helping farmers to prepare land for crops. Plowing assists in incorporating crop residues, preparing the seedbed, alleviating soil compaction in topsoil layers, and decreasing weed, pest, and soilborne plant pathogen load (Tilman et al., 2002; Hobbs et al., 2008). Although plowing is known to increase soil fertility and yield in the short term, it leads to a major soil structure disturbance. The destruction of soil macroaggregates and networks of pores results in severe soil erosion and ecological niche homogenization. Conservation or reduced tilling as well as no-till practices emerged in the 1930s to address the detrimental effects of conventional tillage. With massive improvements in NGS technologies, tilling practices are now increasingly investigated from a microbial perspective.

Indeed, most studies describe plowing as one of the major drivers of soil microbiome diversity along with pedological context and farming practices such as organic management or cover crops (Hartmann et al., 2015; Wang et al., 2017; Alahmad et al., 2019; Babin et al., 2019; Degruene et al., 2019). Tilling regimes impact soil and plant microbial communities in terms of their diversity, structure, and composition.

From a metagenomic perspective, the impact of tilling on soil microbial diversity is usually investigated through  $\alpha$ - or  $\beta$ -diversity indexes describing respective differences within and between communities. Conventional tillage has a low impact on fungal  $\alpha$ -diversity, while increasing prokaryotic  $\alpha$ -diversity has a significant impact on prokaryotic  $\beta$ -diversity favoring opportunistic commensal and copiotroph microbes (Degruene et al., 2017; Hartman et al., 2018; Sommermann et al., 2018; Babin et al., 2019; Banerjee et al., 2019; Piazza et al., 2019; Srour et al., 2020). Tilling facilitates fast organic matter decomposition resulting in a sudden nutrient release that is homogeneously distributed in tilled soil columns, thus increasing the abundance of r-strategists or fast-growing microorganisms (Degruene et al., 2017; Schmidt et al., 2018). A fall in  $\alpha$ -diversity under conservation tillage practices is common and attributable to a reduction in evenness as much as in richness (Degruene et al., 2016; Piazza et al., 2019; Tyler, 2019). Nevertheless, conservation tillage and no-till practices favor slower organic matter degradation and the establishment of less diverse but more oligotrophic, complex, and stable microbial communities (Degruene et al., 2017; Song et al., 2017; Wang et al., 2017; Tyler, 2019; Srour et al., 2020). Babin et al. (2019) consistently observed a higher abundance of predicted genes involved in oligotrophic lifestyles under low tillage conditions. On a long-term basis, decreasing tilling intensity promotes a higher abundance of microbes degrading more complex organic compounds, which enhances soil fertility (Karlen et al., 1994; Souza et al., 2013). Wang et al. (2017) reported concordant results indicating higher soil organic carbon and nitrogen leading to higher nitrogen and carbon plant accumulation under reduced tillage regimes. Low soil disturbance farming systems appear to increase soil nutrient content and stability as well as the number of oligotrophic and structured soil microbiota (Srour et al., 2020; Wang et al., 2020).

The impact of tilling on the structure of microbial communities is often evidenced through  $\beta$ -diversity analyses; however, network analyses are required to investigate its complexity in greater depth. The exploration of such networks requires specific metrics describing their size (nodes and edges), the degree of co-occurrence/exclusion of interacting operational taxonomic units (OTUs), network cohesion (density), centrality, and modularity (Schmidt et al., 2019). Hartman et al. (2018) found that plowing to structure soil bacterial communities by increasing network density also diminishes their size, modularity, and stability. Higher density indicates a larger proportion of interacting prokaryotic OTUs, whether through co-occurrence or co-exclusion relationships. This is consistent with the increases in prokaryotic  $\alpha$ -diversity discussed above. A lower modularity index suggests conventional tilling practices break down the structure of soil prokaryotic communities (Newman, 2006). The effects of tilling on networks of fungal communities



remain unclear but negatively impact the structure of fungal microbiota. Banerjee et al. (2019) identified a significant negative correlation between plowing intensity and fungal network connectivity. They demonstrated that fungal networks were larger and are more densely connected under no till while tilling disrupted hyphal networks.

In addition to the impacts of soil inversion on the complexity and stability of soil microbial networks, tilling has been shown to noticeably affect the composition of microbial communities (Wang et al., 2017; Hartman et al., 2018; Nelkner et al., 2019). Historically, one of the reasons farmers used to plow land was to decrease soilborne pathogen load (Tilman et al., 2002). In fact, by burying crop residues, moldboard plowing helps to control residue-borne or moist-dependent plant pathogens. Reduced tilling or no tilling keeps crop residues in upper soil horizons which favors plant pathogens belonging to *Fusarium* species (Hartman et al., 2018; Sommermann et al., 2018). However, the absence of soil disturbance promotes other fungi forming hyphal networks such as plant-beneficial arbuscular mycorrhizal fungi (AMF) (Degrune et al., 2017; Srouf et al., 2020). Consistent with this, Lienhard et al. (2014) suggest that Actinobacteria exhibiting a mycelia-like growth habit are more sensitive to soil work.

Thus, taxonomical data generated through metagenomic approaches should be considered cautiously depending on the taxonomical level and specific biological and/or ecological functions. While both conventional and conservation tillage increase the abundance of Actinobacteria (Hartman et al., 2018; Babin et al., 2019), Cania et al. (2020) demonstrated that low tilling intensity favors those harboring a higher potential to produce exo- and lipopolysaccharides. These biomolecules are crucial in soil macroaggregate formation and stability and, therefore, play crucial roles in preventing severe erosion events and ecological niche homogenization. Favoring heterogeneous soil microbial niches with overlapping phylogenetic groups and redundant ecological functions contributes to the creation of more resilient and sustainable agroecosystems (Schmidt et al., 2019; Srouf et al., 2020). The sustainability of such agroecosystems also relies on disease-suppressive traits favored under no till conditions as described by Srouf et al. (2020). However, among  $\alpha$ -Proteobacteria, Sphingomonads associated with disease-suppressive traits are favored in till soils, while the abundance of Rhizobiales, encompassing symbiotic nitrogen-fixing bacteria, increases under conservation tilling (Souza et al., 2013; Wang Z. et al., 2016; Degrune et al., 2017; Babin et al., 2019). These findings illustrate the need to consider lower taxonomical levels when investigating soil microbiomes. Ultimately, these studies are paving the way for informed soil work decision-making that will help in recruiting specific microbial guilds and building healthier soils.

## IMPACT OF SOIL COVER ON SOIL MICROBIOTA

In addition to adaptation of the tilling regime, growing cover crops, also known as catch crops, is another efficient way to

prevent soil erosion and increase long-term soil fertility. Abdalla et al. (2019) recently reviewed the impacts of cover crops on the leaching of soil nutrients and crop productivity. They concluded that seeding catch crops significantly decreases nitrogen leaching and increases soil organic carbon sequestration and grain yields when favoring mixed legume–non-legume cover crops.

Indeed, mixing cover crops seems to favor more abundant and specialized microbiomes (Finney et al., 2017). Overall, although the  $\alpha$ -diversity of soil microbial communities is not impacted, specific microbial guilds seem to be recruited when catch crops are implemented in crop rotation (Cloutier et al., 2020; Kim et al., 2020). Cloutier et al. (2020) identified significant changes in soil fungal microbiome with more abundant and distinct arbuscular mycorrhizal fungal communities under cover crop mixtures. Their findings corroborate previous work that shows cover crop mixture and some specific cover crop species (oat and cereal rye) increase AMF abundance in bulk soils (Finney et al., 2017). By contrast, Detheridge et al. (2016) observed lower levels of root endophytic fungi such as AMF under clover cover crops. Because clovers are leguminous crops, the authors argued that this finding might be due to the release of bacterially fixed nitrogen as they identified a negative correlation between high soil nitrate levels and root-associated fungal populations such as AMF. The importance of considering the identity and diversity of cover crops when attempting to benefit from fungal microbiome management has been pointed out by Cloutier et al. (2020). A recent study highlights a positive effect of cover crop diversity on bacterial microbiota evenness. However, Garland et al. (2021) showed this effect to be negligible when considering environmental factors. The low impact of cover crop diversity on microbial biodiversity might be attributed to the occurrence of sampling when one crop is implemented in the field. The authors suggest that diversifying crop systems in a space by intercropping might have a significant impact on overall microbial diversity. By contrast, this study evidences the proportion of time spent using cover crops to be a determinant of taxa-specific and soil microbial diversity.

Soil as much as plant bacterial microbiomes are impacted by cover crops when implemented and destroyed (Fernandez et al., 2016; Finney et al., 2017). As expected, cover crop burial brings fresh organic matter into the soil and increases bacterial diversity and abundance. Some oligotrophic microbes among Acidobacteria and Verrucomicrobia phyla are promoted along with fast-growing bacteria involved in rapid organic matter decomposition among Actinobacteria and Firmicutes (Ramirez et al., 2012; Pascault et al., 2013). Cover crops also contribute to an increased functional redundancy and complementarity in soil prokaryotic communities. The increased functional redundancy derives from the larger soil heterogeneity and niche partitioning provided by the implementation and burial of catch crops (Alahmad et al., 2019). Remarkably, Nivelle et al. (2016) observed that conventional tilling by disrupting macroaggregates diminishes the beneficial impact of cover crops and impairs microbial functional diversity. Alahmad et al. (2019) demonstrated that bacterial communities favored under cover crop regimes are specifically involved in the metabolism of numerous carboxylic acids. Accordingly,

Nivelle et al. (2016) report faster catabolism of carbohydrate and phenolic compounds within microbial communities associated with cover crops. Both studies support the hypothesis advocated by Alahmad et al. (2019) of a functional complementarity within cover crop-associated bacterial microbiomes.

Beyond the beneficial traits harbored by cover crop-recruited soil microbiota, Romdhane et al. (2019) considered how to select an appropriate way to terminate cover crops. According to Alahmad et al. (2019), Cloutier et al. (2020), and Garland et al. (2021), farmers could eventually resort to cover crop diversity and duration to induce shifts in both bacterial and fungal microbiomes in order to reduce fertilization needs while maintaining yields. There is still much work to be done with regard to the low number of studies investigating the impact of cover crops from a metagenomic perspective.

## FERTILIZATION AND AMENDMENTS

Traditionally employed in agriculture, fertilization and amendments have an important impact on soil and plant microbiomes. By modulating the availability of nitrogen or other minerals, carbon, or through modification of the soil structure, these inputs affect the soil and plant life. On the other hand, microbiome can increase the bioavailability of soilborne nutrients. Most nutrients (N, P, S) are results from organic matter degradation and have to be mineralized by telluric bacteria or fungi in order to be available for plants (Van Der Heijden et al., 2008). In natural conditions, microbial mineralization is the key driver of plant growth (Schimel and Bennett, 2004). Plant exudates are known to shape the microbiome and enhance nutrient microbial conversion and bioavailability for plants (Jacoby et al., 2017). Through this molecular dialog, plants established a “microbial nutrient supply chain”. This relation can be unbalanced by fertilization, especially chemical inputs.

### Fertilization

It has been established that nitrogen supply can impact disease development. Indeed, high concentrations of nitrogen, usually in crops, are often positively correlated with an increase in the susceptibility of plants to diseases (Agrios, 2005).

One notable study led by Fierer et al. (2012) investigated soil communities across nitrogen gradients using genomic as well as physiological tools. In terms of diversity, both sites receiving the highest levels of nitrogen differed from others (intermediate and low levels). The authors evidenced a shift to copiotrophic bacterial communities. This shift was confirmed by metagenomic analyses, with high-rate reproducing copiotrophic bacteria exhibiting an increase in DNA, RNA, and protein metabolism and a decrease in urea decomposition, suggesting a diminishing reliance on organic forms of nitrogen. These observations may have an impact on specific organisms such as plant pathogens. Wei et al. (2015) suggested that the invasion of plant roots by pathogens decreases when there is an overlap between the resident communities and invading pathogens due to intensified competition for resources. However, when the results of Wei et al. (2015) and Fierer et al. (2012) are compared, it can be inferred that N fertilization, by limiting resource

competition, enhances phytopathological disorders. Berg and Koskella (2018) confirmed that N also decreases the protective ability of phyllosphere microbiota. After evidencing that a phyllosphere microbiome could prevent leaf colonization by *Pseudomonas syringae*, the authors demonstrated that the use of a fertilizer significantly decreased this protective effect.

Another study found that N fertilization drastically reduced phagotrophic protists in different soil types. These protists are microbial predators and could play a role in the modulation of soil microbial communities (Zhao et al., 2020).

Nitrogen fertilization can also have an impact on the phosphorus cycle. Dai et al. (2020) demonstrated that the long-term use of N fertilization decreased microbial P-solubilizing and mineralizing capacity by modulating microbial communities while P fertilization favored immobilization by microorganisms by altering the functional profiles of soil microbiota. Another study focusing on phosphorus inputs alone also found that acid phosphatase activity was reduced along with the solubility of mineral P (Pantigoso et al., 2018). Similar to the findings of Dai et al. (2020) for N inputs, the authors suggested that P fertilization decreases the P-solubilizing abilities of soil microbiomes.

Few studies have been conducted on K fertilization alone. Although Pan et al. (2014) showed that K fertilization shapes the soil communities but not the functions in grasslands, no other study was found to be relevant in this context. Studies on NPK fertilization in combination revealed the same trends in soil microbiomes (Pan et al., 2014; Chen et al., 2020). Based on such findings, Zhang et al. (2017) suggested that the affected pH rather than the nutrients was responsible for these shifts in microbial communities.

### Organic Amendments

Bonanomi et al. (2018) reported that the suppressive effects of organic amendments have been exhibited in 78 plant pathogens since the 1940s. Although the authors recognized that the results were often inconsistent and difficult to adapt in prediction tools, the comprehension of chemical actions such as glucosinolates in suppressiveness (Larkin and Griffin, 2007) or the link between amendment, suppressiveness, and bacterial communities (He et al., 2012) paves the way to a better management of beneficial microbes through amendments. Nevertheless, numerous studies concerning amendments and microbiomes focus on a single pathology or a single crop (Cesarano et al., 2017; Inderbitzin et al., 2018) or describe microbiomes in different conditions (Bonanomi et al., 2016), without successfully identifying guidelines for microbiome management. A notable point of view on this issue was presented by Bonanomi et al. (2010) in a meta-analysis of 252 papers. The authors primarily explored the characteristics of organic soil amendments linked to a suppressive effect in soilborne diseases. They found that multiple characteristics can apparently be discarded and that six parameters are particularly useful for predicting suppressiveness prediction. These parameters, both enzymatic and microbial, are the FDA (fluorescein diacetate), enzymatic activity, substrate respiration, microbial biomass, total culturable bacteria, and populations of *Pseudomonads* and *Trichoderma* spp.

Another potentially notable mechanism is the determination of the feeding preferences of microbes. Bonanomi et al. (2018) compiled an analysis of recent studies based on  $^{13}\text{C}$  cross-polarized magic angle spinning nuclear magnetic resonance (C-NMR) and evidenced several differences in substrate preferences. Although these data provide valuable insights, the authors insist on the need for an in-depth study in collaboration with laboratories worldwide. Although studies demonstrate the positive effect of a combination of organic amendment and beneficial microorganisms (Latha et al., 2011; Shen et al., 2015), these current solutions cannot be generalized.

Fertilization and organic amendments shape the soil microbiome, principally through nutrient availabilities, but also through pH or modulation of other soil parameters. Although chemical fertilization seems to make the soil microbiome “dependent” of these nutrients, favoring copiotrophic bacteria, and decreases the solubilizing and mineralizing abilities of bacteria of N and P cycles, organic amendments offer more possibilities. In this respect, Ling et al. (2016) found that long-term organic amendments support stronger functional potential and more interactions within soil communities than chemical fertilization, most likely due to better soil stabilities and a good buffering capacity.

## PLANT GENOTYPE AND MICROBIOME

Several factors can influence the composition of plant microbiomes, including genotypes, plant developmental stage, and plant health (Berg et al., 2015). Characteristic root exudates are usually considered the causative factor underlying the recruitment of specific microbial communities and are influenced by plant genotype. The recruitment of the plant-associated microbiome can vary in terms of structure and functionality and depends to a great extent on the physical properties of soil and nutrient availability (Berg and Smalla, 2009).

Numerous studies have linked microbial diversity with a reduction in the incidence of disease (Keesing et al., 2010; Kopecky et al., 2019). A low potato common scab is observed, even in favorable conditions, when high bacterial diversity is present in the soil (Latz et al., 2012; van Elsas et al., 2012). Higher soil microbiome diversity offers better odds of finding a higher abundance of rare species able to bring specific protective functions against pathogens (Latz et al., 2012). As reported by Mendes et al. (2018), the exclusive and abundant presence of a bacterial taxon is a poorer indicator of disease suppression than the relative abundance of bacterial taxa.

The breeding of cultivars resistant to pathogens is a well-known practice in the control of diseases. In some cases, the genetic background of the plant may not be the only driver of such resistance. Wei et al. (2019) demonstrated that the apparent resistance of cotton cultivar to *Verticillium* wilt is partially due to the plant microbiome. An abundance of beneficial microbes in the cotton rhizosphere offers a complementary protection against this soilborne pathogen. Similar findings were reported for cucumber resistance to *Fusarium* wilt (Yao and Wu, 2010) and tomato resistance to *Ralstonia solanacearum* (Kwak et al., 2018).

The relative abundance of well-known beneficial rhizospheric and root endospheric microbial groups can vary significantly between resistant and susceptible cultivars (Wei et al., 2019).

However, plant genotype is not the major driver of the early establishment of a rhizospheric microbiome. Several studies indicate that soil type rather than cultivar determines the composition of the rhizospheric microbiome (Van Overbeek and Van Elsas, 2008; Xu et al., 2009; Chen et al., 2019). This feature has been specifically evidenced for fungi (Nallanchakravarthula et al., 2014), bacteria (Schlemper et al., 2017), and arbuscular mycorrhizal fungi (Santos-González et al., 2011).

Plant genotype becomes an obvious determinant of root-associated microbiomes as plants mature (Inceoglu et al., 2010; Schlemper et al., 2017). For a particular growth stage, different cultivars can have different dynamics in their exudate release dynamics (Micallef et al., 2009; Mönchgesang et al., 2016; Sasse et al., 2018), thereby affecting rhizosphere microbial communities in a particular way.

The effect of the host genotype on microbial populations is much more important in the endosphere (Urbina et al., 2018). This is not surprising as co-evolution processes have selected populations that are well-adapted to this niche. In the very early stages of a plant's life, i.e., around germination, the microbial community of the spermosphere is composed of microbes originating from inside and outside the seed as well as microbes recruited from the soil during imbibition (Lemanceau et al., 2017). At this point, the main factor affecting the colonizer communities of the spermosphere is the seed genotype (Adam et al., 2018; Sahadevan et al., 2019). Microbial communities associated with germinating seeds can have a direct impact on the promotion of plant growth, nitrogen fixation, and disease control (Walitang et al., 2017; Rahman et al., 2018; Sabu et al., 2019). With the transformation of rootlets in the spermosphere into roots creating the rhizosphere, the plant genotype becomes of minor importance in community shaping compared with soil type.

A recent study on tomato root endospheric fungi evidenced a clear difference in the phytohormone profiles between two cultivars harboring different endophytic communities (Manzotti et al., 2020). More work is needed to determine whether the hormonal profile determines the composition of endophytic communities (as with root exudates) or whether it is a consequence of mycobiome composition. For bacteria, it has been shown that most endophytic bacteria can interfere with plant hormonal systems (Jasim et al., 2015, 2016).

Plant genotype can also play a critical role under mixed cropping conditions. It has been shown that two varieties of pea can influence each other in terms of root-associated bacterial and fungal populations (Horner et al., 2019). In this particular study, the root bacterial community of one cultivar remains stable (similar to single-crop community) in response to mixed cropping, whereas the bacterial community of the second cultivar shifts toward the first. Conversely, the root fungal community of the second cultivar remains stable when mixed-cropped, whereas the fungal community of the first cultivar shifts toward the second.

Polyculture has been identified as a way to enhance rhizospheric fungal diversity (LeBlanc et al., 2015), which can be



modulated by the identity of plant species (LeBlanc et al., 2017) and by environmental parameters that can also have a significant influence (Schlatter et al., 2015).

Differential shifts in bacterial and fungal communities can be attributed to microbial interactions, a change in soil attributes or a change in root exudates resulting from competition between intervarieties, plant communication, or a better mineralization of organic matter enhancing nutrient availability (Hinsinger et al., 2011; Reiss and Drinkwater, 2018).

## BIOSTIMULANTS AND MICROBIOMES

Biostimulants can be defined as substances or microorganisms that stimulate natural processes to enhance tolerance to abiotic stress, crop quality, and nutrient uptake and efficiency (du Jardin, 2015). The effect of biostimulants can be attributable to the direct effects of carbon or nitrogen metabolism (Calvo et al., 2014) but also indirectly through the modulation of plant microbiomes through the enhancement of microbial activity (Colla et al., 2015).

Biostimulants are relatively new products and their effects on microbiomes are not well-known, partly due to the great diversity of biostimulant origins. In this paper, we focus on biostimulants obtained from protein hydrolysates from seaweed and on microbial biostimulants.

### Biostimulants Based on Protein Hydrolysates

Tejada et al. (2011) tested four biostimulants on degraded soils. They identified an alteration in microbial community structure and higher microbial activity, which facilitates better plant development on degraded soils. The biostimulant with the best effect in terms of microbial activity and plant development was the one derived from rice bran extract. The authors hypothesized that the effect of this biostimulant was due to its richness in little peptides (<3 kDa), easily assimilable by microorganisms. Other studies focused on the phyllosphere microbiome. Luziatelli et al. (2016) found that a biostimulant from protein hydrolysates modulated the leaf microbiome on lettuce. Notably, microbes isolated from lettuce leaves treated with biostimulant indicated the presence of bacteria enabling phosphorous solubilization or producing phytohormones (IAA). Moreover, the biostimulant favored the presence of *Bacillus* species exhibiting an inhibitory activity against leaf lettuce pathogens *Erwinia amylovora* and *Fusarium oxysporum*. The biostimulant could thus shape the phyllosphere, promoting plant growth.

Regarding protein hydrolysate (PH) biostimulants, Colla et al. (2017) concluded that this kind of biostimulant can help provide better resilience to biotic and abiotic stresses by modulating the microbiome. Given that PH biostimulants can only select beneficial microorganisms present in the rhizo- or phyllosphere, the authors proposed their use in synergy with beneficial microbes.

### Biostimulants Derived From Seaweed

Most investigations of seaweed-derived biostimulants have been conducted under the scope of the functional diversity of the

microbiome, through enzyme activity research rather than an analysis of the microbial community diversity. Ji et al. (2017) reported that the P solubility was higher after a seaweed biostimulant application but without affecting the microbial communities. The positive action of seaweed biostimulants indicated greater activity of hydrogenase (Onet et al., 2019), invertase, urease, proteinase, and phosphatase (Wang Y. et al., 2016). These enzymes, involved in the carbon, nitrogen, and phosphorus cycles, explain the better nutrition status of the plants.

### Microbial Biostimulants

Despite the fact that microbial biostimulants are relatively common in agriculture, usually through PGPR products or vermicomposts, their effect on microbiomes is poorly documented. Berg S. et al. (2020) studied the effect of microbial biostimulants on soil and root microbial communities. Although no significant differences were found in the diversity of these communities, except for the fungi, the tested biostimulants did not increase the yield.

Mahnert et al. (2018) studied the impact of vermicompost on leaf and environment microbiomes under controlled conditions. It appeared that the biostimulant reshaped the microbiomes of the leaves, with an increase in Bacteroidetes and other phyla such as Verrucomicrobia, Acidobacteria, and Thaumarchaeota. Other groups containing beneficial microorganisms also increased. The authors demonstrated that the effect of the biostimulant on microbiome composition could be predicted with an accuracy of 87%. To the best of our knowledge, no other significant research has been conducted on this theme. The two research papers yield different results, maybe lightening the impact of field conditions on plant biostimulation, and that a stronger impact of plant biostimulant products are observed under controlled conditions or synthetic substrates.

## IMPACT OF IRRIGATION/WATER ON MICROBIOME

Irrigation can have an impact on microbial communities and plant microbiomes through the frequency of irrigation or the quality of irrigation water. Due to climatic changes, the irrigated area is predicted to increase to 62% from 2020 to 2070, with an impact on soil and, therefore, microbial communities (Döll, 2002). Without irrigation, soils will alternate between drying and rewetting periods. In these conditions, more active microbes are known to be affected more by these drying–rewetting stresses (Van Gestel et al., 1993). For an active microbial rhizosphere, drying and rewetting periods could be damaging. Fierer et al. (2003) focused on the impact of this alternation on soil bacterial community structure. They found that microbial communities in annual grasslands were less affected in terms of biodiversity by these events than forest (oak) soils. Nonetheless, Wu and Brookes (2005) reported a 44% decrease in microbial biomass in a single dry–rewet cycle in grassland. It is unclear whether these impacts on microbial structure are direct effects of the dry–rewet cycle or indirect effects through the perturbation of physical or biochemical soil processes, such as the C cycle (Schimel, 2018).



Long-term monoculture irrigation has been relatively poorly studied. Nevertheless, Mavrodi et al. (2018) studied the effect of long-term irrigation on wheat. They reported that beneficial phenazine producing (Phz+) *Pseudomonas* spp. were less abundant or detectable in irrigated fields or in higher rainfall areas. Irrigation should alter rhizodeposition and soil properties, disturbing microbiomes. Mavrodi et al. (2018) found that irrigation had a slight effect on the diversity of the wheat rhizosphere microbiome. However, some taxa displayed strong positive and negative responses to irrigation such as Bacteroidetes and Proteobacteria. Some genera, previously identified as phytopathogen antagonists such as *Chryseobacter* spp., *Pedobacter* spp., or *Brevundimonas* spp., were among the bacteria with the highest relative increase in abundance under irrigation.

The quality of irrigation water also seems to have an important impact on plant microbiomes. Cui et al. (2019), from the perspective of water management, tested different water qualities. They found that reclaimed water and piggery wastewater use increased the abundance of Bacteroidetes while decreasing Acidobacteria abundance. Although PGPR were logically more abundant in the rhizosphere microbiome, their response to the different water qualities (distilled, reclaimed, piggery wastewater) was quite variable. Finally, no increase in (phyto)pathogenic bacteria was evidenced after irrigation with reclaimed water or piggery wastewater.

Gu et al. (2019) also evidenced a modulation of the spinach microbiome according to the quality of irrigation water. Although they did not find any increase in foodborne pathogens, they evidenced an increase in potential opportunistic (phyto)pathogens. These two publications highlight the importance of a quality of irrigation water survey for plant and consumer health.

Some authors have studied the resilience of soil microbial communities after irrigation with water of different quality. It appears that a soil microbiome—and to the same extent plant microbiome—is not resistant to irrigation with treated wastewater. Differences have been observed between irrigation with freshwater and treated wastewater. Nevertheless, during the rainy season, the baseline state of microbiomes is recovered, evidencing the resilience of soil and plant microbiomes in the long term (Frenk et al., 2014). Frenk et al. (2018) showed that under conditions of high mineral and organic carbon activities, bacterial communities can change drastically, exhibiting proteobacterial dominance. These changing communities displayed less resistance to environmental stress such as heat disturbance as they have less diversity than soils with low resource availability. However, the authors evidenced a functional resilience after the end of the stress, probably due to the high growth rates of certain groups such as Bacteroidetes or Proteobacteria.

In conclusion, if irrigation and quality of irrigation have a relative impact on diversity, the impact on biomass of different groups can be important. If populations are resilient in the long term, thanks to microbial seed banks (bacteria in dormancy) (Lennon and Jones, 2011), the impact of irrigation and the quality of irrigation have to be considered in the short term,

during one agricultural season. In this respect, observations have been nuanced. Some studies evidenced the positive role of irrigation on PGPR (Mavrodi et al., 2018), while others obtained variable (Cui et al., 2019) or negative results (Phz+ not present in irrigated soils, Mavrodi et al., 2018). Ultimately, even if wastewater did not seem so harmful when applied in the short term, repeated applications of this kind of wastewater have to be studied for a longer period. According to the observations of Frenk et al. (2018), the use of high availability resource irrigation water in the longer term could probably and durably reshape agricultural soil microbial communities. Conversely, plant microbiome management will probably be a future tool employed to better exploit limited water resources (de Vries et al., 2020).

## CROP PROTECTION

The application of pesticides in fields influences microbial populations inside aerial and belowground plant parts, as well as in the soil. The effects can be due to the applied molecule itself, but also the degradation products of the molecule. Degradation can occur through multiple processes: degradation by microorganisms, hydrolysis, photolysis, sorption and binding to organic and soil components, plant uptake, and volatilization (Srivastava et al., 2020). If microorganisms are able to survive in the environment contaminated by the molecule, they can then metabolize and degrade the pesticides (Wołejko et al., 2020). Therefore, microorganisms can play a significant role in plant tolerance to herbicides (Tétard-Jones and Edwards, 2016).

Depending on the chemical, the active ingredient can be a racemic mixture or enantiomer-enriched solution. Sometimes only one of the two enantiomers has a desired effect, with the other having an indirect effect on non-target organisms (Asad et al., 2017). When a pesticide is applied, it usually leads to the eradication of groups of microorganisms sensitive to the active ingredient. Niches are consequently freed and colonized by microbes previously of minor abundance in the community (Chen et al., 2001) or thriving as a result of the release from competition (Roesti et al., 2005; Nettles et al., 2016). It is also possible that treatment has no significant effect on some parts of the community, such as an effect on the fungal rhizosphere community but not on bacterial communities (Nettles et al., 2016). If a microbial population is well-adapted to the pesticide, the treatment can induce a short-term increase in microbial carbon, indicating increasing biodegradation (Astaykina et al., 2020).

In the presence of pollution, microorganisms can enhance their adaptation to the prevailing conditions by altering their metabolism (Sun et al., 2004; Rangasamy et al., 2018). Therefore, groups of microorganisms can take advantage of an active ingredient in the environment (Webber et al., 2015). For example, Newman et al. (2016) showed a shift in a bacterial community toward a tolerant community after long-term glyphosate adaptation. With the destruction of certain groups of microbes involved in the degradation of another molecule, the stability of this second product can increase (White et al., 2010).

A long-lasting product in the environment can be important in ensuing long-term protection for the crop. This can also increase the likelihood of unintended effects on non-target microbiomes (Nettles et al., 2016). Plants are also capable of exuding pesticides absorbed on their aerial parts with their roots, in addition to endogenous exudates (Dinelli et al., 2007). All these mechanisms can influence the microbiota. Regarding the effects of a pesticide application, as reviewed by Wolejko et al. (2020), the effects of fungicide, insecticide, or herbicide on microbial communities varies greatly according to the molecule used and the microbial group studied. Although most studies agree on the lack of impacts on  $\alpha$ -diversity in the rhizosphere (Lupwayi et al., 2004, 2009; Nettles et al., 2016), the effects on microbiome functionality (Fournier et al., 2020), or structure (Nettles et al., 2016) have been reported with shifts in relative abundance and community composition. In the phyllosphere, microbial diversity can even increase after a foliar treatment (Katsoula et al., 2020). Seed treatments can have a more pronounced and dynamic impact on microbial diversity. As shown by Li et al. (2018), the richness of bacteria and fungi species at the seedling stage decreased with a neonicotinoid seed coating. With a decreasing concentration of neonicotinoids when reviving, the growth of microorganisms was stimulated. Overall, general microbiomes recovered at the end of the cultural season.

## DISCUSSION

From this work, a major trend appears to dictate the process followed when investigating microbial communities from a metagenomic perspective. DNA extractions are almost exclusively performed using a FastDNA Soil SPIN Kit (MP Biomedicals, USA) or PowerSoil DNA Kit (MoBio, Qiagen, USA). Consideration of the extraction procedures implemented remains relevant and may induce bias when comparing one study to another (Kennedy et al., 2014).

When studying prokaryotes, most authors target the 16S rRNA gene, focusing on the V4 region or a wider region including V4. Overlapping between V2 and V3 regions is also commonly used. The diversity of eukaryotic communities is often investigated *via* the sequencing of an internal transcriber region (ITS) or 28S ribosomal unit. Ideally, several genetic markers should be considered (Sommermann et al., 2018).

Currently, sequencing usually relies on Illumina technology when 454-pyrosequencing tends to become more anecdotal. When Illumina technology is used, a large majority of researchers make use of the MiSeq platform and, more recently, the HiSeq platform. Third-generation technologies such as PacBio or Ion Torrent continue to be marginal when investigating soil and plant microbiomes but are expected to become a gold standard in metagenomic approaches (Lee et al., 2016; van Dijk et al., 2018).

Most of the studies presented in this paper used barcoded rRNA sequences. Although this approach enables researchers to assess the impact on microbial composition and diversity, it is notable that when shotgun or enzymatic analyses are applied, authors gain better insight into the networks, relationships inside the communities, and the functional aspects of the microbiome.

The interpretation of metadata produced by next-generation sequencing technologies depends mostly on the data management methodologies implemented. Several best practices are required, such as choosing an adapted normalization strategy (Schlatter et al., 2017; Knight et al., 2018). The normalization process should be selected to fit the size and organization of the datasets, as suggested by Weiss et al. (2017). Sufficient technical replicates should be performed in order to quantify sequencing error rates within an assay and between assays (Nguyen et al., 2015; Schloss et al., 2016).

In almost every paper reviewed, the composition and diversity of microbial communities are affected by the agronomic parameters investigated. Networks and functionalities are nearly always impacted, although these attributes have been poorly studied. Of 54 papers, only 14 studies describe networks, 14 consider biological functionalities, and only three address both aspects simultaneously.

A deeper understanding of crop-associated microbiomes and their functionalities requires a more holistic approach that combines data not only from omics technologies. Investigating the fate of microbial communities requires a four-dimension perspective that examines microbiomes in terms of their diversity, structure, composition, and biological functions. Functionalizing microbial communities not only through prediction tools but also through quantification technologies such as qPCR or enzymatic assays will provide relevant insights that illustrate how those microbial communities and the services they provide are affected by farming practices.

Before studying the impacts of any agricultural parameter on microbiota, environmental features should be systematically considered with greater concern when interpreting the results of such studies. Organic carbon content, nitrogen content, pH, soil structure, soil classification, and moisture content are several parameters that vary from field to field and significantly impact microbiomes. Moreover, some authors highlight the indirect activity of certain practices through pH change (e.g., Zhang et al., 2017). For instance, in 2006, Fierer and Jackson found that soil pH is the main parameter influencing microbiome structure (Fierer and Jackson, 2006). This has since been confirmed by numerous studies (Lauher et al., 2009; Rousk et al., 2010; Geyer et al., 2014; Qi et al., 2018; O'Brien et al., 2019; Schlatter et al., 2020; Tan et al., 2020).

Soil pH determines the chemical forms of element in the soil, therefore affecting their bioavailability for plants. This would also be an indirect limiting factor for microbial life, as suggested by Zhalnina et al. (2015). In addition to nutrient availability, pH also exerts effects on catabolic activities, soil structure, and biomass activities (Wakelin et al., 2008). Low pH can have direct toxic effects on microbe cells. Some organisms, better adapted to acidophilic conditions, possess a special membrane structure, proton pumps, or special transporters (Lehtovirta-Morley et al., 2016). The relationship between soil pH and microbial diversity can also be explained by the wide range of optimal pH tolerance for a community in contrast with the rather narrow pH optima for individual species (Fernández-Calviño and Bååth, 2010). Therefore, a shift in pH will affect the survival of some but not all microbes (Santoyo et al., 2017). Numerous

studies also link pH with microbial activities, such as phosphate solubilization or ammonia oxidation (Nautiyal et al., 2000; Hu et al., 2013; Sharma et al., 2013). Indeed, pH is thought to drive the community composition of ammonia-oxidizing organisms by modifying the ammonia to ammonium ratio (Gubry-Rangin et al., 2011; Stempfhuber et al., 2015).

A recent study demonstrated the influence of pH and depth on microbiomes in an agricultural soil configuration (Schlatter et al., 2020). The authors showed that pH decreases from the surface to a 10-cm depth and so do bacterial richness and diversity. Notably, they observed that bacterial richness and diversity did not recover with increasing pH at depths below 10 cm. These results suggest that pH is the main factor affecting diversity at near-surface depths, while other factors (dispersal, nutrient availability) become prevalent at greater soil depths. This lower microbial diversity at around 10 cm deep could reduce the functional redundancy and resilience of the communities in the seed zone (Shade et al., 2012).

Land use history is also an important parameter to consider when investigating soil-associated microbiota. Although the underlying mechanisms are not well-understood, it is known that plants can recruit specific root microbiomes through root exudates, enabling them to select beneficial microbial traits. Time after time, the soil microbiome is enriched in certain specific taxa, as suggested by the concept of a soilborne legacy (Bakker et al., 2018). Recruited microbiomes produce bioactive metabolites or useful resources for plants. Once the crops are harvested, molecular signals and other plant-beneficial compounds might remain in the soil and benefit the next generation. This phenomenon was proposed by Lapsansky et al. (2016) and is called the soil memory effect.

Considering the duration of assays is therefore of tremendous importance as is the implementation of cover crops, intercropping, and crop rotation composition, all of which are among the numerous parameters to examine when studying crop-associated microbiomes. The long-term studies reviewed in this work reported a certain resilience or results different from those in the short term (Mavrodi et al., 2018), probably due to microbial seed banks (Lennon and Jones, 2011).

In this review, we investigated the impacts of farming practices on soil and plant microbiomes. Within the heterogeneity of the reviewed studies, as previously noted, supplementary approaches to omics facilitated a strong comprehension of underlying mechanisms. Levy et al. (2018) reviewed the different advantages and limitations of -omics techniques. They conclude that no -omics approaches provide the necessary causality and argue that, more than amplicon-based studies, a functional metagenomic

approach is needed and can be supplemented by synthetic communities or reverse genomics approaches. Vorholt et al. (2017) also advise using synthetic communities for a better understanding of microbiomes. They argue that the factors shaping microbial communities in soil matrices are not well-understood because of the complexity of environmental samples. For a better understanding of plant microbiome interactions, they recommend the use of multispecies synthetic communities.

If the impact of agricultural practices on microbiomes exists, we wonder whether the microbiome could be farmed as “collaborative crops.” “Seeding” practices through microbial or consortia inoculation, or a selection of varieties promoting positive microbiomes, are likely to be the future of microbiome management (Compant et al., 2019). These inoculations of strains or microbiome engineering in plants can be obtained in different ways. They can occur through host-mediated and multigeneration microbiome selection; inoculation into bulk soil, rhizosphere, seeds, or seedlings; atomization into tissues such as stems, leaves, and flowers; and direct injection into tissues or wounds. Some recent relevant studies involving the aforementioned techniques are reviewed below. Given the lability of existing microbiomes and the fact that soil memory is more active in a low nutrient environment and thus less adapted to conventional agricultural soils (Lapsansky et al., 2016), microbiome engineering consisting of community inoculation and host-mediated “microbiome maintenance” in single (Orozco-Mosqueda et al., 2018) or associated crops (Horner et al., 2019) could be a useful tool for future pest integrated management.

## AUTHOR CONTRIBUTIONS

CB reviewed the paper. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

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# Efficacy of Biostimulants Formulated With *Pseudomonas putida* and Clay, Peat, Clay-Peat Binders on Maize Productivity in a Farming Environment in Southern Benin

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Maize plays a vital role in Benin's agricultural production systems. However, at the producer-level, yields are still low, although the production of this cereal is necessary for food security. The aims of this study were to assess the efficacy of solid biostimulants formulated from the rhizobacteria *Pseudomonas putida* and different binders on maize cultivation in the farming environment in three (03) study areas in South Benin. For this purpose, three (03) biostimulants were formulated based on *Pseudomonas putida* and the clay, peat and clay-peat combinations binders. The experimental design was a randomized block of four (04) treatments with 11 replicates per study area. Each replicate represented one producer. The trials were set up at 33 producers in the study areas of Adakplamè, Hayakpa and Zouzouvou in Southern Benin. The results obtained show that the best height, stem diameter, leaf area as obtained by applying biostimulants based on *P. putida* and half dose of NPK and Urea with respective increases of 15.75, 15.93, and 15.57% as compared to the full dose of NPK and Urea. Regarding maize yield, there was no significant difference between treatments and the different study areas. Taken together, the different biostimulants formulations were observed to be better than the farmers' practice in all the zones and for all the parameters evaluated, with the formulation involving *Pseudomonas putida* on the clay binder, and the half-dose of NPK and Urea showing the best result. The biostimulant formulated based on clay + *Pseudomonas putida* could be used in agriculture for a more sustainable and environmentally friendly maize production in Benin.

**Keywords:** biostimulants, clay, formulations, peat, PGPR, *Zea mays* L.

## INTRODUCTION

In most West African countries, particularly in Benin, maize (*Zea mays* L.) is emerging as a staple food for food security. It is one of the major cereal crops that undergoes more than a hundred different modes of processing (Adjadi et al., 2015). In terms of production, high nutritional value has been attributed to its grains. The grains have been reported to be a notable source of protein, lipids, fiber and sugar (Ignjatovic-Micic et al., 2015). Maize is the most traded cereal on the domestic and subregional market (Gandonou et al., 2019). Despite the importance of this speculation and its increasing demand, its productivity faces many constraints, including the constant decline in the fertility of cultivated soils due to their degradation (Igué et al., 2013). The land is subjected to severe degradation as a result of poor farming practices that destroy the flora, organic matter and soil fauna and microfauna. Cultivated land is being depleted at an accelerating rate, and crop yields are continually declining, thereby dangerously compromising the productivity and sustainability of the entire agricultural system (Alamri et al., 2016). In modern agricultural systems, thousands of millions of synthetic agrochemicals are used to achieve high crop yields. After application, these synthetic chemicals are not entirely used by plants, but persist in the soil in different forms. In addition, excessive use of synthetic agrochemicals, declining soil nutrients, and water-use issues, amongst others, are threats to the ecosystem (Omomowo and Babalola, 2019). These chemicals seep into the soil, and thus disrupt the diversity and performance of the rhizosphere (Ai et al., 2012) and human health via the food chain (Ayala and Rao, 2002). The use of synthetic fertilizers is therefore not considered as good practice because of the high costs and acute environmental risks (López-Bellido et al., 2013). In order to reduce the use of toxic chemicals, one of the safe management options is the use of environmentally friendly solutions (Adesemoye et al., 2009). These alternatives include microbial biostimulants. Biostimulants are substances or microorganisms applied to plants with the aim to enhance nutrition efficiency, abiotic stress tolerance and/or crop quality traits, regardless of its nutrients content (du Jardin, 2015). Those containing microorganism's such as those containing Plant Growth Promoting Rhizobacteria (PGPR) can directly stimulate plant growth through the production of phytohormones (Kang et al., 2019; Ahmed et al., 2020), biological nitrogen fixation (Glick, 2014; Kumar et al., 2020), production of siderophores (Glick, 2014) and phosphate solubilization (Alori et al., 2017; Agbodjato et al., 2018). As biocontrol agents, PGPRs suppress plant pathogens (Bajracharya, 2019). Some rhizobacteria play an important role in improving soil fertility and plant growth by providing various unavailable nutrients. Rhizobacteria secrete organic acids that reduce the pH of the rhizosphere and thus freely produce phosphate available to plants (Kashyap et al., 2020). Alori and Babalola (2018) mentioned that the use of microbial inoculants is a reliable alternative to the use of chemical inputs because these microbial inoculants can act as biofertilizers, bioherbicides, biopesticides and biocontrol agents. The development of plant biostimulants has become the focus of much research interest. Plant biostimulants are

diverse substances and microorganisms used to enhance plant growth. Plant biostimulants also designate commercial products containing mixtures of such substances and/or microorganisms (du Jardin, 2015). In recent years, there has been increasing use of biostimulants (Schisler et al., 2004; Viswanathan and Samiyappan, 2008; Gu et al., 2014). In Benin, several studies have been carried out on microbial biostimulants based on native PGPRs from rhizospheric soils (Adjanohoun et al., 2012; Noumavo et al., 2013; Agbodjato et al., 2015; Amogou et al., 2019; Adoko et al., 2020). Most of this work was carried out with PGPR-based biostimulant suspensions. The work carried out with solid biostimulants formulated based on different binders in Benin has proved the effectiveness of the biostimulant clay + *P. putida* in greenhouse conditions on ferrallitic and ferruginous soil. The aim of the study was to evaluate the efficacy of solid biostimulants formulated from the rhizobacteria *Pseudomonas putida* and different binders on maize cultivation in the farming environment of South Benin.

## MATERIALS AND METHODS

### Study Areas

The trials were set up with 33 producers in three zones of South Benin: 11 producers in Adaplamè (Kétou), 11 producers in Zouzouvou (Djakotomey) and 11 producers in Hayakpa (Torri Bossito) (Figure 1). The sites were flat land with a maximum 2% slope, not flooded, and declining soil fertility is a priority constraint (source). The producers were at least 500 m apart from each other.

### Characteristics of the Bacterial Inoculant and Maize Seeds

- The rhizobacteria *Pseudomonas putida* used was isolated and characterized from the maize rhizosphere in southern Benin by Adjanohoun et al. (2011) and preserved at  $-85^{\circ}\text{C}$  in Muller Hinton broth with added glycerol (10%) at the Laboratoire de Biologie et de Typage Moléculaire en Microbiologie (LBTMM) of the Université d'Abomey-Calavi (UAC). It is recognized as a producer of indole acetic acid and capable of solubilizing phosphate (Noumavo et al., 2015).
- Maize seeds of the variety 2000 SYN EE W were used during the study. They are provided by the Center de Recherche Agricole Nord (CRA-Nord) of the Institut National de Recherches Agricoles du Bénin (INRAB). It is an extra-precocious variety with a vegetative cycle of 80 days. It is resistant to breakage, streak, American rust and blight. It is moderately resistant to drought (MAEP, 2016).

### Preparation of the Inoculum and of the Various Formulations

#### Preparation of the Inoculum

The inoculum was obtained by culture in a nutrient medium (liquid MH) for 24 h at  $30^{\circ}\text{C}$ . The concentration of the bacterial culture was adjusted to about  $10^8$  CFU/ml (OD 0.45 at 610 nm) with a spectrophotometer according to the method described by Govindappa et al. (2011).

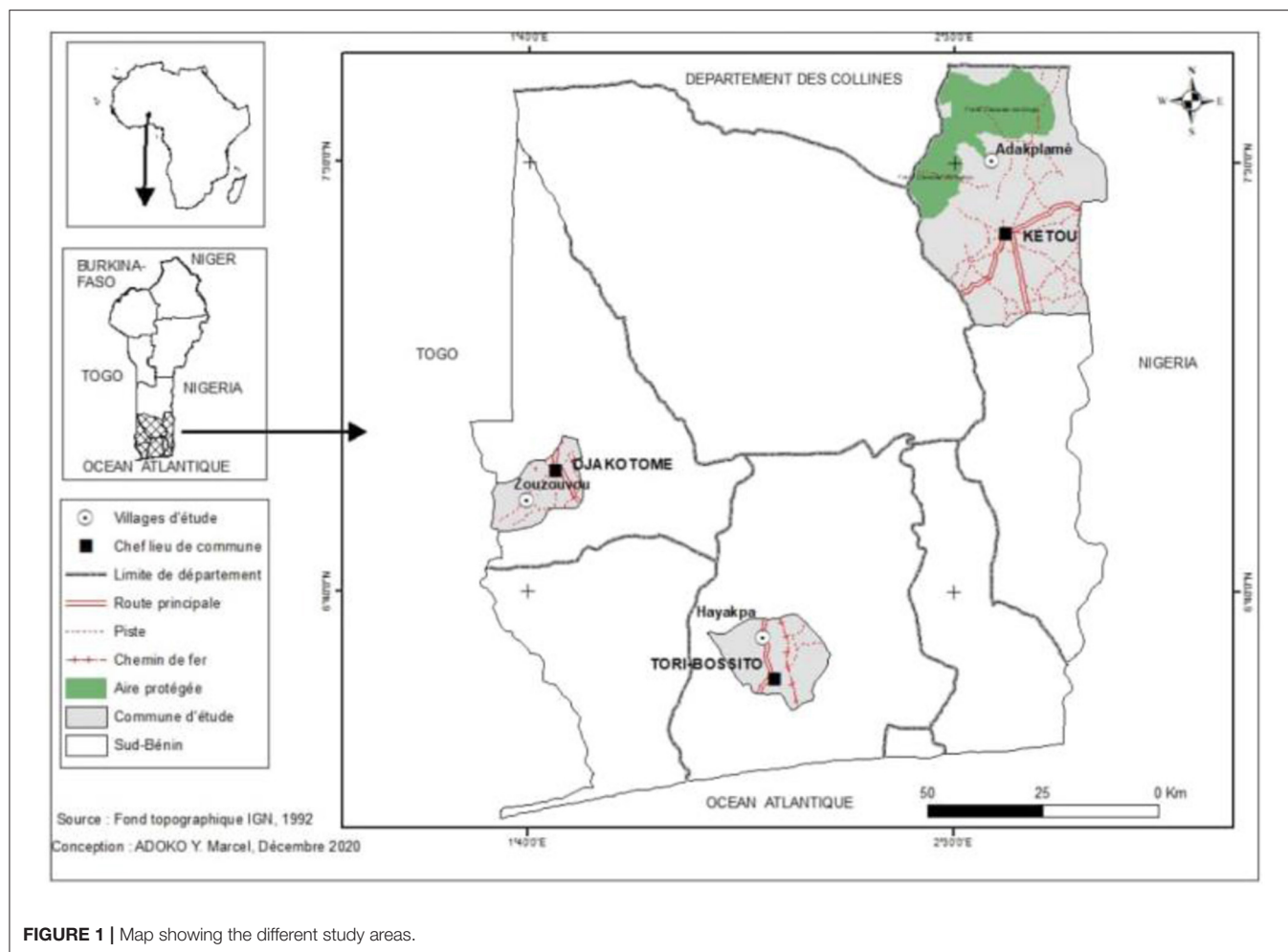


FIGURE 1 | Map showing the different study areas.

### Preparation of the Various Formulations

The modified method of Connick et al. (1991) was used for the preparation of the formulation. Clay, peat and maize flour were separately sterilized for 15 min at 120°C. Thirty-two gram maize flour, 6 g binder (clay, peat and clay-peat), 2 g sucrose and 30 ml bacterial suspension ( $10^8$  CFU/ml) of *Pseudomonas putida* were considered as a ratio for the preparation of the biostimulant. After cooling, the appropriate amounts of each component were mixed with gloved hands under aseptic conditions until a soft paste was obtained. The latter was spread on aluminum foil for 2 days at room temperature (25°C). After 2 days of drying, the paste was crushed in mortar then sieved.

### Soil Sampling and Analysis Prior to Installation of the Tests

Thirty-three (33) composite soil samples were taken at a depth of 0–20 cm from the fields of the various producers. These samples were sent to the Laboratoire des Sciences du Sol Eau et Environnement (LSSEE) of the INRAB for the determination of chemical characteristics. The analyses consisted of the determination of organic carbon by the method of Walkley and Black (1934); total nitrogen by the Kjeldahl (1883) method;

pH water and pH KCl using a pH meter with (1/2.5) as a soil-water ratio; Assimilable phosphorus, by the Bray and Kurtz method (1945); Exchangeable cations (Ca, Mg, K and Na), by the ammonium acetate method using atomic absorption spectrophotometry (Thomas, 1982).

### Installation of the Trials in a Farming Environment and Fertilization Experimental Design

The experimental design was a randomized block of four (04) treatments with eleven (11) replicates per study area. In a study area, each replicate represented one producer. Each elementary plot had a surface area of 40 m<sup>2</sup> and was made up of 5 lines of 10 m long with 0.80 m spacing. The distance separating each plot was 5 m. Sowing was done at a spacing of 0.80 × 0.40 m, i.e., a density of 31,250 plants/ha (Yallou et al., 2010a). The treatments defined as follows:

- T0: peasant practice (100% NPK and Urea);
- T1: clay + *P. putida* + ½ NPK and Urea;
- T2: peat + *P. putida* + ½ NPK and Urea;
- T3: clay-peat + *P. putida* + ½ NPK and Urea.

With: 100% NPK and Urea is recommended dose of mineral fertilizer, and ½ NPK and Urea is half of recommended dose of mineral fertilizer.

### Seed Sowing and Application of the Formulated Biostimulant and Mineral Fertilizer

Three (03) seed holes of about 5 cm of depth and 2 cm apart were realized and 2 maize seeds were put in the central hole. Then, 5 g of formulated biostimulant and half a dose of NPK were applied separately in the other two holes on the day of sowing and the holes were immediately closed. The urea doses were applied on the 46th day after sowing according to each treatment. For the T0 treatment, application of the recommended dose of NPK was made according to the practice popularized to the producers on the 15th day after sowing. Note that the recommended dose of mineral fertilizer (NPK and Urea) for maize cultivation in Benin is 200 kg/ha of NPK and 100 kg of Urea (INRAB, 1995). Note that the NPK used in our study is of formula is  $N_{13}P_{17}K_{17}$ . As for urea, it contains 46% of nitrogen (N).

### Data Collection

At 60 days after sowing, the height was measured with a tape measure. The diameter at the collar of the plants was measured using a caliper, and the leaf area was estimated by multiplying the length and width of the leaves by a coefficient of 0.75 (Ruget et al., 1996). At harvest (80 days after sowing), the ears of the maize plants were harvested. After shelling, the total weight of the maize grains was measured with a precision balance (Highland HCB 3001, Max:  $3000 \times 0.1$  g), and the moisture content was taken with a moisture meter (LDS-1F). Maize grain yield values were obtained using the formula (Valdés et al., 2013):

$$R = \frac{P \times 10.000}{S \times 1.000} \times \frac{14\%}{H} \quad (1)$$

Where:  $R$  is the maize yield, expressed in T/ha;  $P$  is the maize mass per calculated elemental area, expressed in kg;  $S$  is the useful parcel area in  $m^2$ ;  $H$  is the grain moisture rate, in %.

### Statistical Analysis

The various tests were carried out using R 4.0.2 software (R Core Team, 2020). These analyses required the use of the dplyr and DescTools packages for the calculation of descriptive statistics, the ggplot2 and ggpur packages for the creation of mustache boxes, the stats package for the Shapiro-Wilk and Levene tests, the car package for the ANOVA and the multcomp package for the *post-hoc* pair comparison test. The effect of the experimental area and the treatments applied on the growth and yield performance of the plants was assessed by means of a two-factor.

### ANOVA Test

The normality and the homogeneity of the data variances were verified (Glèlè Kakai et al., 2006). As the experimental design was unbalanced, the type III ANOVA test was adopted. Once the ANOVA test was significant, a pair-wise comparison

*post hoc* test using the Tuckey *post hoc* test (Douglas and Michael, 1991) was carried out to assess statistical differences in the means. Besides, descriptive statistics were calculated for each measured parameter. The significance threshold used was 5%.

## RESULTS

### Chemical Characteristics of Soil

Soil chemical properties of the sites before the tests were set up (Table 1) generally showed that the soils at the different sites in South Benin were slightly acidic ( $5.7 \leq \text{pH} \leq 6.4$ ). All soils had low fertility  $12.46 \leq \text{C/N} \leq 15.61$  characterized by high C/N ratios. The soils had low levels of organic carbon ( $8.9 \leq \text{C} \leq 10.6$ ) (g/Kg), total nitrogen ( $0.57 \leq \text{N} \leq 0.72$ ) (g/Kg), exchangeable bases ( $3.3 \leq \text{Ca}^{2+} \leq 5.14$ ) (g/Kg);  $2.3 \leq \text{Mg}^{2+} \leq 3.72$  (cmol/Kg) and  $0.7 \leq \text{K}^+ \leq 1.9$  (cmol/Kg). Generally speaking, assimilable phosphorus ( $28.38 \leq \text{P} \leq 36.8$ ) (mg/Kg), was lower in the soils of the different sites.

### Effect of Biostimulants on Maize Plant Height

The histogram in Figure 2 illustrates the variation in average maize plant height as a function of treatments at DAS 60 in the different study areas. In the Hayakpa and Zouzouvou zones, the biostimulant clay + *P. putida* + ½ NPK and Urea gave the best result with respective increases of 4.18 and 12.41% compared to the peasant practice (100% NPK and Urea). In the Adakplamè area, the peat biostimulant + *P. putida* + ½ NPK and Urea was the highest with an increase of 15.75% compared to 100% NPK and Urea. The results of the analysis of variance showed a significant difference in the effects of the treatments ( $p = 0.01$ ) and the experimental area ( $p < 0.001$ ) on maize plant height. Plants in the Adakplamè zone induced the best performance (15.75% increase) for most treatments than plants in the other zones (Figure 3). Moreover, the interaction between the different treatments and the study areas was significant ( $p < 0.05$ ).

### Effect of Biostimulants on the Stem Diameter of Maize Plants

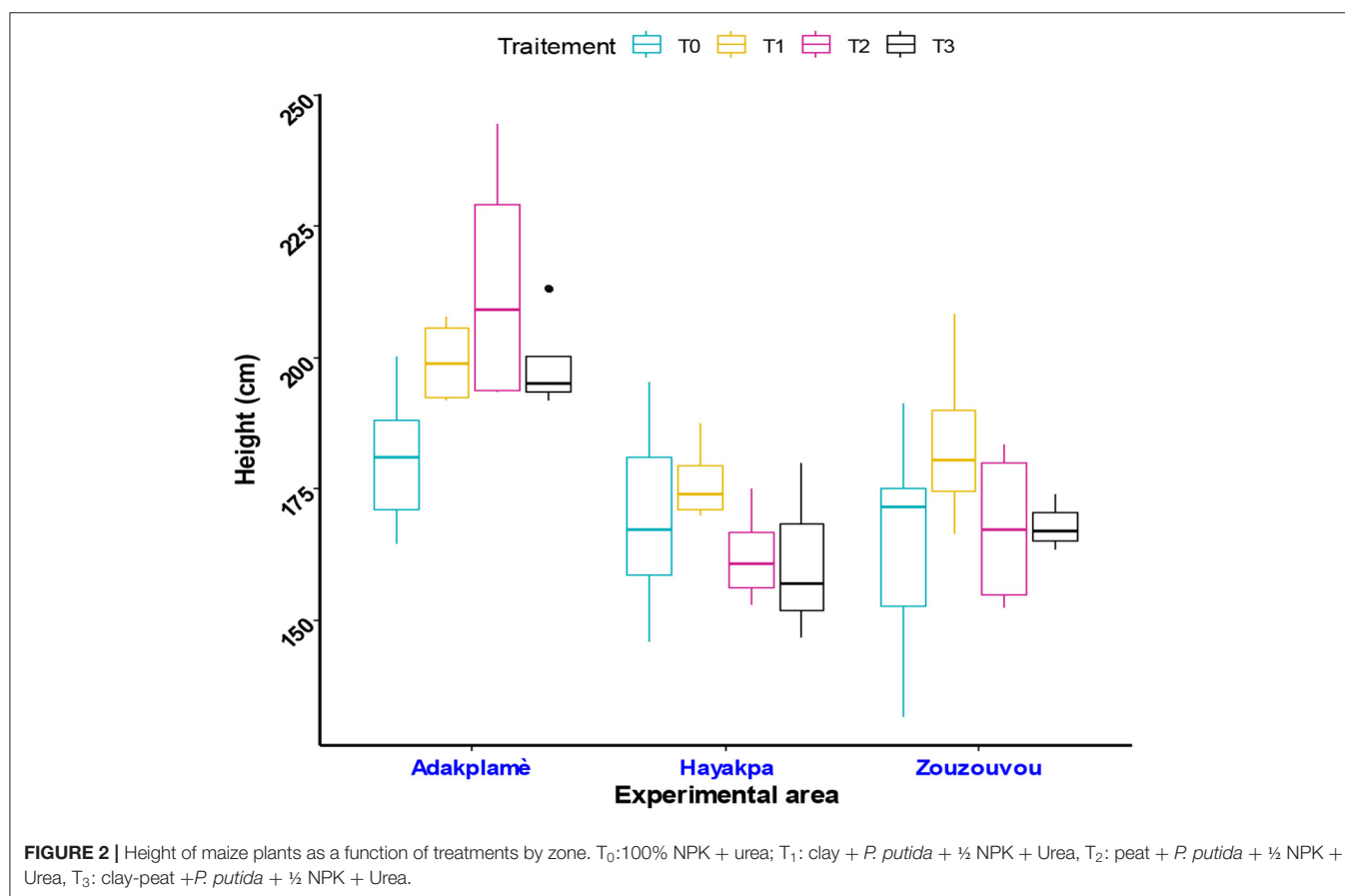
The histogram in Figure 4 shows the variation in the stem diameter of maize plants as a function of the treatments at 60th DAS in the different study areas. In the Hayakpa and Adakplamè zones, the biostimulant clay + *P. putida* + ½ NPK and Urea were in the lead, with an overrun of 0.78 and 9.32%, respectively, compared to the recommended dose of NPK and Urea. In the Zouzouvou area, the peat biofertilizer + *P. putida* + ½ NPK and Urea resulted in a better collar diameter. This better treatment exceeded the recommended dose of NPK and Urea by 15.93%. The results of the analysis of variance showed a significant difference in the effects of the treatments ( $p = 0.01$ ). On the other hand, no difference was recorded between the experimental areas ( $p = 0.12$ ) on the stem diameter of the maize plants. It was also noted that the interaction between treatment and area was also non-significant ( $p = 0.20$ ), indicating that the variation in maize plant crown diameter per treatment does not depend on the experimental site. From the analysis of Figure 4, it appears



**TABLE 1** | Chemical characteristics of soils in different localities.

Sites	Villages	pH (water)	C-org (g/Kg)	N-total (g/Kg)	C/N	P <sub>ass</sub> -Bray1 (mg/Kg)	B.E (cmol/kg)		
							Ca <sup>2+</sup>	Mg <sup>2+</sup>	K <sup>+</sup>
Kétou	Adakplamè	6.4	8.10	0.65	12.46	36.8	33.3	2.3	1.9
Tori	Hayakpa	5.9	10.6	0.72	14.72	33.92	5.14	3.72	0.7
Djakotomey	Zouzouvou	5.7	8.9	0.57	15.61	28.38	5.02	3.39	1.08

C-org, organic carbon; N-total, Azote total; P-Bray1, Phosphorus available; B.E, Base Exchangeable.

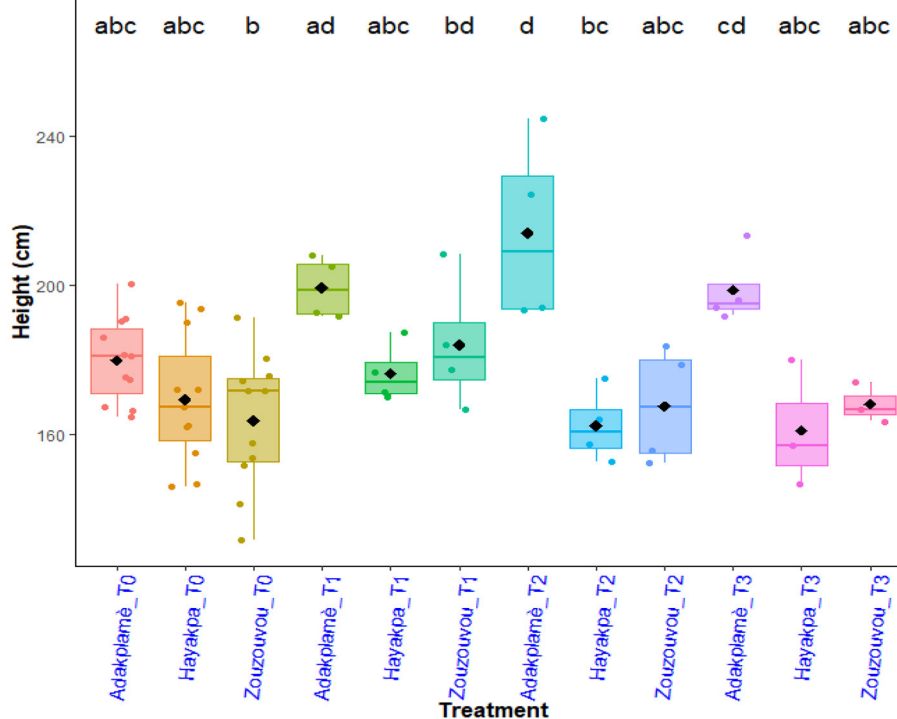


that the plants in the Adakplamè area performed best. The Tukey test carried out confirmed the trend (Figure 5). Thus, the clay + *P. putida* + ½ NPK and Urea treatment in the experimental areas gave the best performance in terms of diameter at the crown, with an increase of 15.93% compared to the extended practice.

## Effect of Biostimulants on the Leaf Area of Maize Plants

The effect of biostimulants on the leaf surface as a function of the treatments and by zone was illustrated by the histogram in Figure 6. In the Hayakpa zone, the biostimulants clay + *P. putida* + ½ NPK and Urea induced a large leaf area. This application resulted in a 5.77% growth rate in relation to the popularized dose of NPK and Urea. In the Zouzouvou area, the

same treatment was better, with an increase of 18.31% in relation to the recommended dose of NPK and Urea. In Adakplamè, with the biostimulants formulated with peat + *P. putida* + ½ NPK and Urea, an increase of 15.57% in relation to the recommended dose of NPK and Urea was recorded. The results of the analysis of variance indicated a non-significant difference in the effects of the treatments ( $p = 0.051$ ) in the same locality. However, a highly significant difference between experimental areas ( $p < 0.001$ ) was observed. It is noted that the treatment-area interaction was non-significant ( $p = 0.08$ ), indicating that the variation in the leaf area of maize plants does not depend on the treatments but on the experimental area. From the analysis in Figure 6, it appears that the plants in the Zouzouvou area performed best. The Tukey test carried out confirms the trend (Figure 7). Thus, the Zouzouvou zone comes first, followed by Hayakpa and Adakplamè.



**FIGURE 3 |** Height of maize plants as a function of treatment and area. T<sub>0</sub>: 100% NPK + Urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.

## Effect of Biostimulants on Maize Grain Yield

Maize grain yields as a function of treatment and area were illustrated by the histogram in **Figure 8**. In the Hayakpa zone, the biostimulants clay-peat + *P. putida* + ½ NPK and Urea performed better in maize grain yield. This treatment has an increase of 2.17% compared to the recommended dose of NPK and Urea. In the Zouzouvou area, the peat + *P. putida* + ½ NPK and Urea application was better with an increase of 3.24% concerning the recommended dose of NPK and Urea. In Adakplamè it is the biofertilizer clay + *P. putida* + ½ dose of NPK and Urea was better with an increase of 10.96% in relation to the recommended dose of NPK and Urea. The results of the analysis of variance revealed that there were no significant differences in the effects of the treatments ( $p = 0.92$ ) and between the experimental areas ( $p = 0.14$ ) on maize grain yield. Treatment and zone interactions were also non-significant ( $p = 0.81$ ), indicating that maize grain yield variations do not depend on treatments and experimental zones.

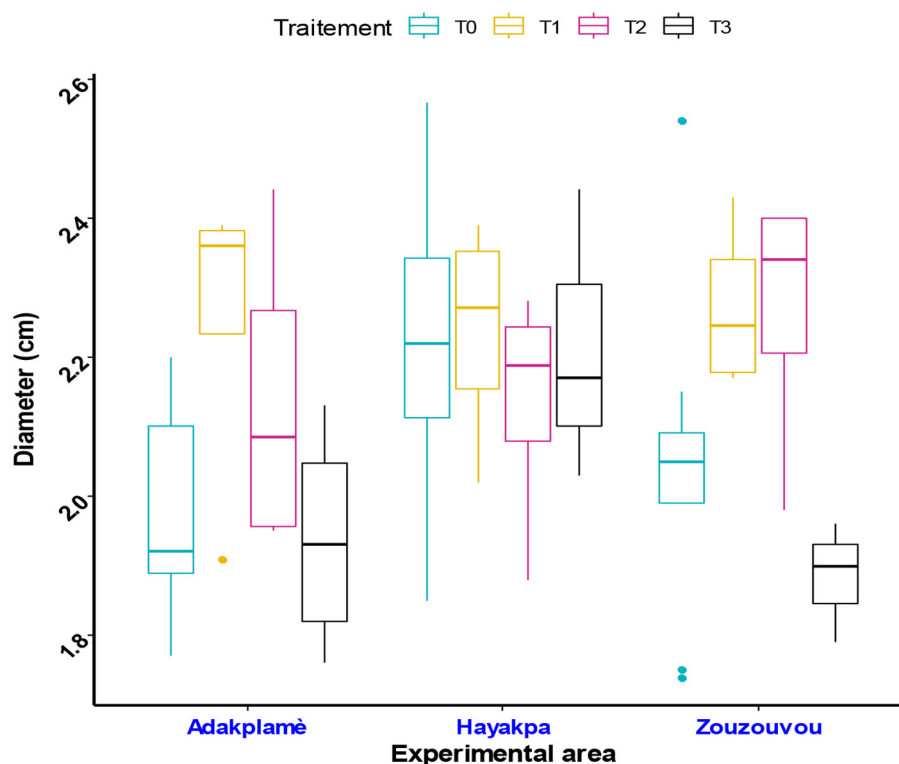
## Correlation Between Growth and Yield Parameters

Principal Component Analysis (PCA) on the different maize plant growth and yield parameters showed that the first two axes retain 80.01% of the total variance (**Figure 9**). Height and grain yield were positively correlated with axis 1, while the area was negatively correlated with the same axis. The collar diameter is

strongly associated with axis 2 as shown by the projection of the variables in the first two axes.

## Classification of Treatments According to Their Performance

The analysis of the projection of the individuals indicates three classes of grouping of treatments (**Figure 10**) discriminated by the variable's height, stem diameter, leaf area, grain yield. The first class (C1) is made up of three practical peasant treatments (100% NPK and Urea); clay + *P. putida* + ½ NPK and Urea; peat-clay + *P. putida* + ½ NPK and Urea from the Zouzouvou zone. The plants maintained under these treatments are characterized by an average height of  $171.91 \text{ cm} \pm 10.68$  and an average grain yield of  $2.14 \text{ T/ha} \pm 0.04$ . The second class (C2) is made up of five treatments, including the four (04) treatments of Hayakpa, a 100% NPK and urea peasant practice; clay + *P. putida* + ½ NPK and Urea; peat + *P. putida* + ½ NPK and Urea; clay-peat + *P. putida* + ½ NPK and Urea and the peat + *P. putida* + ½ NPK and Urea treatment of Zouzouvou. The plants having benefited from the treatments of this class (C2) have an average height of  $167.30 \text{ cm} \pm 6.09$  and an average grain yield of  $2.27 \text{ T/ha} \pm 0.06$ . The third class (C3) consisted of the four treatments of Adakplamè 100% NPK and Urea; clay + *P. putida* + ½ NPK and Urea; peat + *P. putida* + ½ NPK and Urea; clay-peat + *P. putida* + ½ NPK and Urea. The plants subjected to these treatments have an average height of  $197.95 \text{ cm} \pm 14.01$  and an



**FIGURE 4 |** Stem diameter of maize plants as a function of zone treatments. T<sub>0</sub>: 100% NPK + urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.

average grain yield of 2.38 T/ha  $\pm$  0.12. The class (C3) gave the best performance in both height and grain yield of maize.

## DISCUSSION

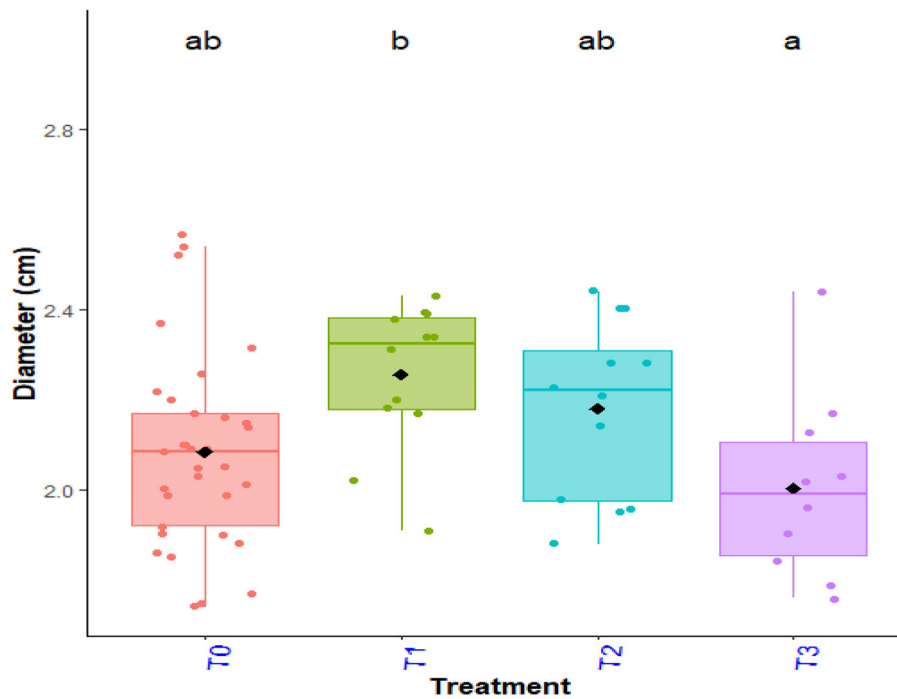
The biostimulants are substances and/or microorganisms that contain living microorganisms and have no toxic effects on the soil. Their use would be inexpensive compared to mineral fertilizers (Amutha et al., 2014). The application of mineral fertilizers in combination with biostimulants could be an effective strategy to improve soil health and nutrient availability for crops. The aim of the study is to test solid biostimulants formulated with the rhizobacteria *P. putida* and various binders in a farming environment in southern Benin. The trials were set up in three different areas in southern Benin on ferrallitic soils.

Analysis of the initial chemical properties of the test soils shows that the soils at the three sites are slightly acidic. The C/N ratio (12.46–15.61) was high in the topsoil. The level of assimilable phosphorus was lower. In general, in the soils of the study areas, the sum of exchangeable bases and the cation exchange capacity are low, which reflects their low fertility (Adjanooun et al., 2011). In the soils of the three study zones, potassium was globally deficient in relation to calcium and magnesium. Better still, imbalances between calcium, magnesium and phosphorus were noted. These results, which were in line with those reported by Igué et al. (2013), showed that it was

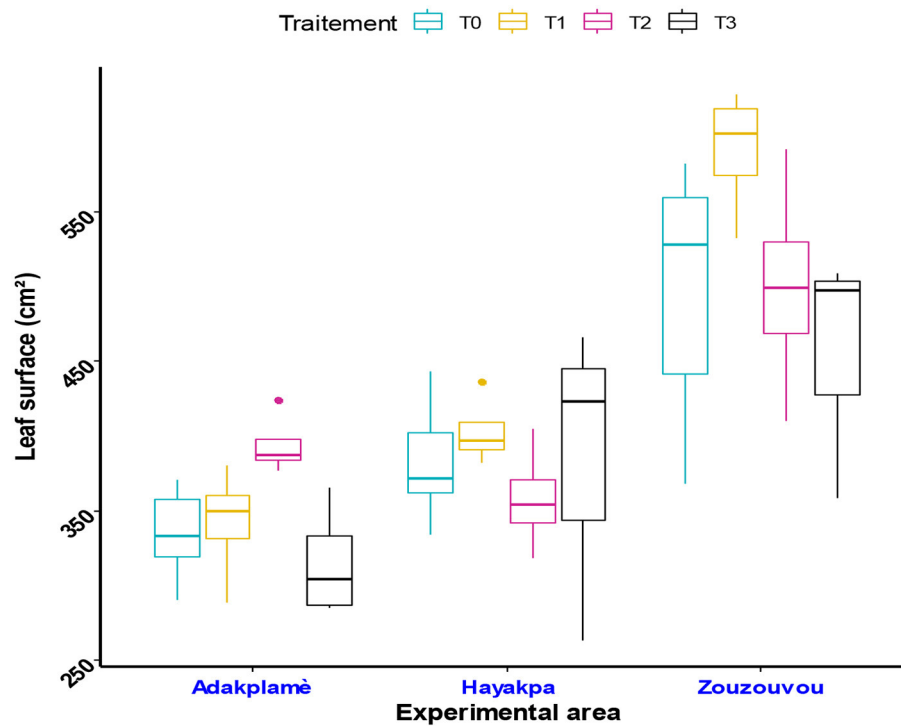
necessary to provide nutrients to the soil because these quantities are insufficient to meet maize's nutritional requirements (Yallou et al., 2010b). Regarding the growth parameters of maize plants on ferrallitic soil, the formulated biofertilizers were expressed differently.

In all three zones, the best height and stem diameter were obtained with biostimulants formulated with clay and peat supports. Significant differences were recorded between these applications and the farming practice on these growth parameters. The same observations were made by different authors in Benin when they combined liquid microbial biostimulants with mineral fertilizers (Agbodjato et al., 2015; Amogou et al., 2019; Adoko et al., 2020). These increases can be explained by the growth stimulating effect of the rhizobacteria *P. putida* (Noumavo et al., 2015) under study, on the one hand, and, on the other hand, the effect of conservation binders (clay and peat) which maintain the bacterial concentration for a long time and which would better protect the PGPR strains against abiotic factors (Brar et al., 2012).

In the same locality, there was no significant difference between treatments for the leaf area of the maize. However, from one area to another, it was highly significant, with the Zouzouvou area leading the way. The best leaf area was obtained with the application of biostimulants T1: peat-clay + *P. putida* + ½ NPK and Urea. This could be explained by the lack of variability in the chemical composition of the soils of the different producers

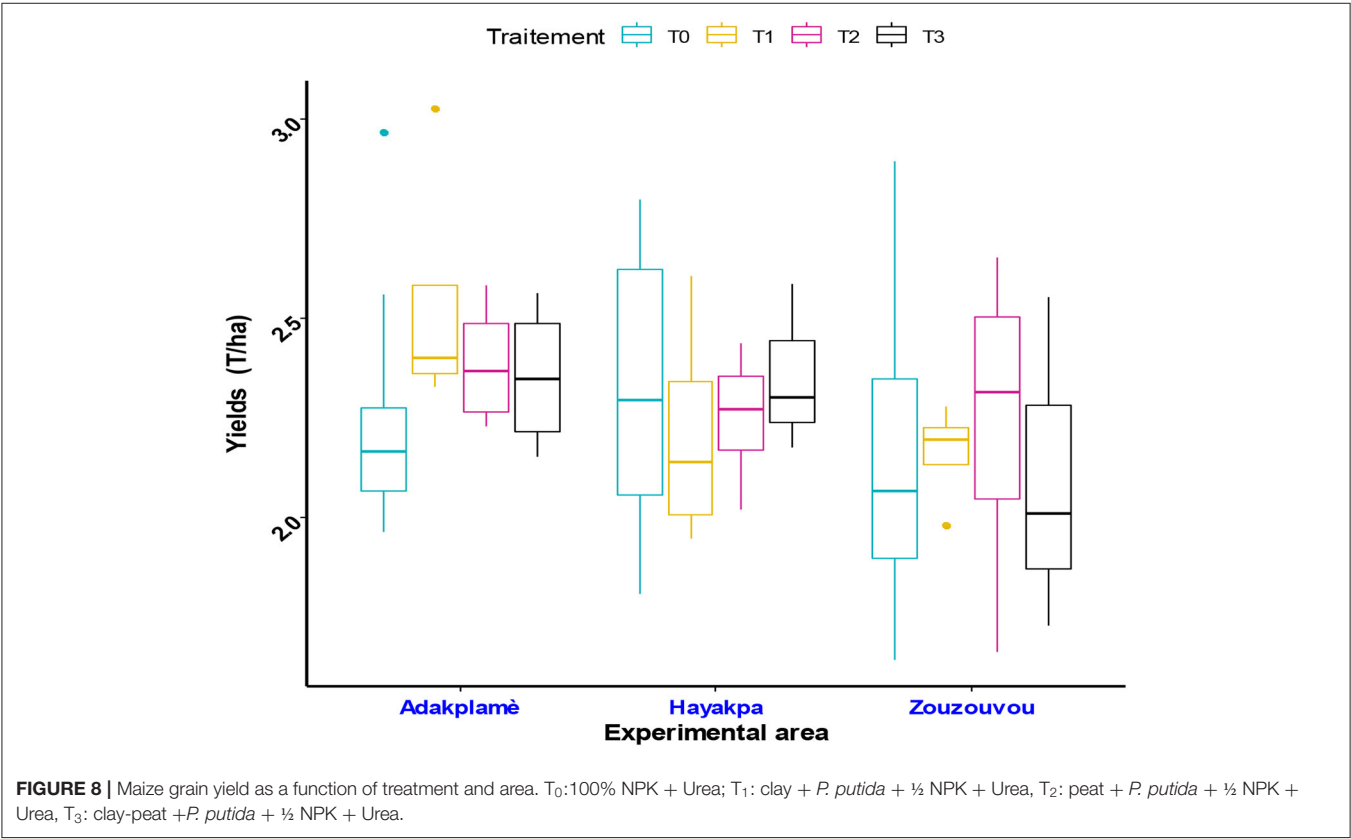
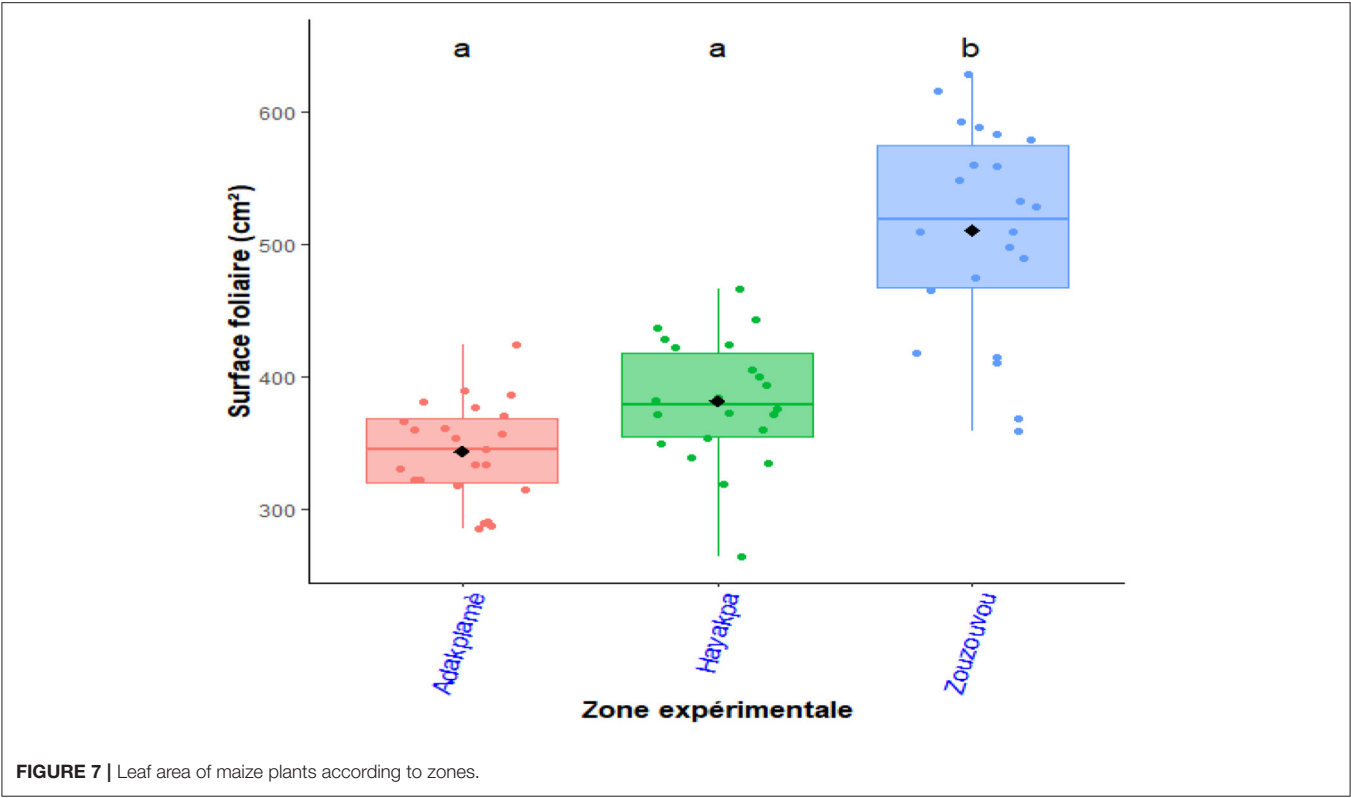


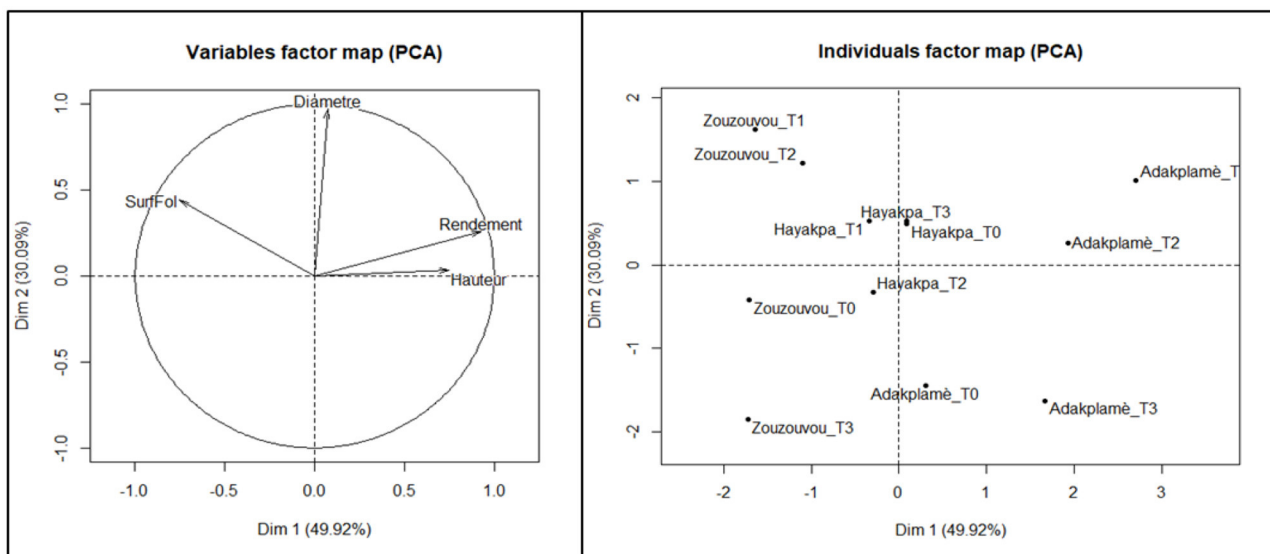
**FIGURE 5 |** Stem diameter of maize plants as a function of treatments. T<sub>0</sub>:100% NPK + urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.



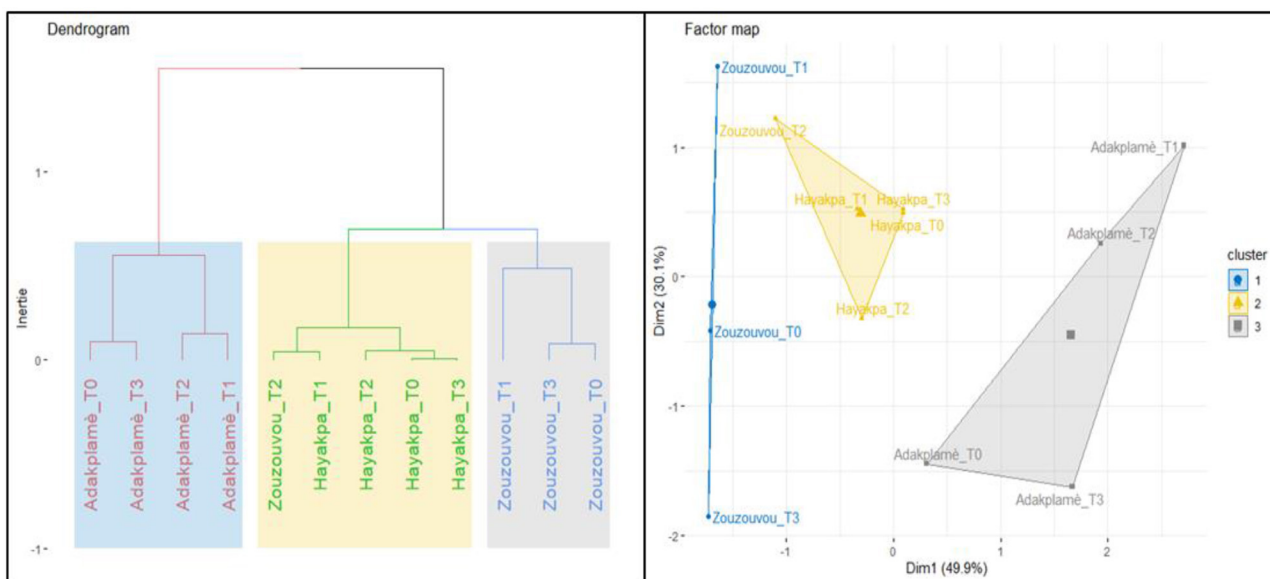
**FIGURE 6 |** Leaf area of maize plants as a function of treatments by zone. T<sub>0</sub>:100% NPK + urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.







**FIGURE 9** | Classification of different growth and yield parameters based on their performance. T<sub>0</sub>: 100% NPK + Urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.



**FIGURE 10** | Dendrogram of classes obtained and projection of treatments in the first two dimensions of PCA. T<sub>0</sub>: 100% NPK + Urea; T<sub>1</sub>: clay + *P. putida* + ½ NPK + Urea, T<sub>2</sub>: peat + *P. putida* + ½ NPK + Urea, T<sub>3</sub>: clay-peat + *P. putida* + ½ NPK + Urea.

that hosted the trials in the same area. In the same way, the farming practices of a locality remain similar. But from one area to another, the soils do not have exactly the same chemical properties. Similar findings were made by Adoko et al. (2020) in their studies of the liquid biostimulants *P. putida* in a farming environment in Benin.

Maize grain yields obtained in this study from all treatments and in all areas were similar. The results of the statistical analysis did not reveal any significant differences between the various

treatments and between the different zones. The formulated biostimulants combined with the half dose of NPK and Urea had comparable effects with the full dose of NPK and Urea in all study zones on maize grain yield. The same findings have been made by several authors who have applied liquid PGPR biostimulants in research stations (Noumavo et al., 2013; Agbodjato et al., 2015; Amogou et al., 2019) and then in farmers' fields (Adoko et al., 2020) in Benin and other countries (Amutha et al., 2014; Sagay et al., 2020). The rhizobacteria *P. putida*

contained in these formulated solid biostimulants was thus able to provide the plants with nutrients from the environment to increase their yield (Kashyap et al., 2020). This rhizobacteria was able to provide maize plants with the maximum nitrogen (N), phosphorus (P) and potassium (K) supplied or available in the soil, necessary for plant growth and yield (Ahmed et al., 2020). Ojuederie et al. (2019) also mentioned that multifaceted PGPRs are potential candidates for biofertilizer production to lessen the detrimental effects of drought stress on crops cultivated in arid regions.

In the present study, the correlation between growth and yield parameters showed that the biostimulant T1: clay + *P. putida* + ½ NPK and Urea expressed itself better than all the others. This biostimulant clay + *P. putida* was, therefore, the best in the farming environment. This result can be explained in part by the capacity of the preservative binder used for the bioformulation to maintain a good bacterial concentration in the rhizosphere for a long time. According to the work of Brar et al. (2012), the clay binder makes it possible to maintain a high population of PGPR for several months, which is favorable to the promotion of plant growth and yield. Earlier work by Noumavo et al. (2015) stated that *P. putida* used in this study is capable of producing growth phytohormones and solubilizing phosphate. The best growth and yield parameters recorded during this study can be explained by the combined effects of *P. putida* and the binder clay. Some strains of rhizobacteria of the genus *Pseudomonas* are capable of producing ammonia, indole acetic acid (IAA), HCN, siderophores, solubilizing potassium (Verma and Pal, 2020), phosphate, zinc and increasing the bioavailability of nutrients for good plant development (Marra et al., 2012; Verma et al., 2015; Shahid et al., 2017; Singh and Jha, 2017; Ullah and Yusuf, 2019; Zaheer et al., 2019). The rhizobacteria *P. fluorescens* have also been reported to colonize the rhizosphere of wheat and sugarcane and stimulate plant growth (Verma et al., 2015). Oteino et al. (2015) attributed the efficacy of *P. fluorescens* on onion yield to its ability to produce indole acetic acid. Similarly, the biocontrol properties of this genus are well documented (Reetha et al., 2014; Khanghahi et al., 2018). PGPRs also secrete several growth phytohormones such as auxins, cytokinins, gibberellins and ethylene which improve both root growth and whole plant growth (Lugtenberg and Kamilova, 2009; Dodd et al., 2010; Wani et al., 2013). Furthermore, work carried out in Senegal by Diagne et al. (2020) has also shown that inoculation with PGPR and/or Arbuscular Mycorrhizal Fungi (AMF) can improve the salinity resistance of *Casuarina obesa* plants by increasing their growth parameters. The use of biologically active natural products and microbial extracts could be an important means of increasing soil nutritional status, absorption and improving the efficiency of nutrient use (NPK) by plants (De Pascale et al., 2017). Phosphorus, potassium and magnesium have been reported to improve root growth, resulting in improved water supply and drought tolerance. Cassán and Diaz-Zorita (2016) showed that the increase in crop yield was due to the ability of

*Azospirillum sp* to provide the plant with nutrients. According to Zeffa et al. (2019) inoculation of maize seed with *Azospirillum brasilense* intensified plant growth and yield by improving nitrogen use in the event of nitrogen deficiency. It is in this same context that Fadji and Babalola (2020) mentioned that the major benefit of embracing the beneficial microorganisms in the field of agriculture is to bring about a reduction in the use of different agrochemicals such as pesticides, chemical fertilizers, other artificial chemicals and this would make agriculture more productive and sustainable.

## CONCLUSION

The results of the experiment show that *Pseudomonas putida*-based biostimulants combined with the half dose of NPK and Urea recommended (100 kg/ha NPK and 50 kg/ha) for maize cultivation in Benin gave the best performance both in terms of growth parameters and maize grain yield. The effects of these microbial biostimulants vary from region to region and according to the type of binder. The application of biostimulants formulated on the basis of clay or peat in combination with the half-dose of NPK and Urea in the different study areas is more favorable to corn plants than the recommended full dose (100% NPK and Urea). The *Pseudomonas putida* strain could be used as biofertilizers for environmentally friendly sustainable agriculture. It would be interesting to continue this study by repeating the trial on a larger area to assess the performance of this rhizobacteria to improve maize growth through the formulations made.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## AUTHOR CONTRIBUTIONS

NA, MA, OA, and FB carried out the experimental work and analysis. NA, MA, PN, AA, OB, and LB-M contributed to the design, supervision, and interpretation of the results. NA, MA, and OB revised the final draft. OB reviewed the final draft. All authors participated equally in the work and approved the final submission.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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# Growth-Promoting Potential of Rhizobacteria Isolated From Sugarcane

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Growth-Promoting Potential of  
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The search for plant growth-promoting rhizobacteria (PGPRs) addresses the ongoing need for new bioinoculants to be used on various agricultural crop species, including sugarcane. Bacterial strains were isolated from the rhizosphere of sugarcane plants and identified by sequencing the 16S ribosomal gene. The main indole acetic acid producers were *Enterobacter* sp. IP11, *Enterobacter* sp. IP14, and *E. asburiae* IP24. *Achromobacter spanius* IP23 presented the highest levels of cellulolytic activity and potassium solubilization. *Bacillus thuringiensis* IP21 and *Staphylococcus saprophyticus* IJ8 showed the highest levels of fixed nitrogen. The levels of calcium phosphate and aluminum phosphate in *B. thuringiensis* IP21 were notable, as this strain solubilized 481.00 and 39.33 mg of phosphorus mL<sup>-1</sup>, respectively; however, for Araxá apatite, the results for *B. anthracis* IP17 were notable (622.99 mg phosphorus mL<sup>-1</sup>), while for iron phosphate solubilization, *Enterobacter* sp. IP14, which solubilized 105.66 mg phosphorus mL<sup>-1</sup> was notable. The *B. thuringiensis* IP21 and *Enterobacter* sp. IP11 isolates promoted the growth of the tallest sugarcane plants, inducing increases of 14.1 and 10.4% relative to the control plants, respectively. For shoot dry matter, root dry matter, and total dry matter, plants inoculated with *Enterobacter* sp. IP14, *B. anthracis* IP17, and *A. spanius* IP23 presented higher values than the controls. Furthermore, plants inoculated with *B. thuringiensis* IP21 presented higher root dry matter and total dry matter values, and those inoculated with *Enterobacter* sp. IP14 also presented higher total dry matter values. These results indicate that bacteria with the potential for use as future inoculants should be investigated since bacteria with plant growth-related characteristics may not impact growth promotion.

**Keywords:** *Saccharum* spp., growth promotion, solubilization, IAA, nitrogen, phosphorus, greenhouse

## INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is one of the most economically important agricultural crop species, mainly as a source of sugar, and is grown worldwide in tropical and subtropical areas. Brazil is the largest producer of sugarcane in the world, and the 2019/20 harvest was estimated at 642.7 million tons (Conab, 2019). Sugarcane growth and performance are directly impacted by the use of fertilizers; fertilizers are expensive, and their continual use causes damage to the environment (Moutia et al., 2010; Leite et al., 2018).

An alternative to fertilizers that can reduce costs and environmental impacts is the use of plant growth-promoting rhizobacteria (PGPRs) (Pérez-Montaño et al., 2014). This group of bacteria inhabits the plant rhizosphere and promotes plant growth (Zhou et al., 2016; Riaz et al., 2021).

A wide range of applications of these beneficial rhizobacteria in sugarcane crops have been proposed, and a considerable number of research studies have focused on their functionality and applicability. The results of several studies have shown that the utilization of PGPRs in sugarcane is a great alternative to the challenges of modern agriculture (dos Santos et al., 2020).

PGPRs promote plant growth by direct and indirect mechanisms. The direct mechanisms include assisting plants in obtaining nutrients and modulating the levels of growth-related plant hormones, whereas the indirect mechanisms include inhibiting certain pathogens and protecting plants from future attacks by acting as a biocontrol agent (Glick, 1995; Miliute et al., 2015; Vurukonda et al., 2018; Estrada-Bonilla et al., 2021).

Regarding the acquisition of nutrients, members of some genera of free-living bacteria, such as *Azospirillum*, *Azotobacter*, *Bacillus*, *Burkholderia*, and *Herbaspirillum*, are capable of converting atmospheric nitrogen into ammonium and supplying it to plants. These PGPRs can provide nitrogen for agronomically important crop species, such as wheat, sorghum, corn, rice, and sugarcane (Pérez-Montaño et al., 2014; Batista et al., 2021).

Some rhizobacteria also have the ability to solubilize inorganic phosphates that are present in the soil in large amounts in an unavailable form that cannot be absorbed by plants, which limits plant growth. PGPRs can convert phosphorus into a soluble form that is usable by plants (Gandhi, 2016; Ramakrishna et al., 2019). Phosphorus solubilization can occur by different mechanisms, most commonly by the production of organic acids such as acetic acid, malic acid, lactic acid, succinic acid, oxalic acid, and tartaric acid (Ahmad and Kibret, 2014; Patel and Desai, 2015). In addition, PGPR inoculation can increase the absorption of various other nutrients by plants, including Ca, K, Fe, Cu, Mn, and Zn (Mantelin and Touraine, 2004).

Several PGPRs can alter root architecture and promote root development by increasing nutrient absorption and accumulation due to their phytohormone-synthesizing capability (Boiero et al., 2007; Dar et al., 2021). The growth of plants inoculated with indole acetic acid (IAA)-producing rhizobacteria is generally significantly greater than that of uninoculated plants (Kaymak et al., 2008).

PGPRs indirectly promote plant growth when they decrease or interrupt the harmful effects of phytopathogens. Under biotic stress, rhizobacteria can trigger the induction of systemic resistance, and under abiotic stress, PGPRs can protect against unfavorable environmental conditions (Jha et al., 2011; Gururani et al., 2013; Vacheron et al., 2013; Glick, 2014; Nadeem et al., 2014) by producing antibiotics, toxins, siderophores, hydrolytic enzymes, and volatile organic antimicrobial compounds (Sheoran et al., 2015).

The success of using PGPRs as inoculants for agricultural crops depends not only on the growth-promoting abilities of rhizobacteria but also on plant-microorganism interactions, which are influenced by various factors, such as the composition

of exudates released by plant roots as well as soil health, gene expression patterns, cell communication, plant genotypes, and rhizosphere colonization capability (Danhorn and Fuqua, 2007; Meneses et al., 2011; Alquéres et al., 2013; Beauregard et al., 2013).

In view of these findings, the present study aimed to identify new bacterial strains isolated from sugarcane that have the ability to promote plant growth for use as inoculants in sugarcane cultivation.

## MATERIALS AND METHODS

### Bacterial Strain Isolation

Sixty bacterial strains were isolated from the rhizospheres of the sugarcane plant varieties IAC95-5000 and RB86-7515 in the municipality of Jaboticabal-SP; 62 colonies were obtained from the rhizospheres of the varieties CTC9 and RB85-5156 in the municipality of Frutal-MG; and 45 colonies were isolated from the rhizospheres of the varieties IAC91-1099 and CTC4 in the municipality of Pirajuba-MG. A total of 167 bacterial colonies were isolated from the three locations, of which 58 could fix nitrogen, 20 could produce indole acetic acid, 53 had cellulolytic activity, and 17, 26, 44, 33, and 51 could solubilize potassium, calcium phosphate, aluminum phosphate, iron phosphate, and Araxá apatite, respectively.

Bacteria from rhizospheric soil samples were isolated by serial dilution (Wollum, 1982; Vieira and Nahas, 2005). After incubation, bacterial colonies were picked, placed in SMA-containing test tubes and refrigerated for later use.

The strains were characterized on the basis of their growth-promoting abilities as described below.

### Biological Nitrogen Fixation (BNF)

For BNF quantification, 650  $\mu$ L of each strain was added to 15 mL of NFb semisolid media, which was then incubated at 30°C for 5 days at 180 rpm. Thereafter, 9.5 mL of the resulting solution (medium + cell content) was poured into tubes for digestion using the semimicro-Kjeldahl method. After this process, the solutions were distilled and titrated to quantify the total nitrogen produced by each bacterial strain. Fixed nitrogen is expressed as milligrams of nitrogen per milliliter (Tedesco et al., 1995).

### Phosphate Solubilization

The phosphate solubilizing ability of the bacterial strains was measured in liquid media (Nahas et al., 1994) comprising 0.1 g of NaCl, 1 g of  $\text{NH}_4\text{Cl}$ , 0.2 g of KCl, 0.2 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.1 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 g of glucose, 0.5 g of yeast extract, and 1,000 mL of  $\text{H}_2\text{O}$  (pH 7) supplemented with a single insoluble/slightly soluble source of phosphorus. Four sources of phosphate were tested, namely,  $\text{CaPO}_4$ , Araxá apatite ( $3\text{Ca}_3(\text{PO}_4)_2 \cdot \text{CaF}_2$ ),  $\text{AlPO}_4$ , and  $\text{FePO}_4$  in amounts of 5, 5, 3.5, and 4.33 g per 1 L of medium, respectively (Silva Filho and Vidor, 2000).

Erlenmeyer flasks containing liquid media were incubated for 48 h at 28°C with shaking at 180 rpm (Nautiyal, 1999). After incubation, 5 mL of each sample was transferred to tubes, which were then centrifuged at 9,000 rpm for 15 min. Thereafter, 1 mL of the supernatant of each strain, 4 mL of distilled water, and

1 ml of an ammonium molybdate-vanadate reagent were added to a new tube and subsequently read by a spectrophotometer at 470 nm (Malavolta et al., 1989). Phosphorus values were obtained using a standard curve generated with known  $\text{KH}_2\text{PO}_4$  concentrations, and the results are presented in milligrams of phosphorus per milliliter.

### Potassium Solubilization

Potassium solubilization was measured using Ekosil® fertilizer, which is an alternative source of K produced from a type of volcanic rock known as phonolite that contains 8% soluble  $\text{K}_2\text{O}$  (Yoorin, 2018).

To quantify potassium solubilization by the bacterial strains, Aleksandrov media (Hu et al., 2006) supplemented with Ekosil® was used. Approximately 1 ml of each strain was inoculated in 50 ml of Aleksandrov media and then incubated at 30°C at 180 rpm for 5 days. Shortly thereafter, the bacterial samples were filtered through No. 1 Whatman filter paper, and the amount of solubilized K was determined by flame photometry (Pachaiyappan and Janarthanam, 2007). The results are expressed in milligrams of K per liter.

### IAA Production

IAA production was measured according to the methods of Sarwar and Kremer (1995) with slight modifications. The strains were incubated in 20 mL of DYGS (dextrose, yeast, glucose, sucrose) media supplemented with 5 mL of L-tryptophan for 48 h at 28°C under constant agitation at 120 rpm in the absence of light. After this period, 5 mL of each bacterial culture was centrifuged at 10,000 rpm for 10 min, and 2 mL of the supernatant was subsequently transferred to a test tube containing 2 mL of 2% Salkowski's reagent (v/v) (0.5 M  $\text{FeCl}_3$  in 35% perchloric acid), which was then incubated for 30 min in the dark.

IAA production was determined by spectrometry at 530 nm, and IAA levels were obtained using a standard curve generated with known concentrations. The results are expressed in micrograms of IAA per milliliter.

### Cellulolytic Activity

The cellulolytic activity of the bacterial strains was measured using Ramachandra media (Ramachandra et al., 1987) supplemented with 5% carboxymethyl cellulose (CMC). The strains were grown for 48 h at 30°C under agitation at 170 rpm and then centrifuged at  $4,000 \times g$  for 10 min. The supernatant was then collected and analyzed using DNS (dinitrosalicylic acid solution). Total cellulase activity was quantified by measuring the amount of reducing sugars released during the degradation of a strip of No. 1 Whatman filter paper measuring  $1.0 \times 6.0$  cm (Ghose, 1987).

The standard curve for enzymatic determination was generated by glucose determination (Miller, 1959). One enzyme activity unit (U) was defined as the amount of enzyme capable of releasing 1 mg reducing sugar per min at 50°C, and enzyme activity is expressed as units per milliliter.

### Molecular Identification of Bacterial Strains

Strains that showed the most consistent results with the previously described properties were identified following the protocol of the Quick-DNA Universal Extraction Kit (ZymoResearch, cat nos. D4068 and D4069) (Sambrook et al., 1989). For identification, PCR products were purified using the Wizard® SV Gel and PCR Clean-up System Kit and sequenced using universal primers. The sequences were edited using the Biological Sequence Alignment Editor – BioEdit (Hall, 1999), and the consensus sequence was obtained using the BLAST® tool (Altschul et al., 1990) and compared with the National Center for Biotechnology Information (NCBI) GenBank database. The resulting phylogenetic trees were constructed using MEGA7® software (Tamura et al., 2004).

Then, the characterized strains were lyophilized and stored at  $-80^\circ\text{C}$ .

### Data Analysis

The data were analyzed by analysis of variance (*F*-test), and the average results for each treatment were compared by Tukey's test at the 5% probability level using AgroEstat version 1.0 software (Barbosa and Maldonado, 2010).

## Plant Growth Promotion Assay at Greenhouse

### Experimental Design

A randomized block design with three replicates and eight treatments was used. A total of 24 pots were used, and each pot was considered one experimental unit. The treatments were described in Table 2.

Initially, ministalks were planted in sprouting boxes in a greenhouse. After 15 days, the plants that had developed were transplanted into pots with a volumetric capacity of 5 liters and maintained outdoors for 60 days.

The experiment used ministalks (the region where the bud is located) of 10-month-old RB 5201 sugarcane plants. The ministalks were planted in eight sprouting boxes (one per treatment). The sprouting boxes were filled with a 1:1 mixture of sand:vermiculite. The ministalks were germinated to ensure that the sugarcane plants would be at the same vegetative stage of development when they were transplanted into pots (Figure 1). The pots were filled with a 2-cm layer of gravel and soil. The soil was classified as a Eutrophic Red Latosol and had the following chemical properties: a pH of 6.9 (in  $\text{CaCl}_2$ );  $10.0 \text{ g dm}^{-3}$  organic matter (OM);  $14.0 \text{ mg dm}^{-3}$  P resin; 0.7, 79.0, and  $13.0 \text{ mmolc dm}^{-3}$  K, Ca, and Mg, respectively; a cation exchange capacity (CEC) of  $104.2 \text{ mmolc dm}^{-3}$ ; a V of 90%; and an SB of  $93.4 \text{ mmolc dm}^{-3}$ .

The precipitation and irrigation during the experimental period totaled 816.4 mm (620.2 and 196.2 mm of precipitation and irrigation, respectively). The average maximum and minimum temperatures during the period were 27.3 and  $13.5^\circ\text{C}$ , respectively. The identifications of sugarcane rhizospheric bacteria are shown in Table 1 and the sugarcane pot treatments are shown in Table 2.





**FIGURE 1 |** Experimental trial showing sugarcane in pots.

**TABLE 1 |** Identification of sugarcane rhizospheric bacteria using NCBI BLAST-N of 16S rRNA gene sequences.

Isolate	Species identification	Identity (%)
IJ8	<i>Staphylococcus saprophyticus</i> NR_114090.1	99.76
	<i>Staphylococcus saprophyticus</i> MG694483.1	100.00
IP11	<i>Enterobacter</i> sp. KR558701.1	99.85
	<i>Enterobacter</i> sp. HM748078.1	99.85
IP14	<i>Enterobacter</i> sp. KR558701.1	96.34
	<i>Enterobacter</i> sp. HM748078.1	96.34
IP17	<i>Bacillus anthracis</i> MK575034.1	99.88
	<i>Bacillus anthracis</i> AF290553.	99.88
IP21	<i>Bacillus thuringiensis</i> NR_112780.1	99.79
	<i>Bacillus thuringiensis</i> KT159186.1	99.68
IP23	<i>Achromobacter spanius</i> MN007235.1	99.29
	<i>Achromobacter spanius</i> NR_025686.1	99.29
IP24	<i>Enterobacter asburiae</i> MG571735.1	99.84
	<i>Enterobacter asburiae</i> KY316493.1	99.84

Query cov. 100%; E-value 0.0 for all sequences.

**TABLE 2 |** Sugarcane pot treatments and accession number of Rhizobacteria's sequences deposited at NCBI.

Treatment	Inoculant	Accession number
T1	<i>Staphylococcus saprophyticus</i> IJ8	MT764797.1
T2	<i>Enterobacter</i> sp. IP11	MT764798.1
T3	<i>Enterobacter</i> sp. IP14	MT764799.1
T4	<i>Bacillus anthracis</i> IP17	MT764800.1
T5	<i>Bacillus thuringiensis</i> IP21	MT764801.1
T6	<i>Achromobacter spanius</i> IP23	MT764802.1
T7	<i>Enterobacter asburiae</i> IP24	MT764803.1
T8	Controle (Sem inoculação)	–

## Soil Fertilization

For soil fertilization, the equivalent of 60 kg ha<sup>-1</sup> urea (0.74 g pot<sup>-1</sup>) and 200 kg ha<sup>-1</sup> potassium chloride (1.89 g per pot) were

applied in two applications: one at the time of planting and another as a topdressing at 30 days after planting. At the time of planting, 140 kg ha<sup>-1</sup> simple superphosphate (4.16 g pot<sup>-1</sup>), 5 kg ha<sup>-1</sup> zinc sulfate (0.15 g pot<sup>-1</sup>), 2 kg ha<sup>-1</sup> boric acid (0.065 g pot<sup>-1</sup>), and 3 kg ha<sup>-1</sup> manganese sulfate (0.057 g pot<sup>-1</sup>) were applied. The soil amendment and fertilization amounts were based on the recommendations of a previous study (Raij et al., 1997).

## Inoculation

All previously characterized lyophilized strains were resuspended in nutrient broth and incubated for 24 h in a BOD oven at 28°C to achieve a final concentration of 1 × 10<sup>9</sup> colony-forming units (CFUs) mL<sup>-1</sup>.

The first inoculation was carried out after transplanting the sugarcane into pots, and inoculation was conducted every 15 days thereafter. Inoculation was performed via the soil with the aid of a graduated pipette with the addition of 15 mL inoculum pot<sup>-1</sup>. In the control treatment, no inoculum was added.

## Biometric Analysis and Bacterial Counts

The number of tillers per pot was counted, and the height of the tillers was measured from the base of the plant to the +1 leaf (in accordance with the Kuijper numbering system) using a graduated ruler. The diameter of the tillers was also measured at the base of each tiller close to the ground with the aid of a caliper. The height and diameter of the main tiller were measured.

For the counting of endophytic bacteria, the plants were separated into shoots and roots, which were washed with a water jet to remove the soil. One gram of each vegetative tissue (shoots and roots) sample was subsequently weighed and subjected to superficial disinfection to eliminate epiphytic microorganisms. During this process, both tissues were sequentially immersed in 70% ethanol for 1 min, 3% sodium hypochlorite solution for 3 min and 70% ethanol for 30 s (Wilkinson et al., 1989). Three rinses were subsequently performed with sterile distilled water.

Finally, the shoots and roots were aseptically macerated with the aid of a mortar and pestle, after which they were placed in an Erlenmeyer flask containing 3 mL of 0.1% NaCl.

For the counting of bacteria present in the soil, 10 g of rhizospheric soil was added to an Erlenmeyer flask containing 95 mL of 0.1% sodium pyrophosphate saline. The contents of all the Erlenmeyer flasks were stirred for 1 h, after which serial dilutions were prepared (Wollum, 1982). One hundred microliters of solutions obtained through triplicate dilution were inoculated into Petri dishes containing nutrient agar medium. The dishes were kept in a BOD oven at 30°C, and the number of CFUs was counted after 24, 48, and 72 h (Vieira and Nahas, 2005).

### Plant Dry Mass and Soil and Plant Nutrients

After removing 1 g of fresh mass to count the endophytic and rhizospheric bacteria, the dry plant mass was measured.

Root dry mass (RDM), shoot dry mass (SDM), and total dry mass (TDM) were measured by separating the plants into shoots and roots, which were washed with a water jet for soil removal. Both types of samples were placed in paper bags and then dried in an oven at 65°C until a constant mass was reached. After drying, the mass was measured on a semianalytical scale. To obtain the total dry mass (TDM), the RDM and SDM were summed.

The resin phosphorus content in the soil was determined using spectrophotometric methodology (IAC, 2001). Total nitrogen levels were measured by sulfuric digestion ( $\text{H}_2\text{SO}_4$ ) followed by distillation and titration according to the method of Tedesco et al. (1995). Shoot and root samples used for dry mass determination were ground and then used to measure phosphorus and nitrogen contents. The phosphorus content was measured by means of nitric-perchloric digestion followed by spectrophotometric analysis (Malavolta et al., 1989), and the nitrogen content was measured by sulfuric digestion followed by titration (Malavolta et al., 1989).

From the nitrogen and phosphorus contents of the sugarcane shoots and roots and the dry mass production values, nutrient extraction was calculated by multiplying the nutrient content in grams per kilogram by the dry mass production (in grams).

### Data Analysis

The data were analyzed by analysis of variance ( $F$ -test), and the average results for each treatment were compared by Duncan's test at the 5% probability level using AgroEstat software version 1.0 (Barbosa and Maldonado, 2010).

## RESULTS

### Bacterial Strain Abilities

#### IAA Production

The highest IAA producers were *E. asburiae* (56.68  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ), *Enterobacter* 1 (55.32  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ), and *Enterobacter* 2 (53.23  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ), followed by *S. saprophyticus* (45.3  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ) and *B. anthracis* (42.1  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ). The lowest IAA producer was *A. spanius* (7  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ), followed by *B. thuringiensis* (30  $\mu\text{g}$  of IAA  $\text{mL}^{-1}$ ) (Figure 2A).

#### Cellulolytic Activity

The highest cellulolytic activity was observed for *A. spanius* (0.61 U  $\text{mL}^{-1}$ ), *S. saprophyticus* (0.58 U  $\text{mL}^{-1}$ ), *Enterobacter* 1 (0.58 U  $\text{mL}^{-1}$ ), *B. anthracis* (0.57 U  $\text{mL}^{-1}$ ), and *B. thuringiensis* (0.56 U  $\text{mL}^{-1}$ ). The lowest cellulolytic activity was observed for *Enterobacter asburiae* (0.54 U  $\text{mL}^{-1}$ ) and *Enterobacter* 2 (0.54 U  $\text{mL}^{-1}$ ) (Figure 2B).

#### Biological Nitrogen Fixation (BNF)

Regarding BNF, the highest levels of nitrogen compounds were observed in plants treated with *B. thuringiensis* (108.07  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ), *S. saprophyticus* (105.07  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ), and *A. spanius* (95.4  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ). The lowest level of nitrogen compounds was observed for those treated with *Enterobacter* 2 (40  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ), followed by *Enterobacter* 1 (51.4  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ) and *Enterobacter asburiae* (61.5  $\mu\text{g}$  of N  $\text{mL}^{-1}$ ) (Figure 2C).

#### Potassium (K), $\text{CaPO}_4$ , and $\text{AlPO}_4$ Solubilization

Regarding K solubilization, the highest values were observed for *A. asburiae* (17.8 mg of K  $\text{L}^{-1}$ ) and *S. saprophyticus* (15.2 mg of K  $\text{L}^{-1}$ ), and the lowest values were observed for *E. asburiae* (3.1 mg of K  $\text{L}^{-1}$ ), *B. thuringiensis* (3.2 mg of K  $\text{L}^{-1}$ ), *Enterobacter* 1 (6.2 mg of K  $\text{L}^{-1}$ ), *Enterobacter* 2 (6.4 mg of K  $\text{L}^{-1}$ ), and *B. anthracis* (12.4 mg of K  $\text{L}^{-1}$ ) (Figure 2D).

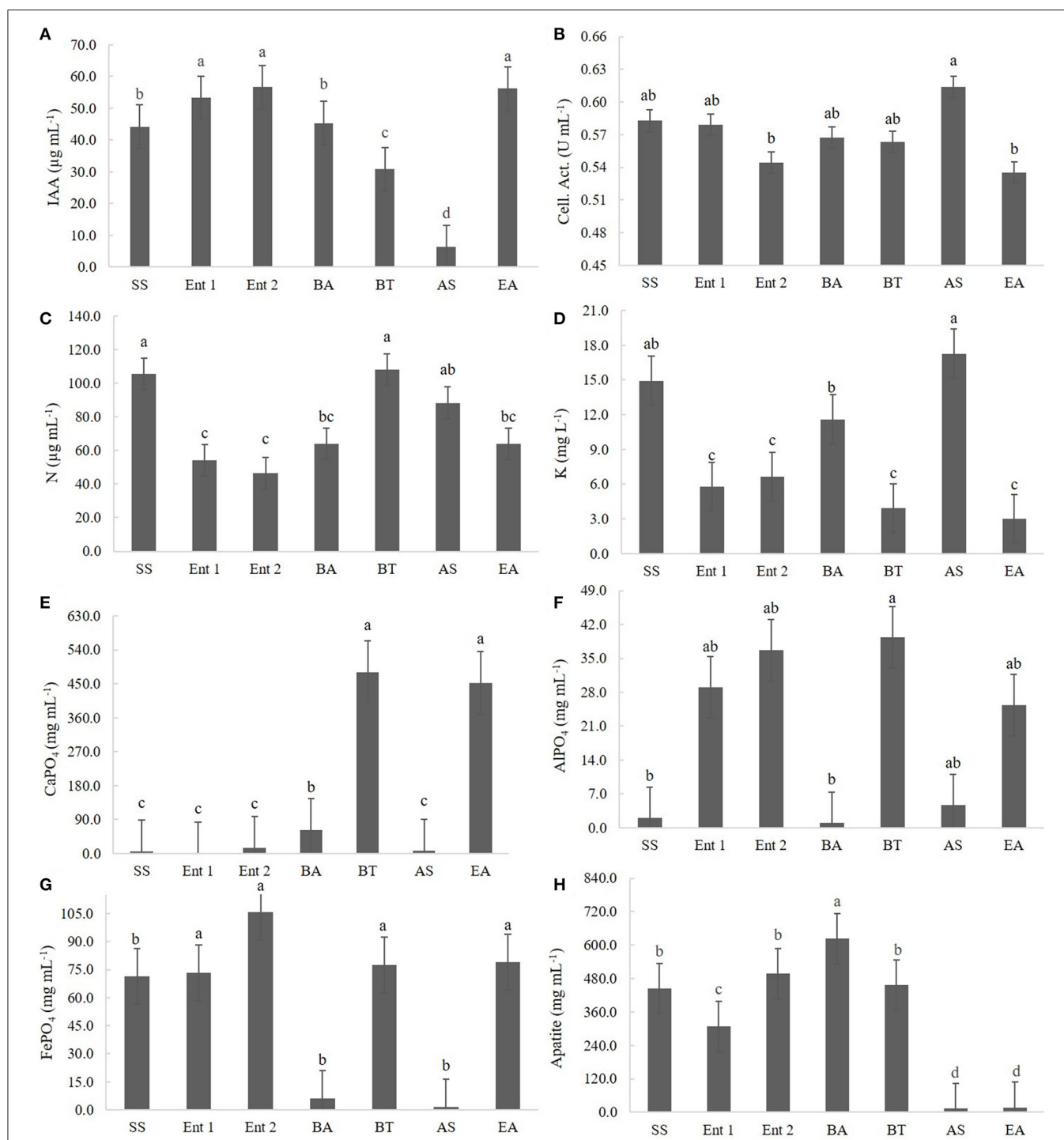
Regarding  $\text{CaPO}_4$  solubilization, the highest levels of P compounds were observed for *B. thuringiensis* (455.1 mg of P  $\text{mL}^{-1}$ ), followed by *E. asburiae* (452.1 mg of P  $\text{mL}^{-1}$ ), and the lowest levels were observed for *Enterobacter* 1 (4.5 mg of P  $\text{mL}^{-1}$ ), *S. saprophyticus* (16.5 mg of P  $\text{mL}^{-1}$ ), *A. spanius* (7.2 mg of P  $\text{mL}^{-1}$ ), *Enterobacter* 2 (9.5 mg of P  $\text{mL}^{-1}$ ), and *B. anthracis* (46.5 mg of P  $\text{mL}^{-1}$ ) (Figure 2E).

Regarding  $\text{AlPO}_4$  solubilization, the highest values were observed for *B. thuringiensis* (41.5 mg of P  $\text{mL}^{-1}$ ), followed by *Enterobacter* 2 (38.7 mg of P  $\text{mL}^{-1}$ ), *Enterobacter* 1 (31.4 mg of P  $\text{mL}^{-1}$ ), and *E. asburiae* (29.4 mg of P  $\text{mL}^{-1}$ ), and the lowest values were observed for *B. anthracis* (5.3 mg of P  $\text{mL}^{-1}$ ), *S. saprophyticus* (5.7 mg of P  $\text{mL}^{-1}$ ), and *A. spanius* (6.1 mg of P  $\text{mL}^{-1}$ ) (Figure 2F).

#### $\text{FePO}_4$ and Araxá Apatite Solubilization

Regarding  $\text{FePO}_4$  solubilization, the highest values were observed for *Enterobacter* 2 (104.5 mg of P  $\text{mL}^{-1}$ ), *B. thuringiensis* (79.3 mg of P  $\text{mL}^{-1}$ ), *E. asburiae* (73.4 mg of P  $\text{mL}^{-1}$ ), *Enterobacter* 1 (73.4 mg of P  $\text{mL}^{-1}$ ), and *S. saprophyticus* (72 mg of P  $\text{mL}^{-1}$ ), and the lowest values were observed for *A. spanius* (4.5 mg of P  $\text{mL}^{-1}$ ) and *B. anthracis* (6.5 mg of P  $\text{mL}^{-1}$ ) (Figure 2G).

Regarding Araxá apatite, the highest values were observed for *B. anthracis* (650 mg of P  $\text{mL}^{-1}$ ), followed by *B. thuringiensis* (465.2 mg of P  $\text{mL}^{-1}$ ), *Enterobacter* 2 (480 mg of P  $\text{mL}^{-1}$ ), and *S. saprophyticus* (450.4 mg of P  $\text{mL}^{-1}$ ), and the lowest values were observed for *A. spanius* (12.3 mg of P  $\text{mL}^{-1}$ ), *E. asburiae* (14.7 mg of P  $\text{mL}^{-1}$ ), and *Enterobacter* 1 (310.2 mg of P  $\text{mL}^{-1}$ ) (Figure 2H).



**FIGURE 2 |** Quantitative results for (A) IAA production, (B) cellulytic activity, (C) BNF, (D) K solubilization, (E)  $\text{CaPO}_4$  solubilization, (F)  $\text{AlPO}_4$  solubilization, (G)  $\text{FePO}_4$  solubilization, and (H) Araxá apatite solubilization. Means followed by the same letters do not differ according to Tukey's test at 5% probability. SS, *S. saprophyticus* IJ8; Ent 1, *Enterobacter* sp. IP11; Ent 2, *Enterobacter* sp. IP14; BA, *B. anthracis* IP17; BT, *B. thuringiensis* IP21; AS, *A. spanius* IP23; EA, *E. asburiae* IP24; Control, no inoculation.

## Greenhouse Tests

### Plant Height

The plants inoculated with the bacteria *B. thuringiensis* (112 cm), *Enterobacter* 1 (111 cm), and *E. asburiae* (111 cm) were taller than

the control plants (108 cm) ( $p < 0.05$ ); no significant differences were observed between the control plants and those inoculated with *S. saprophyticus* (110 cm), *Enterobacter* 2 (109 cm), *B. anthracis* (109 cm), and *A. spanius* (108 cm) (Figure 3A).

## Number of Tillers

There was no significant difference regarding the number of tillers between the treated plants ( $p > 0.05$ ) and the control plants except for plants treated with *Enterobacter* 2, which exhibited fewer tillers than the control plants (**Figure 3B**).

## Diameter of Tillers

There was no difference between the control and treated plants regarding the diameter of tillers, except for plants treated with *S. saprophyticus* (1.4 cm), *Enterobacter* 1 (1.4 cm), *B. thuringiensis* (1.3 cm), and *E. asburiae* (1.3 cm) (**Figure 3C**).

## Root, Shoot, and Total Dry Matter

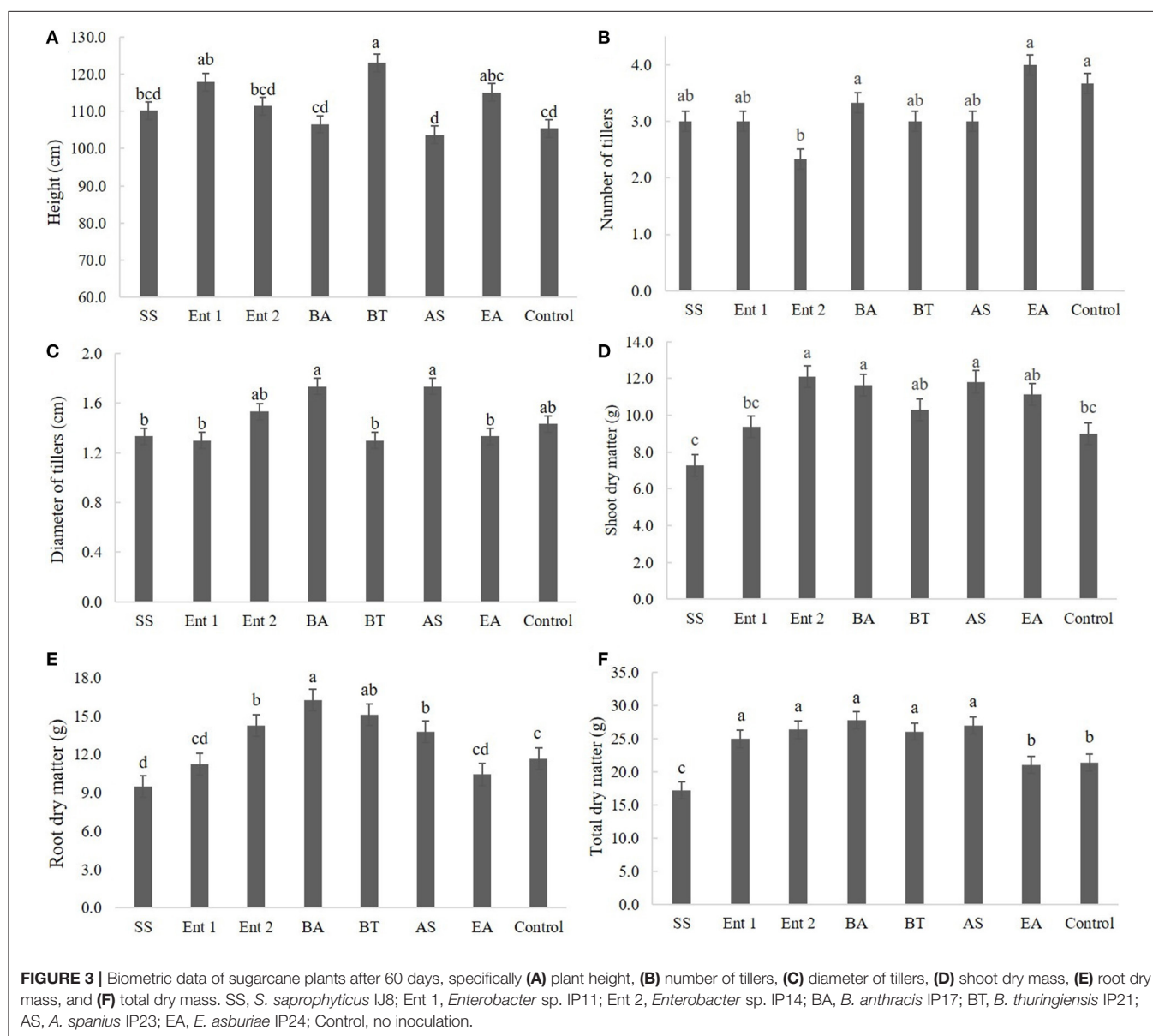
Regarding shoot dry matter, higher values were found for plants inoculated with *Enterobacter* 2 (12 cm), *B. anthracis* (11 cm),

and *A. spanius* (11 cm) than for the control plants (9 cm) ( $p < 0.01$ ), while no differences were found between plants inoculated with *B. thuringiensis* (9 cm), *Enterobacter* 1 (8.5 cm), and *S. saprophyticus* compared to the control (**Figure 3D**).

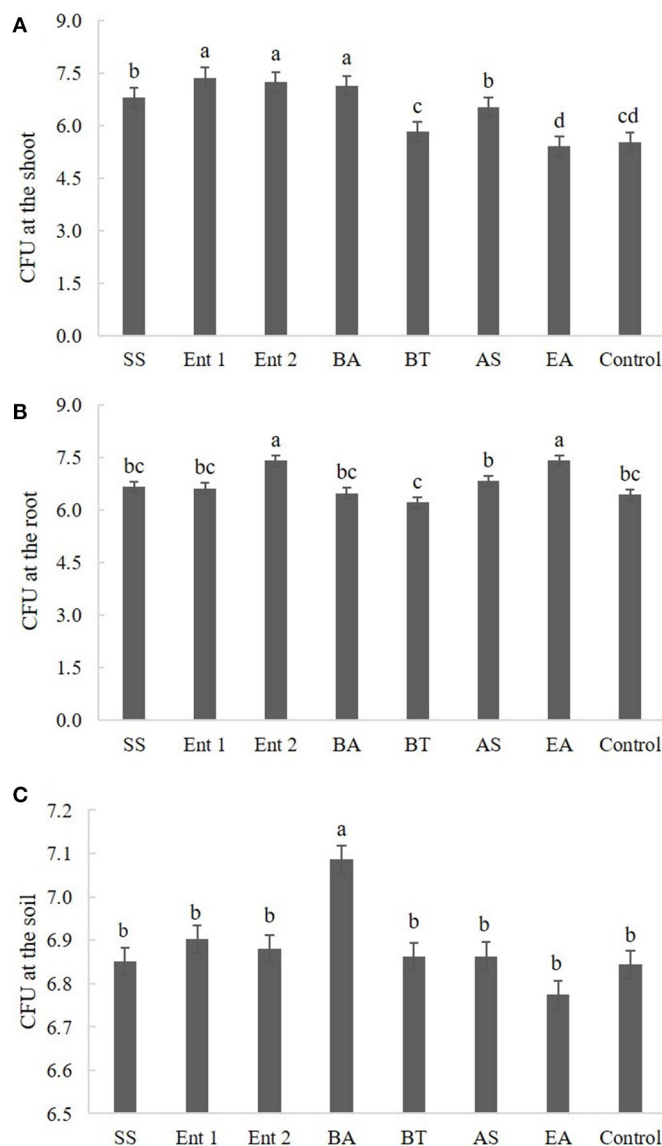
Regarding root dry matter, plants treated with *B. anthracis* (16 cm), *B. thuringiensis* (15 cm), *Enterobacter* 1 (14 cm), and *A. spanius* (13 cm) showed higher values than the control plants (11 cm), whereas plants treated with *S. saprophyticus* (10 cm). *Enterobacter* 1 (11 cm) and *E. asburiae* (11 cm) had lower values than the control plants (**Figure 3E**).

## Total Dry Matter

Regarding total dry matter, higher values were observed for plants treated with *B. anthracis* (27 cm), *Enterobacter* 2 (26 cm), *Enterobacter* 1 (25 cm), and *A. spanius* (25 cm) than for the







**FIGURE 4 |** Number of CFUs **(A)** endophytic in sugarcane shoots, **(B)** endophytic in sugarcane roots, and **(C)** on the soil. The data were transformed to log 10.

control plants (24 cm) ( $p < 0.05$ ), whereas lower values were observed for plants treated with *S. saprophyticus* (17 cm) and *E. asburiae* (17 cm) than for the control plants (**Figure 3F**).

### Colony Forming Units (CFUs) (Log10)

Plants treated with *Enterobacter* 1 (8.0), *Enterobacter* 2 (8.0), *B. anthracis* (7.5), *S. saprophyticus* (6.8), and *A. spanius* (6.8) showed more CFUs at the shoot than the control plants (6.0). The number of CFUs at the shoot for plants treated with *B. thuringiensis* (6.0) and *E. asburiae* (6.0) was not different from that for the control plants (**Figure 4A**).

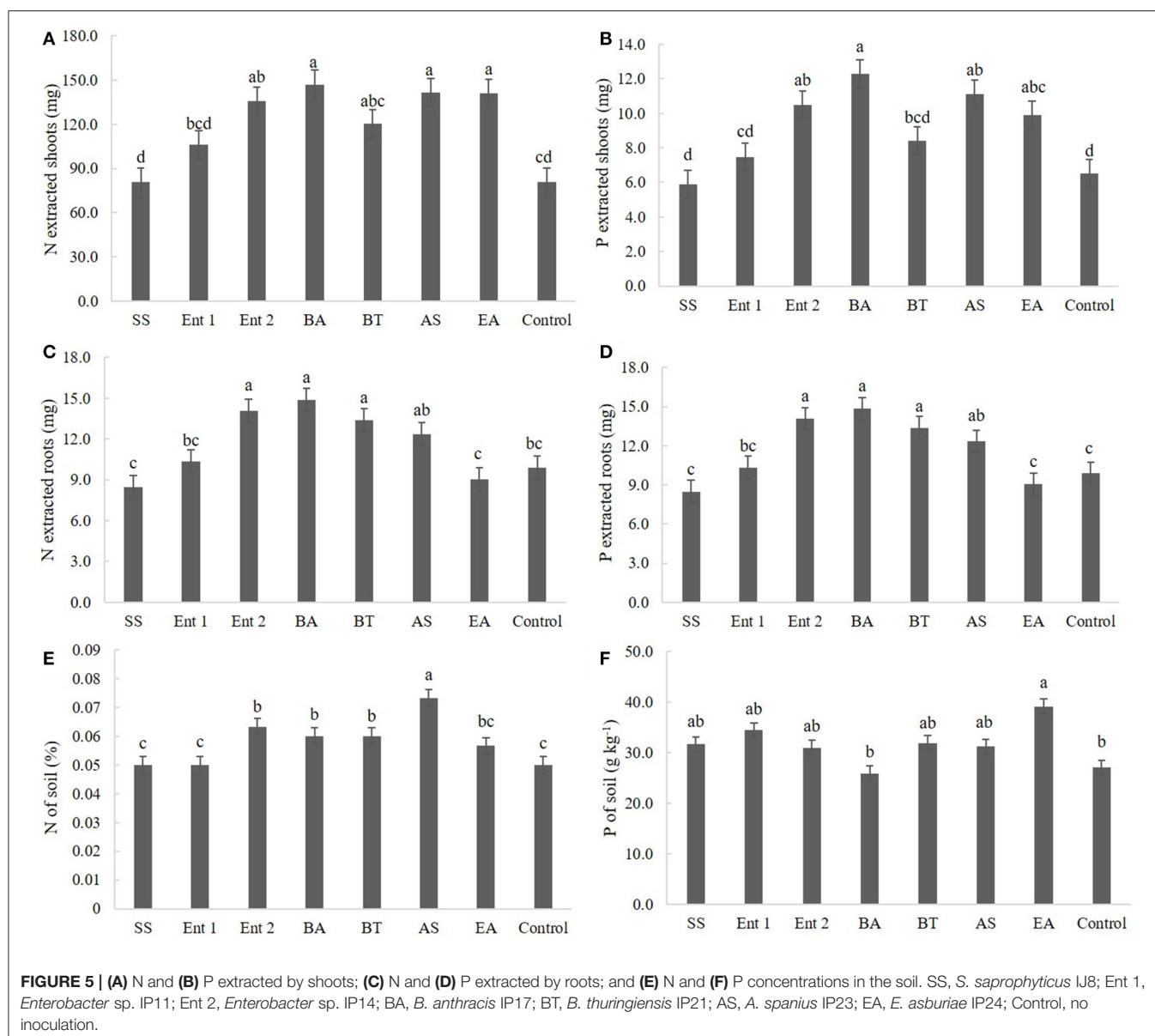
Regarding CFUs at the root, higher values were found for plants treated with *Enterobacter* 2 (7.5) and *E. asburiae* (7.2) than for the control plants (6.5). Plants treated with *S. saprophyticus*

(6.8), *Enterobacter* 1 (6.7), *B. anthracis* (6.4), *B. thuringiensis* (6.5), and *A. spanius* (6.5) did not differ from the control plants in this measure ( $p > 0.05$ ) (**Figure 4B**).

Regarding CFUs in the soil, the only plants that presented higher values than the control plants ( $p < 0.05$ ) were those treated with *B. anthracis* (7.1). The plants treated with other bacteria did not differ from the control plants (**Figure 4C**).

### Nitrogen and Phosphorus Extraction

Regarding nitrogen extracted from the shoot, higher values ( $p < 0.05$ ) were observed for plants treated with *B. anthracis* (140.0 mg), *A. spanius* (135.4 mg), *E. asburiae* (134.2 mg), and *Enterobacter* 2 (133.2 mg) than for the control plants (75 mg). Nitrogen extracted from plants treated with *S.*



*saprophyticus* (80 mg), *Enterobacter* 1 (100 mg), and *B. thuringiensis* (115 mg) did not differ from that extracted from the control plants (Figure 5A).

Regarding phosphorus extracted from the shoot, the plants that presented higher amounts than the control plants (6 mg) were those inoculated with *B. anthracis* (12 mg), *A. spanius* (11 mg), and *E. asburiae* (11 mg). Plants inoculated with *S. saprophyticus* (6.0 mg), *Enterobacter* 1 (7.5 mg), and *B. thuringiensis* (8.0 mg) did not show different levels of extracted phosphorus ( $p > 0.05$ ) compared to the control plants (Figure 5B).

Regarding nitrogen extracted from the root, higher values ( $p < 0.05$ ) were observed for plants inoculated with *B. anthracis* (14 mg), *B. thuringiensis* (13.0 mg), and *Enterobacter* 2 (14.0 mg) than for the control plants (11 mg). The levels of nitrogen extracted from the root did not differ between

plants inoculated with *S. saprophyticus* (8.4 mg), *Enterobacter* 1 (10.0 mg), *A. spanius* (12 mg), and *E. asburiae* and the control plants (Figure 5C).

Regarding phosphorus extracted from the root, higher values ( $p < 0.05$ ) were observed for plants treated with *B. anthracis* (15.0 mg), *B. thuringiensis* (14.0 mg), *Enterobacter* 2 (14.0 mg), and *A. spanius* (120.0 mg) than for the control plants (11.0 mg). The levels of phosphorus extracted from the root did not differ ( $p > 0.05$ ) between plants inoculated with *S. saprophyticus* (80 mg), *Enterobacter* 1 (100 mg), and *E. asburiae* (9.0 mg) and the control plants (Figure 5D).

Regarding nitrogen extracted from the soil, higher values ( $p < 0.05$ ) were observed for plants treated with *A. spanius* (0.07%), *Enterobacter* 2 (0.68%), *B. anthracis* (0.06%), and *B. thuringiensis* (0.06%) than for the control plants (0.05%). The levels of nitrogen extracted from the soil did not differ ( $p > 0.05$ ) between plants

inoculated with *S. saprophyticus* (0.05%), *Enterobacter* 1 (0.05%), and *E. asburiae* (0.05%) and the control plants (**Figure 5E**).

Regarding phosphorus extracted from the soil, higher values ( $p < 0.05$ ) were observed for plants inoculated with *E. asburiae* ( $39.5 \text{ g kg}^{-1}$ ) than for the control plants ( $30 \text{ g kg}^{-1}$ ). The levels of phosphorus extracted from the soil did not differ between plants inoculated with *S. saprophyticus* ( $33 \text{ g kg}^{-1}$ ), *Enterobacter* 1 ( $35 \text{ g kg}^{-1}$ ), *Enterobacter* 2 ( $36 \text{ g kg}^{-1}$ ), *B. anthracis* ( $28 \text{ g kg}^{-1}$ ), *B. thuringiensis* ( $32 \text{ g kg}^{-1}$ ), and *A. spanius* ( $33 \text{ g kg}^{-1}$ ) and the control plants (**Figure 5F**).

## DISCUSSION

Seven bacterial strains isolated from sugarcane were used in the present study, each of which previously showed some ability to promote plant growth.

*E. asburiae* isolated from the mustard rhizosphere showed the ability to solubilize phosphate, to produce siderophores and IAA and to resist fungicides (Ahmad and Khan, 2010). Mahdi et al. (2020) isolated *E. asburiae* from *Chenopodium quinoa* Willd., and this bacterium showed properties related to siderophore production, hydrogen cyanide (HCN), ammonia and extracellular enzymes. Interestingly, the plants that received inoculated *E. asburiae* showed high Na tolerance.

*B. anthracis*, the organism that causes anthrax, derives its name from the Greek word for coal, *B. anthracis*, because of its ability to cause black, coal-like cutaneous eschars (Helgason et al., 2000). As a result, this bacterium cannot be used as an inoculant despite being isolated from the sugarcane rhizosphere and presenting a good ability to promote root growth.

*B. thuringiensis* (Bt) bacteria are insect pathogens that rely on insecticidal pore forming proteins known as Cry and Cyt toxins to kill their insect larval hosts (Bravo et al., 2011). In the present study, the isolate of *B. thuringiensis* showed the capacity to produce IAA. Similar results were found by Raddadi et al. (2008), who found, for the first time, an isolate able to produce ACC deaminase, phosphate enzyme and IAA.

Bacterial isolates able to produce IAA and solubilize phosphorous are interesting because phytohormones promote root cell proliferation and increase nutrient and water absorption through the overproduction of side cells and root hairs (Glick, 2012). In addition, phosphorus-solubilizing microorganisms are important in agricultural ecosystems and directly or indirectly influence physical, chemical, and biological soil properties (Hammer et al., 2011; Cordero et al., 2012; Verma et al., 2017).

*S. saprophyticus* is uniquely associated with uncomplicated urinary tract infection (UTI) in humans and has special urotropic and ecologic features that are different from those of other staphylococci and *E. coli* (Kuroda et al., 2005). Similar to *B. anthracis*, this bacterium cannot be used as an inoculant.

*A. spanius*, a gram-negative, rod-shaped bacterium isolated from members of the *Arabidopsis thaliana* rhizosphere, has been identified in various environments, including freshwater and soil isolates (Coenye et al., 2003; Li et al., 2018). The genome of *A. spanius* has attracted attention because preliminary screening has

shown that it possesses the attributes of plant growth-promoting rhizobacteria, such as phosphate solubilization, indole-3-acetic acid biosynthesis, and siderophore production abilities, as well as actinomycete activity against *Phytophthora cinnamomi* in plants (Rosli, 2016). This is the first report showing the abilities of *A. spanius* to promote the growth of sugarcane crops.

Colonization of the rhizosphere or some plant tissues is the first step required for bacteria to have a plant growth effect. Interestingly, *B. thuringiensis* presented a low plant growth effect at the shoot, root and soil levels. This result suggests that the capacity for interaction with the plant is more important than the bacterial amount. Lobo et al. (2019) reported that some *B. subtilis* strains showed high amounts in the rhizosphere and did not promote plant growth, whereas other strains showed low amounts and promoted plant growth.

The present study has shown that some bacteria isolated from sugarcane are capable of metabolizing unavailable forms of soil nutrients to release them for plant uptake and produce IAA. In natural ecosystems, most nutrients, such as N, P, and S, are linked to organic molecules and therefore are minimally available to plants. To access these nutrients, plants depend on the growth of soil microorganisms such as bacteria and fungi that exhibit metabolic mechanisms for depolymerizing and mineralizing the organic forms of N, P, and S (Jacoby et al., 2017).

Growth promotion of sugarcane could be optimized with appropriate combinations of PGPRs, environmental conditions and plant genotypes. In this sense, efforts must be made in the development of good inoculants.

## CONCLUSIONS

*Enterobacter* sp. IP11, *Enterobacter* sp. IP14, *B. thuringiensis* IP21, *A. spanius* IP23, and *E. asburiae* IP24 have the ability to promote the growth of sugarcane plants under greenhouse conditions. However, because *Enterobacter* sp. IP11, *Enterobacter* sp. IP14, and *E. asburiae* can be potentially pathogenic to humans, *B. thuringiensis* and *A. spanius* are feasible for use as future inoculants in sugarcane cultivation and may increase the potential to achieve production benefits.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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# Potential Use of Beneficial Microorganisms for Soil Amelioration, Phytopathogen Biocontrol, and Sustainable Crop Production in Smallholder Agroecosystems

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Smallholder agroecosystems play a key role in the world's food security providing more than 50% of the food produced globally. These unique agroecosystems face a myriad of challenges and remain largely unsupported, yet they are thought to be a critical resource for feeding the projected increasing human population in the coming years. The new challenge to increase food production through agricultural intensification in shrinking per capita arable lands, dwindling world economies, and unpredictable climate change, has led to over-dependence on agrochemical inputs that are often costly and hazardous to both human and animal health and the environment. To ensure healthy crop production approaches, the search for alternative ecofriendly strategies that best fit to the smallholder systems have been proposed. The most common and widely accepted solution that has gained a lot of interest among researchers and smallholder farmers is the use of biological agents; mainly plant growth promoting microorganisms (PGPMs) that provide essential agroecosystem services within a holistic vision of enhancing farm productivity and environmental protection. PGPMs play critical roles in agroecological cycles fundamental for soil nutrient amelioration, crop nutrient improvement, plant tolerance to biotic and abiotic stresses, biocontrol of pests and diseases, and water uptake. This review explores different research strategies involving the use of beneficial microorganisms, within the unique context of smallholder agroecosystems, to promote sustainable maintenance of plant and soil health and enhance agroecosystem resilience against unpredictable climatic perturbations.

**Keywords:** plant growth promoting microorganisms, biocontrol agents, microbial inoculants, smallholder agroecosystems, soil fertility, food security

## INTRODUCTION

Biological soil fertility restoration techniques within the smallholder agroecosystems, in combination with other agronomic management practices, would provide the much-needed solutions for revitalizing the declining global food production (Raimi et al., 2017). Beneficial soil microbiota such as plant growth promoting microorganisms (PGPMs), comprising of specific groups of bacteria and fungi, provide essential agroecosystem services that support plant growth (Rouphael and Colla, 2020) and ameliorates soil productivity (Santos et al., 2019). PGPMs maintain key agroecological cycles fundamental for soil nutrient enrichment, crop nutrient improvement, plant tolerance to biotic and abiotic stresses, biocontrol of pests and diseases, and water uptake enhancement (Lobo et al., 2019; Goswami and Deka, 2020). They are actively involved in healthy plant development and growth through secretion of hormonal growth regulators, and resistance induction against phytopathogens (Dakora et al., 2015). Besides, versatile PGPMs could be used to bioremediate polluted fields and increase the land available for production as in the case of heavy metals polluted soils (Gouda et al., 2018). These agroecosystem services are primarily important in supporting crop production in smallholder agroecosystems, which are characteristically defined by limited resource inputs.

PGPMs promote plant growth and productivity through various direct and indirect approaches. Several direct mechanisms have been established through previous studies and can be broadly classified into phytostimulants (Babalola and Glick, 2012), biofertilizers (Kalayu, 2019), rhizomediators, or stress regulators (Stamenković et al., 2018). Indirect mechanisms mainly occur in form of biocontrol of phytopathogens through competition for nutrients, enzymatic lysis, antibiosis (Köhl et al., 2019), secretion of volatile organic compounds (VOCs) (Sun and Tang, 2013), and triggering of antioxidative defense mechanism (Sandhya et al., 2010; Malik et al., 2020) and induced systemic resistance (ISR) response in the host plant (Heil and Bostock, 2002). PGPM biofertilizers promote plant growth by enhancing nutrient availability to the plants and the most studied pathways include N fixation (Ahmad and Kibret, 2014; Fukami et al., 2018b), P and K solubilization (Sharma et al., 2013; Soumare et al., 2020), S oxidation, Fe and C sequestration (Kannahi and Senbagam, 2014; Velivelli et al., 2014). PGPMs enhance the availability of P, K, Zn, Se, and Fe in the soil through biochemical processes such as solubilization, chelation, mineralization, oxidation and reduction reactions (Ahmed and Holmström, 2014; Velivelli et al., 2014; Rouphael and Colla, 2020). PGPMs are also known to secrete phytohormones such as auxins (Lin and Xu, 2013; Azizoglu, 2019), cytokinin, abscisic acid, ethylene, brassinosteroids, jasmonic acid, salicylic acid, strigolactones, and gibberellins (Goswami and Deka, 2020; Saad et al., 2020) that act as plant growth stimulators and stress controllers.

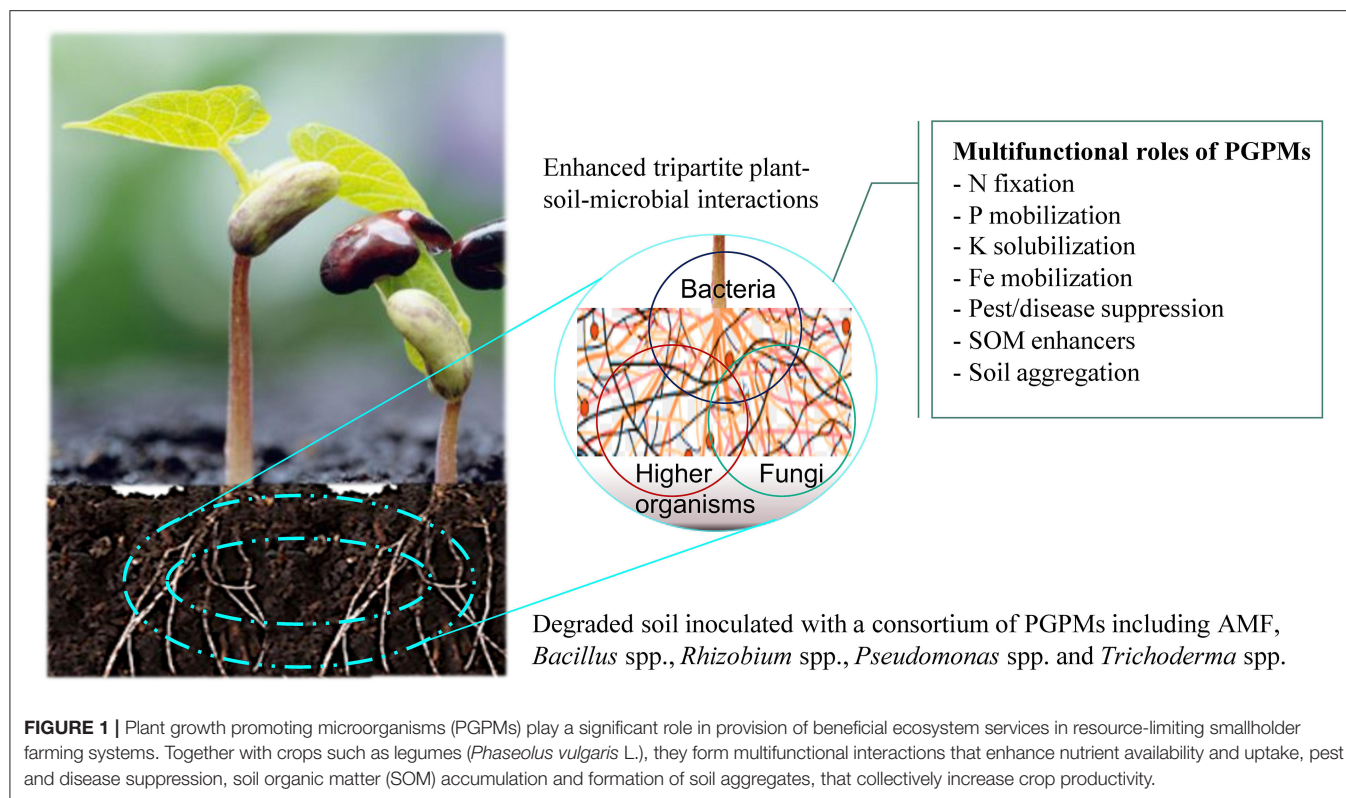
The PGPMs functionality and vigor, however, depend on intrinsic soil properties, environmental and agronomic management factors. Nutrient availability, soil pH, water, temperature, crop genotype, and cultural management are some of the key drivers determining the survival and function of

PGPMs in the soil (Gouda et al., 2018; Gupta et al., 2019). For maximum farm benefits to be realized in a highly heterogeneous smallholder systems (Njeru et al., 2020), tradeoffs in balancing the highlighted determinants have to be considered and appropriate farm management practices are ought to be carried out. For instance, the choice of crop species and diversity is critical in stimulating specific plant-microbe interactions and consequently the intended output (Orrell and Bennett, 2013; Saad et al., 2020). The authors suggest the use of multiple cropping systems that promote synergy and minimize the yield gap between the potential and realized production, a phenomenon that is commonly seen in smallholder systems. Additional methods efficient in augmenting indigenous soil PGPMs can be integrated to enhance microbial bio-functionality. These include the use of organic amendments such as farmyard manure and vermicompost (Mosa et al., 2018; Koskey et al., 2020), biotechnological approaches such as plant breeding (Bakker et al., 2012), crop management practices such as agroforestry, rotation, intercropping, cover cropping, and practicing reduced soil disturbance (Ventorino et al., 2012; Hontoria et al., 2019; Elagib and Al-Saidi, 2020).

The increasing demand among smallholder farmers to cut input costs and the need for sustainable nutrient management practices is driving the growing adoption and use of microbial-rich fertilizers in smallholder setups (Raimi et al., 2017). Commercial microbial inoculants (commonly used as biofertilizers or bioenhancers) containing single species or multiple strains of rhizobia, *Pseudomonas* spp., *Azotobacter* spp., *Bacillus* spp., *Trichoderma* spp., *Aspergillus* spp., and *Glomus* spp. (Figure 1) have been largely used in smallholder agroecosystems for crop production (Koskey et al., 2017; Bargaz et al., 2018; Adeyemi et al., 2019). Previous field researches carried out in different agroecosystems around the world have reported varying levels of successes on the use of PGPMs to support crop performance quantitatively and qualitatively (Pellegrino et al., 2012; Mishra et al., 2019; Saad et al., 2020). However, little has been done focusing on the use of PGPMs to address various challenges facing smallholder agroecosystems in the context of changing climatic conditions. Therefore, this review explores different available strategies involving the use of beneficial microorganisms as biofertilizers, within the unique context of smallholder agroecosystems, to promote sustainable maintenance of plant and soil health, and enhance agroecosystem resilience against unpredictable climatic perturbations.

## ROLES AND CHALLENGES OF SMALLHOLDER AGROECOSYSTEMS IN GLOBAL FOOD SECURITY

Eradicating hunger, poverty, and food insecurity while ensuring sustainable use of natural resources for agriculture, as highlighted in the United Nations Sustainable Development Goals (SDGs), is paramount in a world faced with a myriad of economic, social, political, and environmental challenges (Pérez-Escamilla, 2017). Globally, ~700 million people have no access to sufficient food while about 2 billion people face nutritional deficiencies,



of which about 50% of the food insecure individuals are from Asia, 35% from Africa, and 10% from Latin America (FAO, 2018). This is likely to increase due to the current global health and economic crisis due to the Coronavirus disease 2019 (Covid-19) pandemic. Smallholder agroecosystems, predominantly found in developing countries, are considered critical food security resources that will support food production for the increasing human population in the coming years. Currently, it is estimated that smallholder agroecosystems account for more than 50% of the food produced globally (Herrero et al., 2017). In Africa, they contribute about 75% of the total crop production and 50% of the animal products (Nyambo et al., 2019) and, thus, are significantly involved in rural poverty reduction, economic development, and food security. However, compared to large-scale profit-driven systems, smallholder agroecosystems have limited land size, stringent financial resources, low market sharing, and product range, thus, are faced with more risks and vulnerabilities (Kuivanen et al., 2016; Herrero et al., 2017).

The productivity of smallholder agroecosystems largely depends on the services naturally provided by the ecosystem such as soil fertility, nutrient cycling, water availability, pest control, and pollination (Altieri et al., 2012). Farmers' decision and selection of their appropriate agronomic management practices affect the extent of agroecosystem functioning. External pressures such as poverty, unreliable climatic conditions, and farmer's need for immediate satisfaction exert pressure on land use and cause negative impacts on the ecosystem (IFAD and UNEP, 2013). Agricultural intensification coupled

with the use of harmful agrochemical inputs has negatively impacted on smallholder agroecosystems (Bationo et al., 2012). Their long-term sustainability in the face of new challenges such as the shrinking per capita arable lands, emerging diseases, dwindling world economies, and unpredictable climate change, is on the balance. Notwithstanding their benefits, economic and policy marginalization, low investment support, and the increasing land fragmentation of small farms threaten their contribution to global food security, leaving many farmers vulnerable (IFAD and UNEP, 2013). The rising environmental awareness, depletion of natural resources, and human health nutritional concerns have led to a paradigm shift among the farmers from over-dependence on agrochemical inputs to the use of ecofriendly biological agents for agricultural production (Herrero et al., 2017; Alori and Babalola, 2018). To increase the use and adoption of biological agents in smallholder agroecosystems, more robust integrated pathways that encourage food production based on local innovations, practices, and resources should be established. Tapsoba et al. (2020) emphasize that smallholder farmers should be involved in re-designing agricultural production. This way, farmers are likely to integrate new techniques into their current farm management practices to meet their agrosociological and economic needs. The authors warn that some agricultural initiatives may remain localized and isolated despite evidence of success in other agroecosystems. It is, therefore, necessary to have the right network of stakeholders on a territorial scale to support agricultural initiatives and their implementation.



## USE OF PGPM INOCULANTS IN ENHANCING THE PRODUCTIVITY OF SMALLHOLDER AGROECOSYSTEMS; OPPORTUNITIES AND CHALLENGES

### Use of Nitrogen Fixing PGPMs as Biofertilizers

Nitrogen (N) is one of the essential elements required by plants for proper growth, development, and productivity, and plays a pivotal role in various structural, biochemical, and physiological processes (Giller et al., 2019). Therefore, to achieve good crop productivity and quality, N application in form of nitrogenous-based fertilizers or amendments is inevitable. The production of inorganic N fertilizer through the Haber-Bosch industrial chemical process revolutionized agriculture and significantly increased crop production. However, there are serious human health, economic, and environmental concerns raised on the excessive and continuous use of chemically derived inorganic N fertilizers (Reddy and Saravanan, 2013), and hence the introduction of N biofertilizer formulations as a viable and sustainable alternative. Biological nitrogen fixation (BNF) is a process that naturally involves legumes and rhizobia symbionts, and/or plants and a group of free-living PGPMs known as diazotrophs (Giller et al., 2019). Through the BNF, inert atmospheric  $N_2$  gas is converted via a series of enzymatically regulated complex reaction mechanisms into N containing organic compounds utilizable by the plants (Gupta et al., 2019). In symbiotic association, nodule forming rhizobia produces nitrogenase enzyme complex in the presence of leghemoglobin molecules and convert  $N_2$  into ammonium and nitrate ions which are readily absorbed by the plants. In return, plant hosts the bacteria inside the root nodules and provide photosynthates such as C that rhizobia uses as an energy source (Wang et al., 2013; Choudhary and Varma, 2017).

In smallholder farming systems, the use of microbial inoculants containing diazotrophs and symbiotic PGPMs is on the rise. Major groups of N-fixing bacteria commonly used include *Rhizobium* spp., *Azorhizobium* spp., *Mesorhizobium* spp., *Bradyrhizobium* spp., *Thiobacillus* spp., *Azospirillum* spp., *Sinorhizobium* spp., *Clostridium* spp., *Azotobacter* spp., Cyanobacteria, and *Frankia* spp. (Yeager et al., 2005; Mus et al., 2018; Raimi et al., 2019). It has been demonstrated that inoculating legumes with a single or a consortium of N-fixing bacteria improves soil fertility, plant growth, yield, and nutrition quality (Kawaka et al., 2014; Mabrouk et al., 2018; Menge et al., 2018). Inoculation also enhances root development, nodulation, water stress tolerance, and suppresses pathogenic infestation (Koskey et al., 2017; Alori and Babalola, 2018; Musyoka et al., 2020). Private sectors, research institutions and universities have partnered with smallholder farmers in delivering efficient inoculants. For instance, N2-Africa, a multi-stakeholder project, actively researched on N-fixing rhizobia strains and developed inoculants for use by African smallholder farmers in the production of soybean, common bean, chickpea, ground nut, and faba bean (Giller et al., 2019). In Kenya, the University of Nairobi in collaboration with the Microbiological

Resources Center Network (MIRCEN) partnered with MEA Fertilizer Ltd to produce Biofix<sup>®</sup>, a cheap *Rhizobium* based bioinoculant for use in the cultivation of legumes (Odame, 1997). In South Africa, BioControl Products SA (Pty) Ltd produces *Azospirillum* based N-fixing bioinoculants such as Azo-N<sup>®</sup> and Azo-N Plus<sup>®</sup> for cultivating grain and cover crop legumes (Raimi et al., 2017). Currently, more bacterial species are being identified for use as potential N-fixing bioinoculants (Ouma et al., 2016; Koskey et al., 2018; Gabasawa, 2020; Musyoka et al., 2020). Most of these trials have shown promising results under greenhouse-controlled conditions. Repeated field trials should be done to ascertain their performance under different ecological conditions of smallholder agroecosystems.

Generally, BNF can supply more than half of the plant N needs and can significantly reduce the use and overdependence on external chemical N fertilizers in agriculture (Bado et al., 2018). For instance, in Australia, diazotrophic N-fixation is estimated to provide the annual N demand of 20–80 kg N ha<sup>-1</sup> year<sup>-1</sup> for perennial grasses (Gupta et al., 2019). In Ghana, symbiotic N fixation is estimated to provide up to 16–145 kg N ha<sup>-1</sup> year<sup>-1</sup> for legumes (Kermah et al., 2018). Therefore, BNF could reduce substantially the use of additional basal or top-dresser inorganic N fertilizers and thus cutting the input cost for the smallholder farmers. BNF contribution and N quantification within smallholder agroecosystems, however, remains poorly understood due to the high cost of resources and technical expertise needed, and difficulty to implement at the grassroot level (Mhango et al., 2017; Bado et al., 2018). Despite a large and diverse genetic pool of N-fixing bacteria (Giller et al., 2019) and legume species suitable for different African agroclimatic conditions (Kebede, 2020), their utilization in promoting soil fertility and plant growth has not been achieved. Hence, new sustainable methods that are affordable, simpler, rapid, and easier to implement in smallholder setups should be developed to fill the aforementioned gap.

### Use of Nutrient (P, K, Fe) Solubilizing and Mobilizing Microorganisms as Biofertilizers

Soil nutrients such as phosphorus (P), potassium (K), and iron (Fe) often limit plant growth and development because of their low solubility in the soil (Giovannini et al., 2020). They are firmly fixed and are not readily available for plant uptake, and their shortage could be detrimental to healthy growth and physiological development of the plant (Parani and Saha, 2012). Smallholder farmers rely on the external application of inorganic P-fertilizers whose efficiency declines in the presence of too much rainfall. Granular P-inorganic fertilizers precipitate to form metal-cation complexes in rainy tropical ecosystems and thus become unavailable for plant use (Dissanayaka et al., 2018). Most soils of East and West Africa experience N, P, and K deficiency (Bationo et al., 2012). Therefore, the use of low-cost P and K solubilizing and mobilizing microorganisms that take part in P and K geo-cycles would be of paramount importance to alleviate soil nutrient deficiency and losses. They mineralize organic P and K through a series of complex enzymatic and hydrolytic reactions (Thakur et al., 2014) and also secrete organic acids



**FIGURE 2 |** Application of phosphate solubilizing bacteria (PSB) enhances P availability without disturbing the soil biochemical composition, improves plant growth, photosynthetic activity, crop nutritional values, and yield. The photo shows a greenhouse experiment carried out by the Kenyatta University FLAIR research students on the effect of PSB inoculation on *Zea mays* L. and *Vigna unguiculata* (L.) Walp. **(A)** Un-inoculated *Zea mays* L. plant, **(B)** PSB inoculated *Zea mays* L. plant, **(C)** PSB inoculated *Vigna unguiculata* (L.) Walp, and **(D)** Un-inoculated *Vigna unguiculata* (L.) Walp. Inoculated plants showed improved growth and enhanced photosynthetic activity.

such as gluconic, lactic, and oxalic acids that hydrolyze inorganic P compounds found in the soil (Sharma et al., 2013). **Figure 2** shows the effect of maize (*Zea mays* L.) and cowpea [*Vigna unguiculata* (L.) Walp.] inoculation with P solubilizing bacteria (PSB) under controlled greenhouse conditions. There was an improved growth of maize and cowpea plants inoculated with PSB compared to the un-inoculated controls. Similarly, field studies have reported an enhanced growth, yield and improved nutritional values on crops inoculated with PSBs (Kalayu, 2019; Soumare et al., 2020). The most commonly used P and K solubilizing microorganisms (PSMs) include bacteria such as *Pseudomonas* spp., *Enterobacter* spp., *Burkholderia* spp., and *Bacillus* spp., and fungi such as *Penicillium* spp., *Trichoderma* spp., and *Aspergillus* spp. (Aseri et al., 2009; Sangeeth et al., 2012; Selvi et al., 2017). Although these studies evidently show increase in P solubilization when a single or a combination of bacteria

species are used, the mechanism of action leading to synergism in delivering P to the plants remains unclear.

Arbuscular mycorrhizal fungi (AMF) are also well-known for P mobilization and solubilization and this has led to the development of mycorrhizal inoculants (Tabassum et al., 2017). AMF colonize the roots of nearly 90% of the terrestrial plants and increase the plant root surface area for the absorption of nutrients and water. Primarily, AMF actively participates in P and K mobilization and solubilization and this has been demonstrated both in the greenhouse and in the field with various crops including cereals, legumes, vegetables, fruits and trees (Wu et al., 2005; Njeru et al., 2017; Avio et al., 2018). The most commonly used AMF inoculants include *Funneliformis mosseae*, *Glomus etunicatum*, and *Rhizophagus irregularis* (Giovannini et al., 2020; Musyoka et al., 2020). Iron starvation in the soil causes a specific group of plants and soil microbes to secrete siderophores,

iron specific chelating molecules, that play a vital role in iron transportation and regulating its bioavailability (Novo et al., 2018). Multifunctional biofertilizers containing *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Penicillium chrysogenum*, and *Streptomyces griseus* not only secrete ferric ion-specific chelating biomolecules but also stimulate antagonistic actions against rhizosphere pathogens (Ahmed and Holmström, 2014; Kannahi and Senbagam, 2014).

In Kenya, smallholder farmers utilize Rhizatech® inoculant (Table 1) containing a combination of three AMF species produced and distributed by Dudu Tech Ltd company (Faye et al., 2020). In South Africa, Mycoroot (Pty) Ltd produces a number of Mycoroot® branded AMF inoculants that solubilize P, K, Cu, Zn, and Fe, and improve plant tolerance against abiotic stresses. Organo® biofertilizer produced by Amka Products (Pty) Ltd, South Africa, contains *Bacillus* spp., *Enterobacter* spp., *Pseudomonas* spp., *Stenotomonas* spp., and *Rhizobium* spp (Raimi et al., 2017). These PGPMs secrete siderophores and multifunctional plant growth promoting hormones such as gibberellin, IAA, and cytokinin (Saad et al., 2020). *Pseudomonas fluorescens*, produced by BioControl Products SA (Pty) Ltd as NAT-P®, and *Bacillus subtilis* produced by Ag-Chem Africa SA (Pty) Ltd as B-RUS®, are some of the commonly used multifunctional inoculants available for farmers (Raimi et al., 2017). In addition to P-K-solubilization, they produce siderophores that bind iron ( $\text{Fe}^{3+}$ ) suppressing its availability to phytopathogens, and indole acetic acid (IAA) responsible for stimulating root growth and plant cell elongation (Parani and Saha, 2012).

Despite their novel potential use as agro-inputs, various biotic and abiotic factors may mask the performance of several upcoming commercial bioinoculants in delivering nutrients to the plant (Wahid et al., 2020). Comparative studies on their performance in various smallholder agroecosystems characterized by varying soil typologies should be assessed. Recent studies have suggested that a second generation of multi-trait bioinoculants should be developed based on specific biostimulatory synergism of different PGPMs (Rouphael and Colla, 2020). However, little work has been done to adequately understand the synergistic role of AMF, P-solubilizing bacteria, and siderophore producers and how smallholder farming conditions affect their functional roles. Without scientific experiments to answer these considerations, the economic benefits in the use of bioinoculants in smallholder setups will remain elusive.

## PGPMs in Enhancing the Adaptation of Crops to Abiotic Stresses

Crop production in rain-fed smallholder agroecosystems is mainly limited by various abiotic stresses that interfere with the genetic regulation of key cellular pathways in plants and severely affect the plant's physiological functioning and morphology (Sindhu et al., 2020). High temperatures, water stress, salinity, and floods are some of the important abiotic plant stressors experienced by smallholder farmers in SSA and may cause up to 70% of crop yield losses (Bationo et al., 2012). As the

average global temperatures increase, the risk of widespread desertification heightens and this could traverse across many developing nations, hitting on the majority of the vulnerable smallholder farmers the hardest. The search for new plant breeds that could cope up with the stressors is a long-drawn and costly process considering the unique crop diversity of smallholder agroecosystems. Exploiting the unique environment-tolerant properties of microorganisms, their huge genetic diversity, and interaction with various plants could be crucial in addressing the management of abiotic stress in agriculture (Grover et al., 2011).

Agroforestry is an important practice central to climate change mitigation, soil and water conservation, energy and food sources. There is evidence that farmer-managed agroforestry is responsible for the significant increase in food production, tree diversity, and the greening trends in the Sahel region of Senegal, Niger, and Mali (Elagib and Al-Saidi, 2020). The choice of tree species used in agroforestry depends on various environmental, social, and economic factors. Recently, many development programs in collaboration with the local farmers of the Sahel region have engaged to combat the rising desertification through the use of leguminous trees such as acacia (Sileshi et al., 2020). Legumes such as *Acacia seyal*, *A. senegal*, and *A. albida* have successfully demonstrated their ability to interact with the indigenous AMF and rhizobia species of the Sahel region of West Africa and are considered potential agents for carbon sequestration and land restoration in the region (Fofana et al., 2020). Based on these and other evidence, integration of biofertilization and optimization of agroforestry techniques in the context of an integrated fight against desertification should be considered. The search for potential microbial candidates for ameliorating various abiotic stresses, restoring soil fertility, and enhancing crop productivity should be done in areas vulnerable to the effects of climate change (Goswami and Deka, 2020).

PGPMs exhibiting growth-promoting and stress-tolerant traits such as secretion of volatile organic compounds (VOCs), osmoprotectants (proline, glutamate, trehalose), siderophores, gibberellic and IAA production, P-solubilization, and exopolysaccharides (EPS) (Table 2) could be ideal for use in dryland agroecosystems (Grover et al., 2011; Gouda et al., 2018). For instance, in semiarid regions of India, EPS-producing drought-tolerant strains of *Pseudomonas* spp. with various plant-growth inducing traits, osmoregulation, and antioxidant properties on maize have been identified (Sandhya et al., 2010). EPS cement and stabilizes soil aggregates together creating a biofilm that increases water retention and regulates nutrient and water flow within the plant roots (Grover et al., 2011). In Brazil, an impressive soybean yield enhancement and increased tolerance to water stress have been reported through co-inoculation with *Azospirillum brasilense* and *Bradyrhizobium japonicum* (Hungria et al., 2015). PGPMs such as *Pseudomonas* spp., *Burkholderia* spp., *Funneliformis mosseae*, *Enterobacter* spp., and *Rhizophagus irregularis* stimulate osmolyte regulation mechanisms that control plant cell wall integrity and induce plant tolerance to water and salinity stresses (Agami et al., 2016; Gouda et al., 2018).

High soil salinity, caused by excessive water evaporation and accumulation of chloride salts such as NaCl and  $\text{MgCl}_2$ ,



**TABLE 1** | Selected examples of bio-inoculant products and their roles in smallholder agroecosystems.

Bioinoculant product name	Main PGPMs components (declared or based on previous studies)	Manufacturer /Distributor	Roles declared by the manufacturer or reported through research	References
Bonasol®	A consortium of <i>Azospirillum brasiliense</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas</i> spp., <i>Bacillus subtilis</i> , and <i>Glomus intraradices</i>	Abiosa (Mexico)	Enhance P and K nutrition in tomato, pepper, and chili crops	Jiménez-Gómez et al., 2017
Biofix®	N-fixing bacteria	MEA Ltd (Kenya)	Enhance N fixation and yields of legumes (snow peas, common beans, soybeans)	Odame, 1997; Koskey et al., 2017
Ajay azo/rhizo/azospirillum®	<i>Azotobacter</i> spp., <i>Rhizobium</i> spp., and <i>Azospirillum</i> spp.	Ajay bio-tech (India) limited	N-fixation and P-solubilization in legumes and cereals	Alori and Babalola, 2018; Celador-Lera et al., 2018
Nitrofix®	<i>Azospirillum chroococcum</i>	Agri-Life (India)	Produces phytohormones such as auxins and increases N and P uptake in legumes and vegetables	Wu et al., 2005; Azizoglu, 2019
QuickRoots®	<i>Bacillus amyloliquefaciens</i> and <i>Trichoderma virens</i>	Bayer group-acceleron	Solubilize P, enhance root development, moisture, and NPK uptake in corns	Celador-Lera et al., 2018; Kalayu, 2019; Saad et al., 2020
LegumeFix®	<i>Rhizobium</i> spp.	Legume technology (UK)	N fixation and growth enhancement in various legumes	Masso et al., 2016; Faye et al., 2020
Azotobacterin®	<i>Azospirillum brasiliense</i> B-4485	JSC "Industrial innovations" (Russia)	Increase (up to 20%) in yields of maize, barley, and wheat	Fukami et al., 2018a; Misra et al., 2020
VitaSoil®	A consortium of rhizospheric PGPMs	Symborg (Spain)	Soil nutrient amelioration and growth promotion in cereals and legumes	Celador-Lera et al., 2018; Misra et al., 2020
Pro-soil®	Consortium of <i>Bacillus subtilis</i> , <i>Rhodopseudomonas palustris</i> , and <i>Streptococcus thermophilus</i>	Efficient microbes South Africa	Increase nutrient uptake in grasses and legumes and balances NPK levels in the soil	Iriti et al., 2019
TwinN®	A consortium of N-fixing diazotrophs, P solubilizers and plant growth microbial inducers	Mapleton Agri Biotec Pt Ltd (Australia)	Increase crop growth and productivity, N and P acquisition, auxins secretion and induce disease resistance to plants	Azizoglu, 2019
Rhizatech®	Various strains of AMF <i>Glomus intraradices</i> , <i>G. claroideum</i> , <i>G. etunicatum</i> , and <i>G. mosseae</i>	Dudutech Ltd (Kenya)	Increase the absorption and translocation of P, N, S, Zn, and Cu	Kavoo-Mwangi et al., 2013; Mukhongo et al., 2017
Trichotech®	<i>Trichoderma asperellum</i> strain kd	Dudutech Ltd (Kenya)	Biocontrol of soil borne fungal diseases caused by <i>Fusarium</i> , <i>Pythium</i> and <i>Rhizoctonia</i> in horticultural crops	Preininger et al., 2018
Zander mycorrhiza®	Different strains of AMF	Zander coporation (UK)	Enhance plant nutrition (NPK), growth and health, and induce plant tolerance to water stress in arid zones	Faye et al., 2020
Myco apply®	Various endo-AMF species including <i>Glomus intraradices</i> , <i>G. aggregatum</i> , <i>G. etunicatum</i> , <i>G. mosseae</i> , and ecto-AMF species like <i>Rhizopogon villosulus</i> , <i>R. amylopogon</i> , <i>Scleroderma cepa</i> , among others	Mycorrhizal applications, Inc. (USA)	Increase solubility of P, Zn, Cu, Fe, and Mn and secretion of enzymes and siderophores	Faye et al., 2020
Symbion VAM plus®	<i>Bacillus megaterium</i> var. phosphaticum and two AMF strains <i>Glomus fasciculatum</i> and <i>Gigaspora</i> spp.	T. Stanes and company Ltd (India)	Enhance the absorption of P, water and other micro elements and induce water stress and fungal diseases resistance	Khalid et al., 2017; Mukhongo et al., 2017; Mishra et al., 2019
ECO-T®	<i>Trichoderma asperellum</i>	Plant health products (Pty) Ltd (South Africa)	Biofungicide against <i>Pythium</i> , <i>Fusarium</i> , and <i>phytophthora</i> diseases and promotes the development of healthy root systems	Kavoo-Mwangi et al., 2013
PHC biopak®	<i>Bacillus licheniformis</i> , <i>B. megaterium</i> , <i>Paenibacillus azotofixans</i> , <i>B. subtilis</i> , and <i>B. polymyxa</i>	Plant health care Inc. (USA)	Stimulates NPK uptake and enhance the survival, growth, and productivity of crops	Egamberdiyeva, 2007; Kavoo-Mwangi et al., 2013

(Continued)



TABLE 1 | Continued

Bioinoculant product name	Main PGPMs components (declared or based on previous studies)	Manufacturer /Distributor	Roles declared by the manufacturer or reported through research	References
Mycor®	<i>Glomus intraradices</i>	Iftech (France)	Stimulates root growth and P acquisition and increases plant resistance against climatic stress	Rowe et al., 2007; Kavoo-Mwangi et al., 2013; Mosa et al., 2018
SumaGrow®	A consortium of N-fixers, P-solubilizers, and other plant nutrient-mobilizing microbes suspended in organic humid acid carrier	Bio soil enhancers, Inc. (BSEI), USA	Increase crop yields and nutritional values of food and forage crops, reduce fertilizer dependence, and ameliorates soil pH gradient under extreme environmental conditions	Rivera et al., 2015; Preininger et al., 2018

negatively affect soil microbial biomass, seed germination, and plant development via osmotic potential or ion-specific damage mechanisms (Sindhu et al., 2020). In Uganda, smallholder farmers use Symbion vam plus® biofertilizer produced by T. Stanes and Company Ltd, and contains *Bacillus megaterium*, *Glomus* spp., and *Gigaspora* spp. that improve salinity tolerance and bioavailability of nutrients such as Fe, Cu, Zn, and P (Mukhongo et al., 2016). Gururani et al. (2013) reported increased potato (*Solanum tuberosum*) tuberization, enhanced tolerance to salt, drought and heavy metal stresses upon inoculation with two *Bacillus* spp. that induce changes in the expression of reactive oxygen species (ROS), scavenging enzymes, and proline content. Oxidative stress is commonly associated with drought, salinity and high-temperature conditions and also during plant-pathogen interaction. Fukami et al. (2018a) reported an enhanced induced systemic tolerance (IST) on maize against salinity stress following co-inoculation with *Azospirillum brasilense* and *Rhizobium tropici* that significantly affected antioxidant enzymes and proline content in the leaves.

The area of multi-microbial combinations to equip plants with abiotic stress tolerance is still equivocal and involves many genes of which some have not been identified yet. Thus, further research at the gene regulation level should elucidate the superior performance or lack of additive or synergistic effects that are observed when a combination of certain PGPMs are applied in the field (Ouma et al., 2016; Njeru et al., 2017). Understanding the complex plant-microbe interactions, stress tolerance, response, and adaptation as influenced by the changing soil and environmental factors will be important. Advanced biotechnological tools for identifying the potential microbial candidates with abiotic stress-tolerant properties should be employed and tested for their efficiency under different smallholder agroecosystems.

## Use of Microorganisms in Suppression of Pests and Diseases

Evidence-based concerns against the use of synthetic chemical pesticides are increasingly pushing for the need to develop environmentally friendly pest and disease management strategies. Teratogenic and carcinogenic effects of chemical pesticides have been well-documented (Nicolopoulou-Stamati

et al., 2016; Bonner and Alavanja, 2017). Notwithstanding the injurious effects, the development of synthetic pesticides is a complex process and requires rigorous regulatory approval demands. Besides, they are often costly and beyond the reach of the resource-strained smallholder farmers that contribute immensely to global food security (Mburu et al., 2016; Constantine et al., 2020). In smallholder agroecosystems, pests and diseases cause agricultural losses ranging from 45 to 100% (McDonald and Stukenbrock, 2016), depending on the infestation level. Fall armyworm alone can potentially cause losses of up to 13 billion USD in the smallholding family units of Sub-Saharan Africa (Harrison et al., 2019). Other than significantly limiting yields, most fungal pathogens are well-known producers of mycotoxins that negatively impact human health (Zhou et al., 2018). Development of alternative green technologies in pest and pathogen control is a need of the hour that should be fast-tracked to boost crop production that could feed the growing human population with minimalist disturbance to the already shrunk natural ecosystems. Microorganisms have been widely used as biological control agents (BCAs) for a long time and have been established to antagonize and suppress destructive entomopathogens in several ways (Köhl et al., 2019).

Most bacterial BCAs are of the genus *Bacillus*, with *Bacillus thuringiensis* being the most widely used bacterial biocontrol agent against common fungal pathogens and insects. Its derivatives are found in over 70% of bacterial biopesticides (Melo et al., 2016; Liu et al., 2019). *Agrobacterium*, *Arthrobacter*, *Burkholderia*, *Azotobacter*, *Rhizobium*, *Serratia*, *Thiobacillus*, and *Pseudomonas* are other bacteria genera with antibiotic attributes *in-vitro* and *in-vivo* (Saxena et al., 2000; Raaijmakers et al., 2002). Bacterial biopesticides, like most BCAs, are environmentally friendly and are inexpensive to develop and can be as effective as synthetic pesticides (McDonald and Stukenbrock, 2016; Köhl et al., 2019). *Pseudomonas fluorescens* has been established to be as effective in controlling the root decay agent *Aphanomyces cohlloides* in sugar beet as the commercial fungicides (Kristek et al., 2006). Most fungal BCAs are of the genera *Aspergillus*, *Penicillium*, *Beauveria*, *Metarhizium*, and *Trichoderma* (Abbey et al., 2019; McGuire and Northfield, 2020). *Trichoderma* species have been extensively studied for their antagonism against common soil-borne pathogens such as *Rhizoctonia* and

**TABLE 2 |** Plant growth promoting rhizobacteria (PGPR) and their plant growth promoting traits and mechanisms.

Plant growth promoting rhizobacteria (PGPR)	Plant growth promoting traits and mechanisms	Host/associated plant	References
<i>Achromobacter marplatensis</i> and <i>Achromobacter xylosoxidans</i>	Production of phytohormones IAA and promote vegetative growth and yield	Tomato ( <i>Solanum lycopersicum</i> L.)	Salem, 2016; Abdel-Rahman et al., 2017
<i>Aeromonas veronii</i> and <i>Acetobacter diazotrophicus</i>	Production of phytohormone indole-3-acetic acid (IAA)	Rice ( <i>Oryza sativa</i> L.)	Etesami et al., 2015
<i>Azospirillum brasilense</i>	Promote secretion of nod-gene inducing flavonoids	Common bean ( <i>Phaseolus vulgaris</i> L.)	Coniglio et al., 2019
<i>Azospirillum</i> spp. and <i>Azotobacter</i> spp.	Nutrient uptake	Maize ( <i>Zea mays</i> L.)	Abdel Latef et al., 2020
<i>Azotobacter chroococcum</i>	Enhance resistance against armyworm	Maize ( <i>Zea mays</i> L.)	Song et al., 2020
<i>Bacillus amyloliquefaciens</i>	Induces systemic resistance against tomato leaf curl virus disease	Tomato ( <i>Lycopersicon esculentum</i> L.)	Guo et al., 2019
<i>Bacillus aryabhattai</i>	Production of phytohormones	Soybean ( <i>Glycine max</i> (L.) Merr.)	Park et al., 2017
<i>Bacillus cereus</i>	Biotic stress resistance against bacterial speck disease caused by <i>Pseudomonas syringae</i> pv. tomato	Tomato ( <i>Solanum lycopersicum</i> L.)	Niu et al., 2012
<i>Bacillus pumilus</i> and <i>B. subtilis</i>	Resistance against downy mildew disease	Millet ( <i>Pennisetum glaucum</i> L.)	Kushwaha et al., 2020
<i>Bacillus subtilis</i>	Absorption of K <sup>+</sup>	Tobacco ( <i>Nicotiana tabacum</i> )	Ding et al., 2020
<i>Bacillus thuringiensis</i>	Bio-pesticide	Maize ( <i>Zea mays</i> L.)	Sanchis, 2011; Melo et al., 2016
<i>Bradyrhizobium</i> spp.	Biological nitrogen fixation	Soybean ( <i>Glycine max</i> (L.) Merr.)	Mburu et al., 2020
<i>Enterobacter</i> spp.	Production of phytohormones IAA	Lettuce ( <i>Lactuca sativa</i> L.)	Park et al., 2015
<i>Gluconacetobacter diazotrophicus</i>	Enhance nitrogen fixation and induce tolerance to NaCl	Wheat ( <i>Triticum aestivum</i> L.) and sorghum ( <i>Sorghum bicolor</i> L.)	Velázquez-Hernández et al., 2011
<i>Klebsiella varicola</i> , <i>Enterobacter roggenkampii</i> , and <i>Pseudomonas koreensis</i>	Nitrogen fixation	Sugarcane ( <i>Saccharum officinarum</i> L.)	Wei et al., 2014; Li et al., 2017; Guo et al., 2020
<i>Paenibacillus alvei</i> and <i>Bacillus velezensis</i>	Confer resilience to water stress and crown rot disease caused by <i>Fusarium pseudograminearum</i>	<i>Sorghum bicolor</i> (L.)	Carlson et al., 2020
<i>Pseudomonas fluorescens</i>	Production of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase to confer resilience against salinity	Groundnuts ( <i>Arachis hypogea</i> L.)	Saravanakumar and Samiyappan, 2007
<i>Pseudomonas aeruginosa</i> and <i>P. fluorescens</i>	Cytokinin production	Soybean [ <i>Glycine max</i> (L.) Merr.]	Kumawat et al., 2019
<i>Pseudomonas fluorescens</i>	Promotes early plant development and enhances yield and leaf nutrition	Canola ( <i>Brassica napus</i> L.), tomato ( <i>Solanum lycopersicum</i> L.), and banana ( <i>Musa</i> spp.)	Gamez et al., 2019; Premachandra et al., 2020
<i>Pseudomonas putida</i> , <i>P. fluorescens</i> , <i>Bacillus megaterium</i> and <i>B. polymyxa</i>	Phosphate solubilization	Potato ( <i>Solanum tuberosum</i> ) and legumes	Browne et al., 2009; Dawwam et al., 2013
<i>Pseudomonas</i> spp.	Promote nodulation in mung bean ( <i>Vigna radiata</i> L.)	<i>Vigna radiata</i> (L.)	Kumari et al., 2018
<i>Pseudomonas</i> spp., <i>Burkholderia</i> spp., and <i>Acidithiobacillus</i> spp.	K solubilization	Soybean [ <i>Glycine max</i> (L.) Merr.]	Jaiswal et al., 2016
<i>Rhizobium leguminosarum</i> and <i>Bradyrhizobium japonicum</i>	Amelioration of arsenic toxicity	Legumes	Seraj et al., 2020
<i>Sinorhizobium meliloti</i>	Confer acid tolerance	Alfalfa ( <i>Medicago sativa</i> L.)	Draghi et al., 2017
<i>Streptomyces cellulosae</i>	Confer resistance against tobacco mosaic virus	Tomato ( <i>Solanum lycopersicum</i> L.)	Abo-Zaid et al., 2020
<i>Trichoderma erinaceum</i>	Biocontrol agent, stress resilience inducer, and promotes plant growth	Rice ( <i>Oryza sativa</i> L.)	Swain et al., 2018
<i>Trichoderma harzianum</i>	Enhances production of auxins and biomass production	Cucumber ( <i>Cucumis sativus</i> L.)	Zhang et al., 2013

*Fusarium* (Haldar and Sengupta, 2015; Köhl et al., 2019). Most destructive arthro-pests have also been successfully suppressed in smallholder agroecosystems through the use of *Steinernema* and *Heterorhabditis* nematodes (Arthurs and Heinz, 2006), baculoviruses and protozoa, such as *Nosema* (Sarwar, 2015; Hatting et al., 2019). BCAs act against pests and pathogens in several established ways, which can be direct or indirect. Understanding the modes of action of BCAs are integral in determining their efficacy in field conditions since *in-vitro* antagonism is not often reflected *in-vivo* (Köhl et al., 2019).

Direct mechanisms of antagonism involve parasitism, antibiosis, and predation (Figure 3). Mycoparasitism is the primary mode of action of most strains of *Trichoderma* and *Clonostachys* spp. against fungal pathogens (Abbey et al., 2019). *Bdellovibrio bacteriovorus* is a biocontrol agent unique in its ability to invade and derive nutrients from the cytoplasmic contents of other pathogenic gram-negative bacteria (McNeely et al., 2017). The direct antagonistic mechanisms of microbial BCAs are aided by the agents' ability to secrete cell wall degrading enzymes; chitinases, proteases, cellulases, glucanases, esterases, and catalases (Alori and Babalola, 2018). These hydrolytic enzymes facilitate the penetration of pathogen's cell wall and pest's tissues. *Bacillus thuringiensis*, the prime entomopathogenic bacteria, produces endotoxins that disrupt insect cell structures, inducing osmotic cell lysis that causes significant ion leakage and functional integrity loss (Melo et al., 2016; Azizoglu, 2019). *Steinernema* and *Heterorhabditis* nematodes secrete lytic enzymes that enable them to invade and release bacteria into the insect's haemocoel (Arthurs and Heinz, 2006). The infected insects consequently die of septicemia. *Trichoderma asperellum*, *Trichoderma virens*, *Trichoderma atroviride*, and *Trichoderma harzianum*, are well-known to possess a high level of chitinolytic activity against common soil-borne pathogens such as *Fusarium*, *Aspergillus*, *Rhizoctonia*, and *Puccinia* (Panwar et al., 2014; Abbey et al., 2019). At humidity of at least 60%, BCA *Ampelomyces* germinates its pycnidia on host surfaces and penetrates powdery mildew hyphae resulting in cytoplasm degeneration (Kiss, 2008).

Other than parasitism, most BCAs also suppress pathogens directly through the production of antibiotic compounds that impede the proliferation of the target pathogens. Bassiacridin and beauvericin produced by *Beauveria* have insecticidal property against entomopathogens (McGuire and Northfield, 2020); bioactive lipopeptides produced by *Bacillus subtilis* necrotizes insect epithelial cells causing death (Melo et al., 2016; Liu et al., 2019). Microbial antagonists also suppress pathogens through indirect mechanisms, notably through competition and induced systemic resistance (Heil and Bostock, 2002). Most antagonist microbes possess aggressive colonization ability suppressing the pathogens hindering their establishment through competition. This mode of action is incredibly effective in controlling necrotrophic pathogens that require exogenous nutrients for their establishment (Tewari et al., 2019). *Trichoderma* strains can produce siderophores and out-compete pathogens for iron, which is essential for the pathogens' normal physiology (Ahmed and Holmström, 2014). Some secondary metabolites produced by *Trichoderma* strains are associated with systemic resistance in *Lycopersicon esculentum* against *Leptosphaeria macularis* and

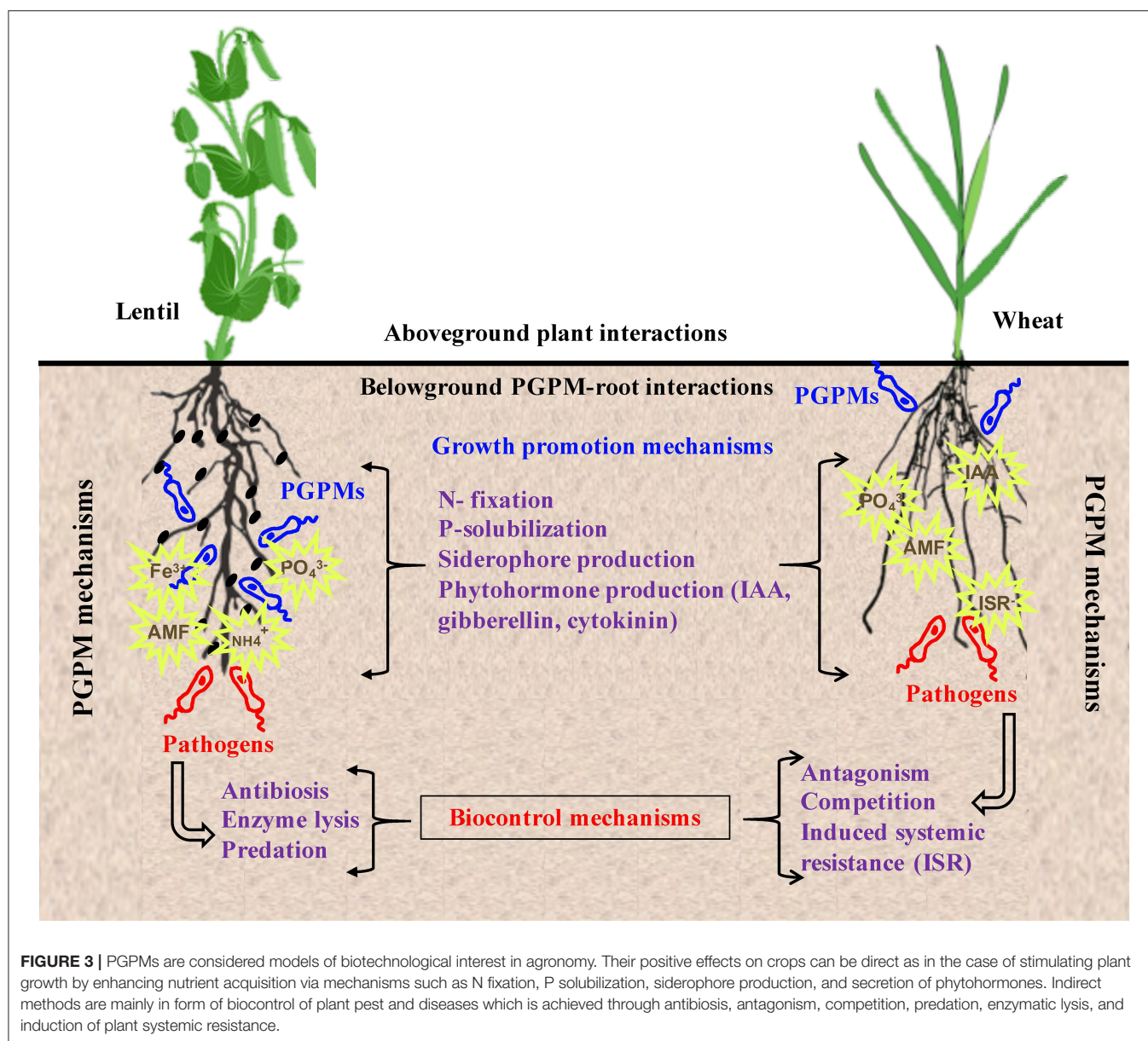
*Botrytis cinerea* (Abbey et al., 2019). It is imperative to note that the antagonistic mechanisms are complex, and microbial BCAs may suppress a pathogen through several mechanisms. Synergistic effects of co-inoculation of certain BCAs have led to higher efficacy in field conditions (Köhl et al., 2019), which can be explored further in smallholder agroecosystems for more significant results.

## MICROBIAL FUNCTIONAL IDENTITY VS. FUNCTIONAL DIVERSITY IN DELIVERING AGROECOSYSTEM SERVICES

Soil microbial communities mediate nearly every biogeochemical process occurring on earth crust controlling the functionality of an ecosystem (Escalas et al., 2019). Their ubiquitous nature, diversity richness, and ability to establish multiple interactions with higher organisms and among themselves, make them the best candidates in delivery of essential agroecosystem services (Brussaard et al., 2007). Understanding the importance of having a specific functional species or group of PGPMs here referred to as "functional identity" or "functional diversity," respectively (definitions adapted from Barberi, 2015) is not well-established in smallholder agroecosystems. This drives the rising research demand for soil biodiversity in the quest for the delivery of beneficial agroecosystem services. In the previous decades, most of the commercial inoculants contained a single microbial species or strain targeting a specific crop genotype (Kaminsky et al., 2019). Economically, this no longer favors the smallholder agroecosystems that are nowadays characterized by a wide range of crop production.

Currently, through research, various microbial species and strain combinations have been produced targeting a broad range of crop species depending on the market requirement and species compatibility. For instance, SumaGrow<sup>®</sup> bioinoculant (Table 1) produced by Bio Soil Enhancers Inc. (USA) contains a consortium of polyfunctional PGPMs comprising of N fixers, P mobilizers and solubilizers, micronutrient mobilizers, growth hormones, and organic humic acid, and enhance growth and yield in a wide range of crops including vegetables, cereals, legumes, trees, and fruits (Preininger et al., 2018). Co-inoculation or mixed inoculation of diverse multifunctional microbial groups as a single inoculant could maximize the chances of strain functional performance (Escalas et al., 2019) in particular the functional biodiversity effect when functional identity effect is suppressed. For instance, a mixture of PGPMs that enhance P solubilization (e.g., *Bacillus* spp.), phytopathogenic biocontrol (e.g., *Pseudomonas* spp.), BNF (e.g., *Rhizobium* spp.), and phytohormone production (e.g., *Azospirillum* spp.) could have synergistic or additive functional biodiversity effect on crops (Hungria et al., 2015; Rashid et al., 2016). However, the performance of co-inoculation is not always the case as its efficiency is affected by several factors including strain compatibility, concentration ratios, inoculation methods, plant genotypes, soil factors, and environmental conditions at the time of application (Kaminsky et al., 2019). Considering all these factors and the changing climatic conditions, the performance of





the current inoculants may not be guaranteed in the near future. Thus, research should be done to deepen the understanding of complex interactions associated with mixing various inoculants to come up with new formulations for use with different crops. Efficient delivery methods of inoculant application in the context of the changing climate should also be investigated.

## PGPMS CONTRIBUTION TO CROP YIELD AND NUTRITIONAL QUALITY

Studies have shown that there are significant benefits of using PGPM inoculants as biofertilizers or biostimulants in crop production (Chavoshi et al., 2018; Aliyu et al., 2019; Santos et al., 2019). A global meta-analysis of biofertilizer efficiency

in enhancing crop nutrients and yields showed an average of 16 % increase in yield of all crops, with legumes showing a superior response to inoculation (Schütz et al., 2018). The beneficial effects of bioinoculation could be more in nutrient-limited soil conditions which reflects the case of SSA smallholder agroecosystems. According to Singh et al. (2017), *Rhizobium* biofertilizer inoculum when applied in semiarid and arid environments can supplement nutrient requirement in legumes and hence improving crop yield. Chavoshi et al. (2018) reported a maximum biomass accumulation of 7,985 kg ha<sup>-1</sup> in red beans and a higher water use efficiency after inoculation with Bio-P and Bio-K fertilizers in limited water resources. In desert areas of the Sahel region, double inoculation of indigenous rhizobia and AMF isolates improved the survival rate and growth of *Vachellia seyal* (acacia) plantations (Fofana et al., 2020). The



authors attribute the improved plant growth to the adaptation of the native bioinoculants to the pedoclimatic conditions of the region and the microbial synergism in delivering plant nutrients under water and heat stress conditions. The fact that PGPM inoculants mitigate water stress in crops and enhance crop health and productivity ensures that there is consistency in yields for smallholder farmers.

The use of effective PGPMs enhances leaf photosynthesis, seed quality traits, and yield of legumes such as *Phaseolus vulgaris* L.) (Iriti et al., 2019). High-quality agricultural products are easily marketable and fetch high prices, therefore, bringing more fortune to smallholder farmers resulting in improved livelihoods. The choice and selection of superior native strains over exotic strains is often encouraged (Aliyu et al., 2019). For instance, the application of native strains of *Bacillus* spp. as PGPM in the cultivation of cumin (*Cuminum cyminum* Linn.) increased the seed oil content and yields compared to uninoculated control (Mishra et al., 2019). Studies have revealed that it is economical for smallholder farmers to apply polyfunctional microbial inoculants with multiple plant growth-promoting traits such as P solubilization, N fixation, and biocontrol compared to the use of single-trait inoculants (Reddy and Saravanan, 2013). However, some studies favor the promotion of functional identity over functional diversity when dealing with specific crop genotypes and environments (Njeru et al., 2017). Further studies to elucidate this disparity should be done in the context of smallholder agroecosystems paying attention to proper management of microbial inoculants as this could significantly affect microbial functioning, abundance, and effectiveness.

The global demand for healthy food and fiber is expected to rise by 2050 to 70% (Singh and Trivedi, 2017). The increase in food demand needs to be satisfied from the existing arable land, which is already under pressure from the rising human population, harsh climatic conditions, and the decline in soil fertility and water availability. In addition, there is a need to safeguard farm produce from emerging and re-emerging pests and diseases. Despite the success of chemically associated conventional farming practices in increasing agricultural productivity, their future reliability is on the balance due to various health concerns arising from food contamination and disease resistance (Alori and Babalola, 2018). Harnessing natural resources such as plant-associated microbiome (PAM) could be one of the most effective strategies to improve agricultural productivity and future food security in a more sustainable way. The PAM technology has a better potential to minimize environmental hazards and increase food quality and quantity while lessening resource inputs compared to the conventional farm practices. Additional plant-microbiome discoveries and technological improvements are emerging and embracing a shift in the paradigm toward next-generation microbial applications could lead to better food production systems (Nezhad et al., 2015). *In situ* plant-microbiome engineering, high throughput sequencing, and plant breeding will be integral to enhancing the understanding and development of efficient microbial inoculants. Evidence shows that more attention has been given to rhizosphere and root microbiota that play a key role in plant productivity (Goswami

and Deka, 2020; Nuzzo et al., 2020; Sousa et al., 2020), while other plant sections such as leaf, stem, seeds, and flowers remains largely unexplored. Yet, they could be playing important roles in plant defense system and response against abiotic and biotic stress (Singh and Trivedi, 2017).

## AGRONOMIC MANAGEMENT PRACTICES AND INTERVENTIONS THAT OPTIMIZE PGPM FUNCTIONALITY

Modern farming encourages the integration of bio-inoculants with other farm management practices and this has been shown to have complementary and synergistic effects in improving growth and yield quality characteristics in apple fruit farming (Mosa et al., 2018). The positive association between agronomic management practices and ecosystem functioning could be exploited to enhance soil fertility amelioration and plant productivity (Costanzo and Barberi, 2014). One possible approach is to increase the above-ground litter and below-ground root traits that host and provide energy to the PGPMs responsible for decomposition and nutrient recycling (Bakker et al., 2012). This approach encourages farmers to utilize agronomic practices that increase genetic, species, and habitat diversity within the field scale hence increasing farm's overall productivity and agroecosystem resilience (Moonen and Barberi, 2008; Mburu et al., 2016). Conserving a high diversity of indigenous microbial functional communities in the soil ensures continuous maintenance of critical soil functions amidst the changing climatic conditions. This could provide production-related insurance to the farmers on ecosystem productivity and stability upon any ecological perturbations (Yachi and Loreau, 1999; Shanafelt et al., 2015).

Multiple cropping systems coupled with rotational practices is demonstrated to sequester more carbon to the soil (Hontoria et al., 2019). Obligate PGPMs, that would not survive without a plant host, utilize carbon as the sole energy source (Ventorino et al., 2012). Therefore, farmers should critically choose cropping systems that favor carbon sequestration in order to conserve the functioning of beneficial obligate microbial communities. However, for maximum benefits to be achieved in multiple cropping systems, choosing complementary plant genotypes known to host multiple beneficial PGPMs would be ideal. For instance, intercropping of cereals with legumes such as lentils, faba bean, and chickpea, which are considered (Faucon et al., 2017; Lazzaro et al., 2018). Cereal-legume intercropping system creates a microclimate that regulates heat stress, moisture, wind stress, weed and pest infestation (Lazzaro et al., 2018). The use of PGPMs can enhance the interspecific plant-microbial interactions in intercropping system (Figure 3). The addition of organic amendments such as vermicompost, manure, and biochar that are rich in PGPMs and nutrients would help to sustain high energy demanding soil processes and promotes microbial decomposition and nutrient recycling (Cobb et al., 2018; Nyamwange et al., 2018; Koskey et al., 2020). Non-chemical weeding and pest control methods promote the build-up of beneficial plant-soil biodiversity which are considered the

main drivers of an ecosystem (Marzaioli et al., 2010). On the contrary, intensive cultivation, a common practice in smallholder agroecosystems, reduces soil biodiversity, organic matter, and increase CN ratio, therefore, reducing the overall microbial functionality (Ventorino et al., 2012). Therefore, a change in cultivation practices to more sustainable agroecological practices in smallholder systems would be inevitable if food security is to be realized.

Plants are considered naturally as selective agents that continuously shape rhizospheric soil microbiome and rhizosphere engineering is slowly emerging as a new research field to potentially address crop production (Haldar and Sengupta, 2015). AMF are important ecosystem promoters and are recognized as key elements in low-input agroecosystems; however, their structural composition, diversity and performance are highly influenced by plant genotype (López-García et al., 2017). Certain plant taxonomic families are known to be poor AMF hosts while others such as legumes are known to be excellent AMF hosts. A few plant taxa are non-host. Currently, plants are selectively bred to produce root exudates that enhance rhizosphere plant-microbiome interactions and possibly promote agroecosystem sustainability and productivity (Bakker et al., 2012). To embrace this strategy and apply in smallholder agroecosystems, it will require farmers no infrastructural changes other than the selection of their preferred crop cultivars or species bred to enhance root exudation. However, more knowledge in this area should be generated to deepen the understanding of their mechanisms, pros and cons, cost-benefit analysis, and their applicability to resource-strained smallholder farmers. Enhancing farmers' knowledge of agroecosystem functionality would optimize soil fertility restoration successes, agroecosystem sustainability, and crop productivity.

## DEVELOPMENT OF MICROBIAL INOCULA FOR SMALLHOLDER FARMERS

Currently, there is a rising global market demand for microbial inoculants that can be used as biofertilizers or biostimulants in crop production (Lobo et al., 2019). Bioinoculants contain one or more PGPMs (bacteria, algae, or fungi) packaged in a carrier material. A carrier material refers to the delivery "vehicle" that is packaged to transfer the bioinoculum to the plant rhizosphere. It determines the form (either liquid or solid), shelf life, and the application or delivery methods of the microbial inoculant (Reddy and Saravanan, 2013). According to Soumare et al. (2020), a good microbial inoculum should be packaged in a carrier material that provides an optimum microenvironment (pH, water, and carbon content) for microorganisms, maintain longer shelf life and microbial viability without the need for a special storage facility. Besides, the carrier material should be cost-effective, readily available, eco-friendly, acquiescent to nutrient supplement, and not harmful to the user (Alori and Babalola, 2018). It is interesting to note that very few studies have focused on the selection and development of carrier material and their effect on bio-inoculum as most studies emphasize on the performance of microbial strains (Herrmann

and Lesueur, 2013). Encapsulation of bioinoculants is a newly emerging technique that utilizes polymer beads to enclose one or more microbial species. This technique allows the incorporation of other organic bio-effectors such as humic acid and strigolactones, protects the microbial life from desiccation, and allows slow release of the components into the soil (Gouda et al., 2018). Despite its biotechnological potentials as bioinoculant carriers, nano- and micro-encapsulation methods have not been exploited commercially particularly by entities targeting smallholder systems (Herrmann and Lesueur, 2013). Therefore, with advancing technologies, more studies that could lead to the development of versatile carrier inoculants suitable for use in smallholder agroecosystems should be done.

The fact that the majority of microbial inoculants in SSA are imported (Babalola and Glick, 2012) raises a question if they are tailored to match the varying smallholder farmers' agroecosystems, shelf-life needs, local storage, and soil conditions. A short inoculant shelf-life constraints inoculant supply chain and significantly reduces inoculant reliability, viability, and availability (Deaker et al., 2012). Inoculant viability determines the success of its use and continuous adoption by the farmers, who are in most cases production-oriented rather than agroecology conservationists. Therefore, effective bioinoculants should be able to competitively and successfully establish themselves in the soil within the shortest time possible amidst the presence of already established native microbes. Various studies have tried to unveil the fate of microbial inoculum and their effect on the native microbial communities upon their introduction into the soil (Sharma et al., 2012). For instance, Nuzzo et al. (2020) demonstrated that some PGPM formulations have no impact on plant growth but significantly affect the diversity and structure of native microbial communities. On the contrary, PGPM inoculation improved plant growth but had no influence on species diversity and richness of native microbes in the host plant roots (Piromyou et al., 2011). These inconsistencies are likely to be common in smallholder agroecosystems and, therefore, calls for selection and development of microbial strains that interact well with native microbial communities.

Soil conditions such as pH, presence of organic matter, water availability, and other physicochemical properties affect the infectivity of microbial inoculants (Njeru et al., 2020; Saad et al., 2020). Some PGPM inoculants can confer resilience against such harsh local environmental conditions (Agami et al., 2016). This explains why microbial inoculants are recommended even in soils with less water and where most nutrients are immobile (Sindhu et al., 2020). In some instances, seed companies and researchers have solved this challenge by producing crop cultivars that customarily favor colonization of specific PGPMs under a wide range of soil conditions (Faye et al., 2020). Through this approach, Gitonga et al. (2021) investigated how organic and conventional smallholder farming systems and soybean cultivars (promiscuous vs. non-promiscuous) affect native *Bradyrhizobium* spp. diversity. Similarly, Sinong et al. (2021) found P-solubilizing microbial isolates that could be exploited to enhance the growth of two rice cultivars under low-input cultivation than conventional practices. The authors aimed at optimizing smallholder agroecosystem at the farm level

that potentially harbor diverse soil microbiota that enhances crop productivity.

In other cases, seed manufacturers have partnered with farmers to establish ‘custom seed inoculation’ where on farmer’s request, seeds are inoculated with specific PGPMs by the manufacturer prior to packaging and delivery to the farmers for planting (Deaker et al., 2012). These two approaches can be easily replicated in smallholder agroecosystems, but more research and partnerships on bioprospecting for effective PGPMs compatible with various local crop cultivars should be initiated. Researchers should keep in mind the need for new PGPM inoculants adapted to the current and incoming stressful climatic conditions as the future performance of the inoculants currently in the market may not be guaranteed.

## CHALLENGES OF WIDESPREAD UTILIZATION OF PGPMs IN SMALLHOLDER AGROECOSYSTEMS

Notwithstanding their importance in upscaling agriculture, the use of PGPM inoculants in smallholder settings is largely unaccountable (Oruru and Njeru, 2016) and more research should focus on quantifying their use, adoption, and their overall effect on soil, crops, and farmers’ livelihood. Farmers are used to the application of ‘blanket’ solutions in solving their day-to-day field challenges and the adoption of particular techniques providing specific solutions in certain locations should be encouraged. Industrially, there is a challenge in the production of bioinoculants that could be used for a broad range of crops grown in geographically and climatically diverse territories (Santos et al., 2019). PGPMs, unlike the broad-spectrum agrochemicals, are highly selective in their use and only target specific plant hosts. Their viability is short and the cost of maintaining PGPMs during storage is very high especially in rural setups where electricity and modern storage facilities are limiting resources (Tabassum et al., 2017). Thus, the search for innovative microbial solutions based on farmers’ needs should be done with geographical considerations, increasing episodes of climatic and environmental stresses.

The risk of toxicity arising from inoculant contamination is high if proper quality control standards and storage measures are not taken into consideration. Raimi et al. (2019) reported 67% of the South African biofertilizers, analyzed through sequencing, showed high levels of toxins and contaminants affecting the quality of the inoculants, therefore, jeopardizing their potential benefits. The intentional movement of bioinoculants containing various exotic microbial species or strains to new agroecosystems is growing, but the possible negative ecological consequence of their introduction is poorly understood (Schwartz et al., 2006). This may lead to unintended negative invasion and the establishment of microbes in new agroecosystems. Non-sterile and contaminated inoculum can result in the emergence of phytopathogens that are parasitic to the native PGPMs and plants and can potentially cause significant crop losses. Controlling the cases of invasive species would be costly to the farmers

and thus, the need for inoculation particularly with imported inoculants should first be carefully evaluated and where possible, the use of high-quality local indigenous species should be recommended. In some of the African countries where economic and technological policies have been put in place to support the use of biofertilizers, there is hardly any evidence of the successful implementation of the approaches. This failure is linked to financial misappropriation, policy management and lack of investment interest among the stakeholders (Abdullah and Samah, 2013). Additionally, unreliable climatic conditions, variation in soil factors and poor agronomic management practices need to be addressed especially in the SSA region where the impact of bio-inoculation would be profound if fully adopted (Ngetich et al., 2012).

## FUTURE PERSPECTIVES

The increasing demand for safe food and better nutrition, advancing research technologies, and interest in sustainable agriculture has further renewed global interest on PGPM bioinoculants. For instance, by 2019, China registered more than 800 inoculant related patents while India surpassed 100 patents (Santos et al., 2019). Therefore, it is expected that, through advanced research innovations, more bioinoculants will be produced in the following years. The effects of climate change present a major challenge to industrial bioinoculant producers, and research on PGPMs that are more effective under a broader range of stressful conditions and induce plant tolerance would increase. The challenges on microbial shelf life, storage and viability losses should be addressed and new technologies of seed coating that deliver stable formulations able to withstand harsh storage conditions should be developed (Bargaz et al., 2018). Plant breeders, seed producers, and farmers could overcome these challenges through the initiation of “tailor-made” products that could address specific challenges. However, further scientific research and economic benefit analysis in this area should be done.

More knowledge and deeper understanding are needed on how agronomic practices under changing climatic conditions affect the composition, abundance, and bio-functionality of PGPMs in delivering multiple agroecosystem services. Farmers need to know how PGPM communities are managed at spatial and temporal scale to promote synergies, effectiveness, and avoid trade-offs. Fostering proximity to smallholder farmers in redesigning agroecosystems and policy making should be prioritized. This can be achieved by involving them in-field research demonstrations, data collection, reporting, and policy recommendation drafting (IFAD and UNEP, 2013). These approaches will enhance farmers’ knowledge and technological capacity in the use of bio-inoculants, therefore, ensuring a continuous adoption of techniques that take into account their local ecological conditions and knowledge.

## CONCLUSION

The use of PGPMs as biostimulants, biofertilizers, or biocontrol agents by smallholder farmers has substantially grown owing to their impressive performance, economic benefits, and environmental safety associated with their use. They provide beneficial agroecosystem services such as soil nutrient amelioration, crop nutrient, and yield improvement, plant tolerance to biotic and abiotic stresses, biocontrol of pests and diseases, and water uptake. The adoption of PGPMs in smallholder agroecosystems is on the rise with the increasing number of patents and new inoculants being observed in developing countries. Multisectoral research on the use of PGPMs involving smallholder farmers is encouraged and its output should capture the aims of both the “productionist” and “agroecologist” paradigms. More knowledge on the effects of climate change and agronomic practices on the bio-functionality of PGPMs in delivering multiple agroecosystem services should be generated. Robust technologies are needed to enhance the PGPM production and most importantly to improve the effectiveness, stability, and reliability of the products under environmentally stressing conditions. Putting into consideration the collective experience, needs, and indigenous knowledge of smallholder farmers, PGPM inoculation could be a key pillar in addressing SDG 2 goal of ending hunger, promoting food security, and environmental sustainability.

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## AUTHOR CONTRIBUTIONS

GK drafted the outline structure and prepared the manuscript with contributions from SM and RA. EN and JM provided technical guidelines and reviews during manuscript preparation. All authors approved the final draft of the manuscript for submission.

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# Biological Nitrogen Fixation and Denitrification in Rhizosphere of Potato Plants in Response to the Fertilization and Inoculation

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The study aim was to evaluate the potential nitrogen fixation and denitrification in the rhizosphere soil of potato plants, crop yield and output quality in response to the different fertilization systems and the inoculation with *Azospirillum brasilense* 410. Field stationary experiment was conducted between 2016 and 2019 with potato in a crop rotation system on leached chernozem soil. Farmyard manure, 40 t/ha, applied prior to potatoes planting promotes nitrogen fixation (0.8–2.0 times compared to control). However, it has also affected denitrification (in 1.4–2.2 times higher compared to control). The lowest rate of mineral fertilizers used in the experiment, N<sub>40</sub>P<sub>40</sub>K<sub>40</sub>, was shown as most environmentally feasible. Under its use the increase of soil nitrogenase activity and low denitrification levels were observed. Same trends were also noted for the medium fertilizer rate, N<sub>80</sub>P<sub>80</sub>K<sub>80</sub>. The highest doses of mineral fertilizers, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, substantially affected the denitrification process and reduced the nitrogen fixation activity (in 1.9–2.2 times). The combination of manure with the medium fertilizers rate has also resulted in high denitrification levels, while the soil nitrogen fixation activity has restored only at flowering stage. Crop inoculation with *A. brasilense* combined with the manure application, has not affected studied processes. However, crop inoculation after the green manure intercropping has shown the growth of nitrogenase activity. Used on the mineral fertilizers background inoculation has activated nitrogen fixation and has ensured the decrease of denitrification levels, subject to the fertilization background. High fertilizer rates have hampered the inoculation efficiency. Inoculation has promoted crop yields on unfertilized and mineral backgrounds or following green manure. Crop inoculation following organic and the organo-mineral backgrounds had no significant effect, probably due to the competition for *A. brasilense* from microorganisms that have created a competitive environment for *A. brasilense*. Despite its environmental expediency, inoculation combined with the low fertilizer doses underperforms the action

of inoculation combined with the medium fertilizer rates showing the latter as the compromise between the environmental requirements and crop productivity. The use of inoculation has promoted the accumulation of starch and ascorbic acid and has contributed to the reduction of nitrate contents in the tubers of inoculated plants.

**Keywords:** nitrogen fixation, denitrification, inoculation, fertilizers, potato, starch, nitrates, ascorbic acid

## INTRODUCTION

Traditional crop fertilization systems do not take into account rising environmental requirements and concerns. The major attention is brought to the determination of the mineral fertilizer rates based on the nutrient removal values and planned harvest indicators while leaving aside the variability of the utilization rates of active ingredients from fertilizers. However, the later, according to various scientific data, should always be considered as the degree of assimilation of the active substance from the fertilizers remains low for crop macrolelements, with the nitrogen uptake being within 35–50%, phosphorus – not exceeding 20%, and potassium staying within 25–60% range, subject to the soil type, fertilizer origin, and used crop production system (Korenkov, 1990; Tilman, 1998). Applied mineral fertilizers cannot be assimilated by crops in full, resulting in the application of higher rates to achieve the planned productivity. At the same time, despite the availability of different practices for coherent crop fertilization systems (Juchenko, 1990; Johnston and Bruulsema, 2014; Sposari and Flis, 2017), they are rarely followed in practice. That results in significant environmental pollution issues related to fertilizer residuals, including mineral nitrogen among the largest contaminant of croplands (Luis et al., 2014; Khan et al., 2018), as more than 50% of the total amount of applied nitrogen can be lost from agricultural systems (Vitousek et al., 1997; Tilman, 1998). In addition to the nitrate pollution, nitrogen fertilizers are the main source of N<sub>2</sub>O emissions from agriculture, accounting for 60–80% of emissions globally (Dalal et al., 2003; Signor et al., 2013; Mazzetto et al., 2016; Millar et al., 2018).

Nitrogen fertilizers, in theory, should only be used within the range of their physiological and ecological feasibility to minimize the risk of environmental pollution with nitrogen compounds. However, most fertilization systems account only for the agrochemical and economic indicators for the calculation of nitrogen fertilization doses. The ability to include environmental indices in the design of crop fertilization systems appeared with the development of biological testing methods, in particular using indicators of functional activity of nitrogen-fixing bacteria associated with the roots of cultivated plants (Umarov et al., 1985; Ladha et al., 1986; Volkogon, 2013).

Nitrogen fixing microorganisms fix nitrogen from the atmosphere only in the absence of the excess amounts of mineral nitrogen compounds in the environment (including the soil) (Shah et al., 1972; Lvov, 1989). Knowing the dynamics of nitrogenase activity in the root zone of plants grown on different fertilization backgrounds (from deficit to excess nitrogen in the soil) the physiologically justified from crop standpoint doses

of mineral nitrogen that will not reduce the nitrogen fixation activity can be determined (Ladha et al., 1986).

Under the excess nitrogen fertilizers conditions diazotrophs cease atmospheric nitrogen fixation and switch to available mineral nitrogen compounds, as they are more energy-efficient for the bacterial cell. For this reason, the assimilation of mineral nitrogen used for constructive metabolism in bacteria is accompanied with the denitrification in soil (Eskeu et al., 1977; Bothe et al., 1981). Consequently, the increase of the denitrification activity in the rhizosphere under the influence of mineral nitrogen fertilizers will also indicate on an excess of nitrogen compounds for the crops. Understanding the activity of N<sub>2</sub>O emission in the rhizosphere soils of croplands in response to different doses of mineral nitrogen will allow the selection of the most appropriate amount of mineral nitrogen, accounting for the minimal losses of gaseous nitrogen compounds from the soil compared to the unfertilized control. Based on the comparative analysis of two processes – nitrogen fixation and denitrification in croplands, the physiologically appropriate doses of mineral nitrogen can be selected (Volkogon, 2013).

Cavigelli and Robertson (2000) have stated that due to the high importance of soil microbiota in the nitrogen cycle, changes in its composition and number can change the rate of nitrogen transformation in the soil. Therefore, changes in groups of soil microorganisms caused by chemical compounds or seeds inoculation with specific microorganisms can potentially alter nitrogen transformation processes in the soil. Such microorganisms capable to stimulate the processes of transformation of nitrogen compounds in croplands are called Plant Growth Promoting Bacteria (PGPB) (Kloepper and Schroth, 1978; Bashan and Holguin, 1998). Among these bacteria, representatives of the *Azospirillum* genus have been studied in great detail. The stimulating effect of *Azospirillum* sp. on plant growth and development is explained by several mechanisms, including the synthesis of plant hormones and other biologically active substances, biological nitrogen fixation, and enhancement of mineral compounds absorption by plants (Bashan and Levany, 1990; Okon and Itzigsohn, 1995; Kennedy et al., 1997; Ruppel and Merbach, 1997; Saubidet and Barneix, 1998; Mirza et al., 2000; Rodrigues et al., 2008; Bashan et al., 2014; O'Callaghan, 2016; Zeffa et al., 2019).

It is rational to assume that *Azospirillum* sp., like other PGPBs promote the assimilation of nitrogen compounds by inoculated plants and establish conditions for the reduction of the chemical fertilizer rates and N<sub>2</sub>O emissions from the soil through the more efficient use and application of lower rates of mineral fertilizers. That was confirmed by various studies showing a significant increase in crop production efficiency in response to inoculation

(Freitas and Germida, 1990; Okon and Itzigsohn, 1995; Bashan and Holguin, 1998; Shaharooma et al., 2008; Martins et al., 2017).

The present study focuses on the characteristics of nitrogen fixation and biological denitrification (as the specific biological testing criteria) in crops rhizosphere under the use of different fertilization systems and microbial inoculation, as well as on the influence of the studied processes on the potato yield and output quality.

## MATERIALS AND METHODS

### Experimental Design

The research was conducted during 2016–2019 in a field stationary experiment of the Institute of Agricultural Microbiology and Agroindustrial Production of the National Academy of Agrarian Sciences of Ukraine (established in 2009) on leached chernozem soil [pH salt – 5.2; soil organic matter – 3.01%; easily hydrolyzed nitrogen – 109 mg/kg; labile forms of phosphates (as  $P_2O_5$ ) – 168 mg/kg; exchangeable potassium (as  $K_2O$ ) – 58 mg/kg of soil].

The potatoes of the Bellarosa variety were grown in crop rotation: potatoes – spring barley – peas – winter wheat under the seven different fertilization systems: farmyard manure, three different doses of complex mineral fertilizer, combination of farmyard manure and the mineral fertilizers, green manure and unfertilized control (see **Table 1** for detailed test variants description), each studied into blocks with and without crop inoculation.

The experimental design was the same across the years of studies, with the only shifting factor of plots' distribution following the crop rotation scheme (potatoes always planted after winter wheat). The test plots ( $7.2 \times 12.0$  m each) were randomly spread across the experimental field in 4-fold repetition following the described crop rotation, accounting total of 56 test plots.

### Crop Inoculation

*Crop Inoculation* was performed with the microbial preparation Biogran (Technical Specifications of Ukraine 24.1-00497360-006:2009, State registration certificate A 05575), created based on the active nitrogen fixing bacterium *Azospirillum brasilense* 410 [deposited in the collection of microorganisms of the All-Russian Institute of Agricultural Microbiology (ARIAM) under the number VNIISHM B-36, and in the Depository of the Institute of Microbiology and Virology of the National Academy of Sciences of Ukraine, under the number IMV B-7222] (Volkogon and Dimova, 2004). Potato tubers were manually treated prior to planting with the aqueous suspension of the microbial preparation at the rate of 2 L per 4 tons of potatoes ( $1.5 \times 10^9$  of bacterial cells in 1 ml of suspension).

### Soil Sampling

Rhizosphere soil samples for analysis were taken from the crop roots within 0.3 cm range around the roots of sampled plants after shuddering. The samples obtained from all plant roots in a specific test plot were mixed and used for preparation of average sample.

### Potential Nitrogenase Activity

To study the differences in the potential nitrogenase activity in the rhizosphere soil of potato plants in response to the action of fertilizers and inoculation the acetylene method developed by Hardy et al. (1968) in the modification of Umarov (1976) was used. For this purpose, the average sample of the rhizosphere soil without crop residues was taken and used for subsequent analyses. Soil samples weighing 10 g were placed in 40 cm<sup>3</sup> glass vessels. 1 cm<sup>3</sup> of 20% D-glucose solution was added to the soil samples. The soil moisture level was determined separately and adjusted with sterile distilled water to achieve 70% of the full soil moisture-holding capacity. All samples were mixed thoroughly, closed with cotton plugs and kept for 72 h in a dark room with controlled temperature regulated with a thermostat at 26°C. After the incubation the cotton plugs were replaced with rubber ones. Acetylene was injected into each vessel in the amount of 10% from the gaseous phase volume (3 cm<sup>3</sup>). After 1-h exposure of the samples with acetylene, gas samples were taken with a syringe and analyzed on a gas chromatograph with a flame ionization detector [3 m steel column filled with Parapak Q 60-80 mesh sorbent (Waters Corporation, USA), thermostat temperature 40°C, gas flow: hydrogen – 15 cm<sup>3</sup>/min, nitrogen – 100 cm<sup>3</sup>/min, air – 500 cm<sup>3</sup>/min]. All measurements were performed in 5-fold repetition.

Potential nitrogenase activity (PNA) in rhizosphere soil, expressed in moles of C<sub>2</sub>H<sub>4</sub> per one gram of soil per hour was calculated using the next formula:

$$PNA = E \times V_1 \times \frac{K}{V_2} \times m \times t \quad (1)$$

*E*, amount of ethylene in analyzed gaseous sample; *V*<sub>1</sub>, volume of gaseous phase in vessel, cm<sup>3</sup>; *K*, soil moisture coefficient; *V*<sub>2</sub>, analyzed sample volume injected in chromatograph, cm<sup>3</sup>; *m*, soil sample weight, g; *t*, acetylene exposure time, hours.

### Potential Denitrification Activity

Taking into account that biological denitrification with the formation of N<sub>2</sub> as the final product is observed only in a limited number of microorganisms, so-called true denitrifiers, and naturally does not always end with the formation of N<sub>2</sub> (Conrad, 1996) but is often suspended at the stage of N<sub>2</sub>O formation the acetylene blockage method developed by Zviagincev (1991) was used to determine the potential denitrification activity in rhizosphere soil of potato plants in response to the action of fertilizers and inoculation. This method is based on the ability of acetylene to inhibit the nitric oxide reductase and suspend the process of NO<sub>3</sub><sup>−</sup> and NO<sub>2</sub><sup>−</sup> dissimilation at the stage of nitric oxide reduction, thus allowing the determination of the N<sub>2</sub>O emissions from denitrification (N<sub>2</sub>OD) and assess the activity of all denitrifying microorganisms in soils, the ones that reduce nitrogen compounds to N<sub>2</sub>O and those that perform complete denitrification – to N<sub>2</sub>. To determine the potential denitrification activity, samples of rhizosphere soil, weighing 10.0 g, were prepared and placed in 40 cm<sup>3</sup> vessels. 1 cm<sup>3</sup> of 20% D-glucose solution and 1 cm<sup>3</sup> of 2% KNO<sub>3</sub> solution were added to the samples. Soil moisture was adjusted with sterile



**TABLE 1** | The description of the fertilization systems used in the experiment.

Fertilization system name	Description
Organic No. 1	40 t/ha of farmyard manure (recommendation based on the manure nutrient content: N – 5 kg/t, P <sub>2</sub> O <sub>5</sub> – 25 kg/t, K <sub>2</sub> O – 6 kg/t) applied in late November prior to plowing
Mineral low	Complex mineral fertilizer N <sub>40</sub> P <sub>40</sub> K <sub>40</sub> (250 kg/ha), applied prior to pre-planting cultivation (in form of N16-P16-K16), P as P <sub>2</sub> O <sub>5</sub> , K as K <sub>2</sub> O
Mineral medium	Complex mineral fertilizer N <sub>80</sub> P <sub>80</sub> K <sub>80</sub> (500 kg/ha), applied prior to pre-planting cultivation (in form of N16-P16-K16), P as P <sub>2</sub> O <sub>5</sub> , K as K <sub>2</sub> O
Mineral high	Complex mineral fertilizer N <sub>120</sub> P <sub>120</sub> K <sub>120</sub> (750 kg/ha), applied prior to pre-planting cultivation (in form of N16-P16-K16), P as P <sub>2</sub> O <sub>5</sub> , K as K <sub>2</sub> O
Organo-mineral	Combination of 40 t/ha of farmyard manure and medium rate of complex mineral fertilizer (N <sub>80</sub> P <sub>80</sub> K <sub>80</sub> ) in form of N16-P16-K16 (P as P <sub>2</sub> O <sub>5</sub> , K as K <sub>2</sub> O)
Organic No. 2	Green manure (narrow leaf lupine grown as intermediate crop after winter wheat), disked and soil incorporated with shallow plowing in fall (late November), approximate annual production accounts to 13 t/ha

water to 70% of the full soil moisture-holding capacity. To create anaerobic conditions vessels were closed with rubber stoppers and filled under the pressure with helium to displace the air. The rubber stopper was punctured with two injection needles, one of which was attached to the helium source, while the other was used to release gases and balance the pressure in vessels. Vessels were filled with helium for 30 s, after which the needles were simultaneously removed. 3 cm<sup>3</sup> of the gas mixture was taken from each vessel followed by the injection of 3 cm<sup>3</sup> of the acetylene (to maintain the normal partial pressure of gases in the vessels). Prepared soil samples in vessels were kept in a dark room with controlled temperature regulated with a thermostat at 26°C for 24 h. At the end of the exposure period, gas samples were taken with an injection syringe and analyzed on a gas chromatograph with an electron capture detector [columns temperature – 40°C, evaporator temperature – 120°C, detector temperature – 330°C. Carrier gas (argon with methane 95/5) consumption rate – 35 cm<sup>3</sup>/min, 3 m steel column filled with Parapak Q 60-80 mesh sorbent (Waters Corporation, USA)]. All measurements were performed in 5-fold repetition.

Potential denitrification activity (N<sub>2</sub>OD) in rhizosphere soil, expressed in nmoles N<sub>2</sub>O per gram of soil per day was calculated using the next formula:

$$N_2OD = E \times V_1 \times \frac{K}{V_2} \times m \times t \quad (2)$$

*E*, amount of N<sub>2</sub>O in analyzed gaseous sample; *V*<sub>1</sub>, volume of gaseous phase in vessel, cm<sup>3</sup>; *K*, soil moisture coefficient; *V*<sub>2</sub>, analyzed sample volume injected in chromatograph, cm<sup>3</sup>; *m*, soil sample weight, g; *t*, acetylene exposure time, days.

## Quantitative Analysis of Microorganisms

To estimate the number of ammonifiers in the rhizosphere soil of potato plants the dilution culture of soil suspensions on meat-peptone agar plates was used (Gerhardt, 1981). The number of nitrogen fixation microorganisms was determined using a semi-liquid medium with malate (Dobereiner and Baldani, 1979) and acetylene test (Villemin et al., 1974). The number of denitrifiers was determined on Giltay liquid medium with Gris reagent (nitrite test) (Zviaginicev, 1991).

The final numbers of nitrogen fixing bacteria and denitrifiers were calculated using the McCrady table based on the growth of the microorganisms in the extreme dilutions (Gerhardt, 1981).

Taking into the account that the concentration of mineral nitrogen compounds in the soil is subject to change during the growing season influencing, respectively, the interrelation of the studied processes all experiments were conducted in dynamics. For this all soil samples and the analysis of the potential nitrogenase activity, the denitrification activity and microorganisms' counts were performed during three main crop growth stages: budding (BBCH 51), flowering (BBCH 61) and crop senescence (BBCH 91).

## Crop Productivity

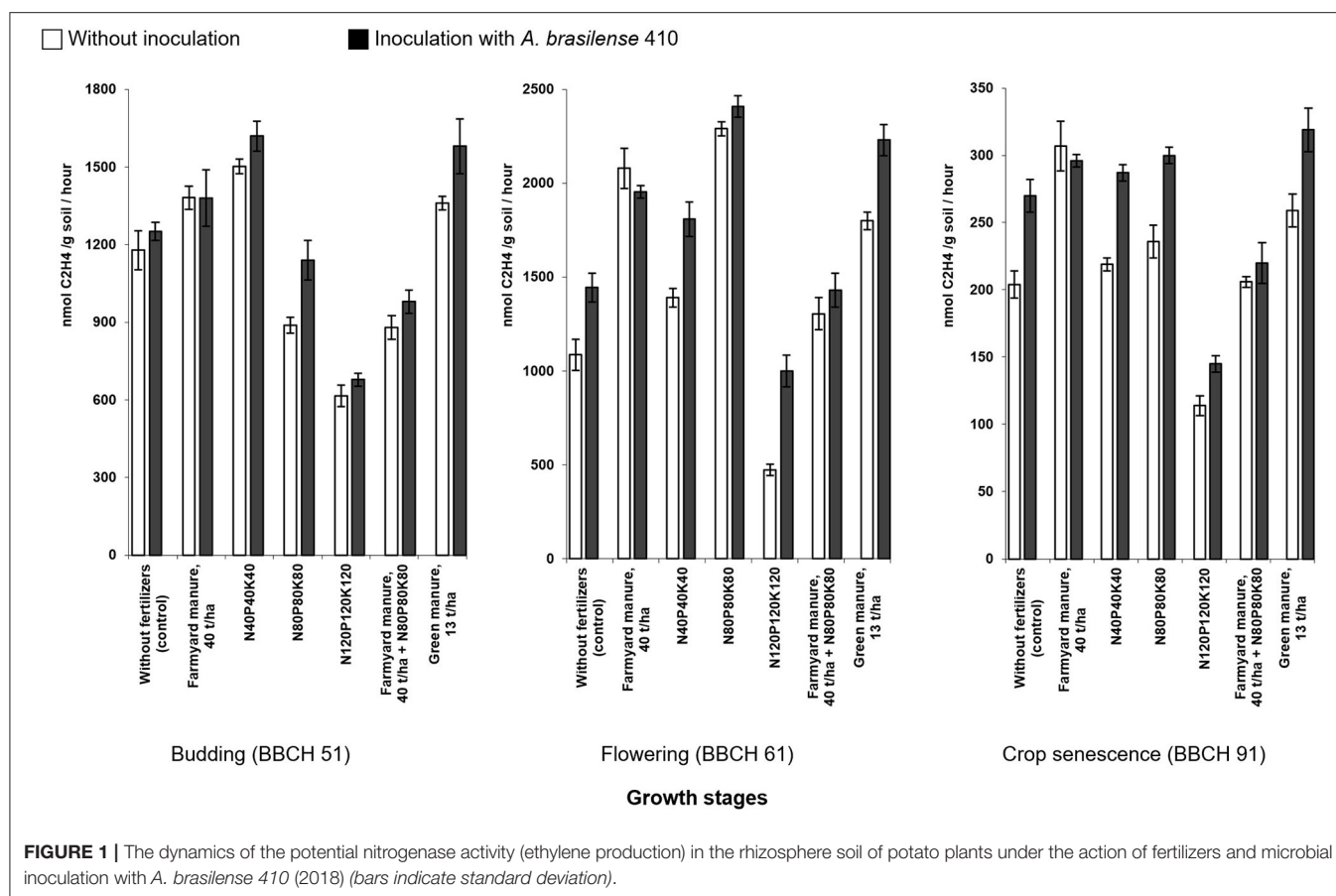
Potato yield was determined by weighing all tubers harvested from the single test plot for the experimental seasons of 2016–2019.

## Product Quality Analysis

The starch content in tubers was determined using the Evers method (Ermakov, 1972), the ascorbic acid – using the method based on the reduction properties of the vitamin C (Ermakov, 1972), the nitrates content – using the potentiometric assay (Gorodniy, 2005).

## Statistical Analysis

The differences between the variants in the potential activity of nitrogen fixation (potential nitrogenase activity), the potential denitrification activity, product quality indices were analyzed using the arithmetic mean and standard deviation values calculated with MS Excel build-in Analysis ToolPak. The dynamics of nitrogen fixing microorganisms and denitrifying bacteria in the rhizosphere soil of potato plants were statistically processed using the McCrady tables. Crop yields were analyzed using the two-way ANOVA algorithm with two probability levels (<5 and <1%) using Statistica 6.0 software (StatSoft Inc., USA). The least significant difference was calculated for (a) the whole experiment, (b) only for different



fertilizer type), (c) only for inoculation with *A. brasilense*) and their interaction.

## RESULTS

Taking into the account that the course of the nitrogenase and denitrification activity in the rhizosphere of potato plants was uniform throughout the research years (2016–2019) the data discussed further represents the results obtained in 2018.

The dynamics of the potential activity of nitrogen fixation in the rhizosphere soil of potato plants indicates its stable increase in response to farmyard manure application. High levels of nitrogenase activity were also observed in the variants with green manure, while the use of organo-mineral fertilizer system has reduced the potential nitrogen fixation activity for a prolonged time, with the slight performance increase starting from the flowering stage (BBCH 61) and beyond (Figure 1).

The lowest studied doses of mineral fertilizers, N<sub>40</sub>P<sub>40</sub>K<sub>40</sub>, have stimulated nitrogen fixation activity, especially at the beginning of the growing season. The intensification of the mineral nutrition doses, N<sub>80</sub>P<sub>80</sub>K<sub>80</sub>, has reduced the potential nitrogen fixation at initial growth stages but gained higher levels later – at the flowering stage (BBCH 61) and the beginning of crop senescence (BBCH 91). The use of the highest rates of mineral fertilizers, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, leads to the

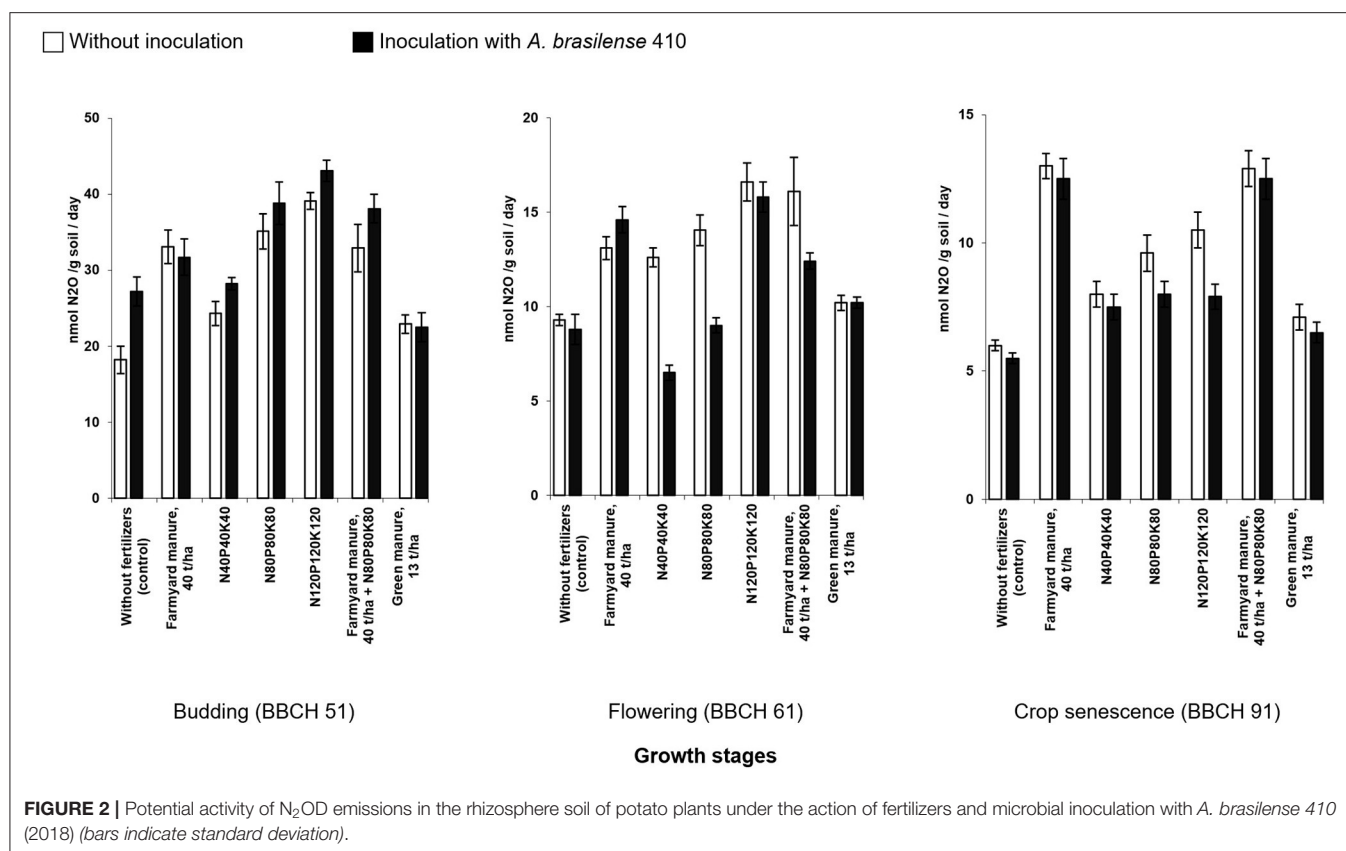
reduction of nitrogen fixation throughout the growing season (Figure 1).

Crops inoculation has stimulated the nitrogenase activity in the rhizosphere soil in the variants with low and medium rates of mineral fertilizers. The combination of inoculation with the highest studied rate of mineral fertilizers, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, has also stimulated the nitrogen fixation activity, although the numbers have remained below the control values.

Inoculation of potato tubers had no effect on the potential nitrogen fixation activity in the variants with the farmyard manure, unlike in the variants with green manure where inoculation with *A. brasilense* has significantly stimulated the nitrogenase activity in the rhizosphere soil of potato plants. A slight increase in nitrogenase activity was observed at the flowering stage (BBCH 61) and the beginning of crop senescence (BBCH 91) in response to crop inoculation on the organo-mineral fertilization background. However, the obtained values were below the numbers observed in the test plots with low and medium fertilizer rates.

## Potential Denitrification Activity

All types and doses of fertilizers used in the given research led to the intensification of the biological denitrification in the rhizosphere of potato plants (Figure 2). Test plots with the farmyard manure were characterized by significant N<sub>2</sub>OD losses



throughout the growing season. The combination of farmyard manure with the mineral fertilizers has induced these losses. In the case of mineral fertilizers, the potential denitrification activity was proportional to the applied rates of fertilizers. Even crop cultivation on the background of green manure has a slight increase in the N<sub>2</sub>OD emissions activity.

Inoculation with *A. brasilense* has induced a significant reduction in N<sub>2</sub>OD emissions in variants with low and medium rates of mineral fertilizers (Figure 2) starting from the flowering stage (BBCH 61). At the end of the growing season (crops senescence, BBCH 91), the denitrification intensity under the influence of the inoculation has decreased even in the variant with the highest studied dose of fertilizers, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>.

## Quantitative Analysis of Microorganisms

The use of farmyard manure has significantly increased the number of ammonifiers, nitrogen fixers and denitrifiers whereas inoculation practically had no impact on the number of microorganisms in the rhizosphere soil of plants grown on the farmyard manure background (Table 2). At the same time, the inoculation of potatoes grown after green manure has stimulated the development of both ammonifiers and diazotrophs while the number of denitrifying microorganisms has remained stable.

Inoculation of plants grown on low and medium mineral backgrounds, N<sub>40</sub>P<sub>40</sub>K<sub>40</sub> and N<sub>80</sub>P<sub>80</sub>K<sub>80</sub>, respectively, has significantly contributed to the increase of the diazotrophs number and decrease of the number of denitrifying

microorganisms in the rhizosphere soil. The highest used rate of mineral fertilizers, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, has reduced the effect of inoculation as the number of diazotrophs in the rhizosphere of plants remained unchanged. At the same time, crop inoculation with *A. brasilense* has promoted the growth of denitrifying bacteria at the early stages of crop development with a gradual decrease in their number starting from the flowering stage (Table 2).

## Crop Productivity

Potato yield records indicate the highest increase in crop productivity with the use of farmyard manure, 40 t/ha, organo-mineral fertilizers and intense mineral fertilization backgrounds. The application of low and medium fertilizer rates has stimulated crop productivity by 24.6 and 93.2%, respectively. At the same time, the combination of the inoculation with mineral fertilizers has the highest impact on output results (Table 3). In particular, the introduction of *A. brasilense* has increased the crop yield by 42.4% on the N<sub>40</sub>P<sub>40</sub>K<sub>40</sub> background, and by 116.9% on the N<sub>80</sub>P<sub>80</sub>K<sub>80</sub> background, compared to the control. The combination of the highest mineral fertilizer rate, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, with inoculation has shown that with the increasing agrochemical load, the efficiency of inoculation decreases.

Crop inoculation has practically no effect on its productivity in variants with farmyard manure, 40 t/ha, application. No significant difference was observed in the variants with combined inoculation and organo-mineral background.

**TABLE 2 |** The quantitative dynamics of ammonifiers, nitrogen fixing microorganisms and denitrifying bacteria in the rhizosphere soil of potato plants under the action of fertilizers and inoculation (2018).

Variant	Ammonifiers, mln CFO/g dry soil*			Nitrogen fixing bacteria, mln/g dry soil			Denitrifying bacteria, mln/g dry soil		
	I	II	III	I	II	III	I	II	III
<b>Without inoculation</b>									
Without fertilizers (control)	13.4 ± 0.7	12.0 ± 1.0	10.8 ± 0.8	0.82	0.58	0.52	2.22	2.85	3.05
Farmyard manure, 40 t/ha	40.3 ± 1.3	44.7 ± 2.0	39.1 ± 1.2	1.06	0.89	0.92	8.25	5.13	4.96
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	18.0 ± 1.0	15.1 ± 1.9	12.4 ± 1.6	0.91	0.94	0.82	4.91	2.85	3.07
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	19.3 ± 0.8	16.8 ± 1.5	14.2 ± 0.7	0.43	1.19	0.96	7.15	8.55	4.96
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	22.0 ± 1.2	18.5 ± 0.9	16.4 ± 1.3	0.03	0.03	0.44	8.33	10.55	7.44
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	52.7 ± 2.2	50.3 ± 0.8	48.4 ± 2.8	0.49	0.62	0.65	8.40	13.22	9.30
Green manure, 13 t/ha	35.2 ± 3.1	37.1 ± 2.7	27.3 ± 1.1	0.94	1.12	0.92	2.45	3.15	3.40
<b>Inoculation with <i>A. brasilense</i> 410</b>									
Without fertilizers (control)	14.1 ± 0.9	11.5 ± 0.9	11.0 ± 0.3	1.26	0.81	0.69	2.78	2.85	3.10
Farmyard manure, 40 t/ha	41.0 ± 1.1	43.9 ± 1.7	38.7 ± 1.9	1.05	0.83	0.92	8.33	5.13	5.58
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	17.6 ± 2.1	16.2 ± 0.9	12.9 ± 1.3	1.12	1.20	7.10	5.24	2.45	2.48
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	18.3 ± 2.0	16.5 ± 1.1	14.8 ± 1.0	0.84	1.91	9.00	7.89	2.65	3.18
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	23.5 ± 3.1	17.9 ± 1.3	16.5 ± 1.8	0.04	0.31	0.53	9.35	8.70	6.99
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	51.9 ± 1.8	52.0 ± 1.5	49.3 ± 2.0	0.50	0.92	0.64	10.55	10.83	5.62
Green manure, 13 t/ha	34.9 ± 1.6	35.0 ± 2.5	25.8 ± 1.3	1.10	1.20	1.27	2.30	3.15	3.15

Growth stages.

I, Budding (BBCH 51); II, Flowering (BBCH 61); III, Crop senescence (BBCH 91).

\*Mean ± standard deviations.

## Product Quality

Analysis of output quality parameters has shown that the inoculation of potato plants has substantially influenced the quality of the obtained products (Table 4). Thus, the application of microbial preparation has stimulated the accumulation of starch in potato tubers while reducing the nitrate contents.

The study of ascorbic acid content has shown a significant increase in the variants with mineral fertilizers, green manure and organo-mineral background (up to 14%). The increase was even more significant in response to inoculation (Table 4).

## DISCUSSION

The dependence of associative nitrogen fixation activity on nitrogen fertilizer doses was first shown by Balandreau and Villemain (1973). Umarov et al. (1985) formulated the concepts of optimal doses of mineral nitrogen for the process of associative nitrogen fixation as “the ones that do not exceed the physiological needs of plants in nitrogen.” The same conclusion was reached by Ladha et al. (1986). In the development of these studies, we proposed (Volkogon, 2013) to determine not only the optimal doses of mineral nitrogen (the ones that promote the highest rates of nitrogen fixation), but also the physiologically, or environmentally, acceptable doses [the ones that do not reduce the nitrogen fixation activity below control (without fertilizers) indicators]. In our opinion, such biological approach can be a significant addition to the practice of agrochemical justification of crop fertilization systems.

Since the introduction of the acetylene reduction method by Hardy et al. (1968) for the determination of the nitrogenase

activity of diazotrophs, a number of its modifications have been developed. Particularly, in 1976 Umarov proposed a modification of Hardy's method for determining the potential nitrogenase activity in the soil. Notwithstanding that the method cannot be used for the evaluation of the activity and productivity of nitrogen fixation, its application allows locating differences in the functional activity of rhizosphere diazotrophs in response to various agricultural techniques, including fertilizers application and inoculation.

The changes of the ecologically acceptable doses of mineral nitrogen can be achieved upon the introduction of certain PGPBs into the crop lands, as inoculated plants uptake larger amounts of nitrogen to ensure a constructive metabolism. At the same time, crop inoculation expands the range of environmentally acceptable doses of fertilizers (Volkogon, 2013; Volkogon et al., 2014). So, higher nitrogenase activity in rhizosphere soil of experimental variants compared to unfertilized control is indicating the ecological feasibility of the selected level of nitrogen nutrition while the reduction of nitrogenase activity, on the contrary, indicates the inhibition of the nitrogen fixation process and possible environmental risks.

Thus, the analysis of the nitrogenase activity dynamics in the rhizosphere soil of potato plants demonstrates that mineral fertilizers in the doses that are not exceeding N<sub>80</sub>P<sub>80</sub>K<sub>80</sub> can be considered physiologically and ecologically expedient. Intensification of mineral nutrition, as well as the combination of farmyard manure with the mineral fertilizers, were shown to be environmentally unfavorable, as the nitrogenase activity indices in these variants were dramatically lower compared to the control numbers. Apparently, the high concentration of



**TABLE 3 |** Potato crop yield under the action of fertilizers and inoculation.

Variant	Crop yield, t/ha					Increment from fertilizers		Increment from inoculation	
	2016	2017	2018	2019	Mean	t/ha	%	t/ha	%
<b>Without inoculation</b>									
Without fertilizers (control)	12.6	12.9	13.6	8.2	11.8	–	–	–	–
Farmyard manure, 40 t/ha	21.8	25.0	21.6	13.6	20.5	8.7**	73.7	–	–
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	15.8	15.0	16.3	11.7	14.7	2.9**	24.6	–	–
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	22.6	25.2	25.2	18.4	22.8	11.0**	93.2	–	–
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	24.8	32.5	29.4	21.3	27.0	15.2**	128.8	–	–
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	28.2	30.0	30.2	24.0	28.1	16.3**	138.1	–	–
Green manure, 13 t/ha	15.1	15.4	14.2	8.8	13.4	1.6*	13.6	–	–
<b>Inoculation with <i>A. brasilense</i> 410</b>									
Without fertilizers (control)	14.8	14.0	15.4	8.9	13.3	–	–	1.5**	12.7
Farmyard manure, 40 t/ha	22.0	25.5	21.8	13.8	20.8	9.0**	76.3	0.3	1.5
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	18.0	18.2	17.5	13.4	16.8	5.0**	42.4	2.1**	14.3
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	25.6	27.9	27.4	21.4	25.6	13.8**	116.9	2.8**	12.3
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	26.4	34.0	30.1	22.3	28.2	16.4**	139.0	1.2*	4.4
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	28.4	30.9	30.7	24.6	28.7	16.9**	143.2	0.6	2.1
Green manure, 13 t/ha	17.5	16.8	16.5	10.3	15.3	3.5**	29.7	1.9**	14.2

\*Significant difference in increments with  $P < 0.05$ .\*\*Significant difference in increments with  $P < 0.01$ .

nitrogen compounds in the soil reduces the synthesis of the nitrogenase nitrogen-fixing complex of diazotrophic bacteria. To collect more evidence on the joint use of mineral nitrogen with the farmyard manure the application of lower doses of mineral nitrogen fertilizers should be tested for the organo-mineral fertilization system.

It is known that the denitrification process does not always end with the dinitrogen formation. Under natural conditions, this process can result in incomplete reduction of nitrates to nitrite ( $\text{NO}_2^-$ ), nitric oxide (NO), or nitrous oxide ( $\text{N}_2\text{O}$ ) (Conrad, 1996). Complete denitrification with the formation of dinitrogen is observed only for a limited number of microorganisms, so-called true denitrifiers. To reveal the activity of all denitrifying microorganisms in the rhizosphere of potato plants we used acetylene, as a specific inhibitor of nitrous oxide reductase, to block the reduction of  $\text{N}_2\text{O}$  to  $\text{N}_2$ .

At the same time, it should be emphasized that the determination of the potential denitrification activity in the rhizosphere soil does not characterize the total loss of  $\text{N}_2\text{O}$  from a certain area, but only reflects the reaction of rhizosphere microorganisms to a certain number of mineral nitrogen compounds, and thus corresponds to the crop response to the shortage or excessive amount of mineral nitrogen.

With  $\text{N}_2\text{OD}$  emission values exceeding control in all studied combinations throughout the growing season, farmyard manure, 40 t/ha, used stand-alone and in combination with mineral fertilizer have demonstrated the highest increase. The losses of gaseous nitrogen in variants with mineral fertilizers have corresponded to the applied rate as they have increased with the fertilization intensity. This data confirms the previously reported findings of Barton et al. (1999) and Shcherbak

et al. (2014) on denitrification rates and  $\text{N}_2\text{O}$  emissions from croplands.

The use of an inoculant based on *A. brasilense* 410 in potato growing technology significantly influences the processes of nitrogenase activity and biological denitrification in the rhizosphere of plants, but the effect of inoculation depends on the fertilization background. High levels of nitrogenase activity at the beginning of the growing season were observed in variants with  $\text{N}_{40}\text{P}_{40}\text{K}_{40}$  and, starting from the flowering phase, for  $\text{N}_{80}\text{P}_{80}\text{K}_{80}$ . Under the use of the highest rate of mineral fertilizer,  $\text{N}_{120}\text{P}_{120}\text{K}_{120}$ , inoculation, even though it stimulates the nitrogenase activity, has not reached the control values. One of the highest indices of the nitrogenase activity in the rhizosphere of potato plants was observed in rhizosphere soil under the combined action of inoculation with green manure.

At the beginning of the growing season, inoculation has contributed to the growth of  $\text{N}_2\text{OD}$  emissions in all variants except for the farmyard manure. Microorganisms introduced into the crop soils can also contribute to the reduction of the excess amount of nitrogen in the soil as even a small amount of mineral nitrogen in the soil can be considered excessive for crop seedlings with the *Azospirillum* sp. involved in both denitrification and nitrogen fixation processes (Bashan and Levanony, 1990). After the utilization of a certain amount of applied mineral nitrogen by potato plants, initiated with the inoculation, bacteria reflect the changes in the soil environment and reveal its nitrogen-fixing function. This, in turn, significantly reduces the denitrification activity in the rhizosphere of inoculated plants. Thus, in the flowering stage, the  $\text{N}_2\text{OD}$  emissions were even below the control levels in the variants with the lowest rate of mineral fertilizers,  $\text{N}_{40}\text{P}_{40}\text{K}_{40}$ , and crop

**TABLE 4 |** The effect of fertilizers and inoculation on the product quality indicators (2018).

Variant	Starch content*, %	NO <sub>3</sub> <sup>-</sup> content*, mg/kg	Vitamin C content*, mg/100 g
<b>Without inoculation</b>			
Without fertilizers (control)	13.56 ± 0.05	43.8 ± 2.7	13.87 ± 0.57
Farmyard manure, 40 t/ha	13.26 ± 0.04	57.8 ± 1.4	14.22 ± 0.31
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	14.07 ± 0.04	70.6 ± 0.5	15.30 ± 0.58
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	14.33 ± 0.05	76.6 ± 3.0	15.42 ± 0.00
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	13.94 ± 0.03	127.8 ± 5.5	15.79 ± 0.33
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	13.83 ± 0.00	124.7 ± 4.4	15.59 ± 0.35
Green manure, 13 t/ha	13.48 ± 0.08	39.5 ± 3.8	15.20 ± 0.39
<b>Inoculation with <i>A. brasilense</i> 410</b>			
Without fertilizers (control)	13.59 ± 0.03	42.8 ± 0.5	15.25 ± 0.18
Farmyard manure, 40 t/ha	13.32 ± 0.03	54.7 ± 2.8	15.92 ± 0.35
N <sub>40</sub> P <sub>40</sub> K <sub>40</sub>	14.26 ± 0.00	65.8 ± 0.8	15.60 ± 0.70
N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	14.80 ± 0.00	66.5 ± 1.0	16.55 ± 0.33
N <sub>120</sub> P <sub>120</sub> K <sub>120</sub>	14.58 ± 0.01	104.4 ± 3.6	17.13 ± 0.07
Farmyard manure, 40 t/ha + N <sub>80</sub> P <sub>80</sub> K <sub>80</sub>	14.02 ± 0.03	107.6 ± 1.2	16.55 ± 0.24
Green manure, 13 t/ha	14.25 ± 0.06	35.0 ± 1.7	16.04 ± 0.45

\*Mean ± standard deviations.

inoculation. The numbers observed in the variants with the medium fertilizer rate, N<sub>80</sub>P<sub>80</sub>K<sub>80</sub>, were similar to the control.

The reduction of N<sub>2</sub>O emissions in response to crop inoculation with PGPB-based preparations used under mineral nitrogen fertilizer background was reported earlier by Calvo et al. (2013) based on the results obtained in a greenhouse experiment. These findings were confirmed in the following experiment, which showed that the use of inoculants in corn production reduced the emissions of N<sub>2</sub>O from 15 to 49%, subject to the type of nitrogen fertilizer (except for urea) and microorganisms. In general, they demonstrated that PGPBs enhance mineral nitrogen uptake following the fertilizer application while reducing N<sub>2</sub>O emissions (Calvo et al., 2016). Our findings have confirmed these findings. However, the introduction of the biological preparation in the cultivation of potatoes on the background of high rates of mineral fertilizer has increased the emission of N<sub>2</sub>OD. It can be related to the ability of *Azospirillum* sp. to perform different functions depending on the availability of the mineral nitrogen in the soil. Since bacteria of *Azospirillum* genus are capable of denitrification, it can be assumed that at high nitrogen background and presence of introduced or present bacteria in the soil, they are involved in the natural regulation process of the nitrogen cycle, accounting for N<sub>2</sub>OD emissions aimed to reduce the excess of mineral nitrogen forms in soil. This can be avoided through the introduction of a sufficient amount of fresh organic matter into the soil with a broad C/N ratio or reducing nitrogen rate application. It would promote the active transformation of mineral nitrogen compounds into organic forms and reduce nitrogen losses.

So, under inoculation, plants utilize mineral nitrogen compounds to a much greater extent, which is extremely important from the ecological point of view. In fact, inoculants can serve as an inhibitor of the denitrification process since their introduction to the “plant-soil” system increases nitrogen utilization coefficients from mineral fertilizer.

The effect of *A. brasilense* 410 on the studied processes was leveled under potatoes growing on the background of farmyard manure, 40 t/ha. Both the nitrogenase activity and the emission of N<sub>2</sub>OD in the rhizosphere of inoculated plants remained unchanged on a farmyard manure background. That is related to the fact that a significant number of microorganisms are introduced into the soil with manure, creating a prevailing competitive environment for nitrogen fixing bacteria from biological products. Under these circumstances, the positive effect of inoculation is offset. These assumptions are indirectly confirmed by the counts of microorganisms of individual ecological and trophic groups in the rhizosphere soil of potato plants (Table 2). Thus, the introduction of farmyard manure into the soil has significantly increased the number of ammonifiers, nitrogen fixation microorganisms and denitrifying bacteria. Inoculation practically has not significantly influenced the number of microorganisms in the rhizosphere soil. In this case, farmyard manure can ensure non-specific bacterization of the soil, which prevents the successful introduction of *A. brasilense*.

Scientists have already paid attention to the abundant number of microorganisms in the farmyard manure. Thus, one of the founders of scientific soil science –Dokuchaev (1948), wrote: “Along with manure, a vast number of microorganisms are introduced into the soil, the role of which is no less important than fertilizers.” Our results, to some extent, confirm these findings and expand the knowledge about the effectiveness of inoculation upon the cultivation of the crops on the farmyard manure background.

Unlike with the farmyard manure, potato inoculation following the green manure incorporation has promoted the growth of ammonifiers and diazotrophs in the rhizosphere soil of plants. The number of denitrifying microorganisms has remained unchanged. Therefore, the incorporation of green manure, lupine, in particular, creates optimal conditions for *A. brasilense* efficiency effect.

The biological preparation applied on low mineral fertilizer backgrounds has considerably increased the number of diazotrophs, which confirms the creation of favorable soil conditions for the development of the introduced microorganism.

Regardless of the fertilization system, an increase in crop productivity was observed for all studied variants. Therewith, the inoculation ensured the highest gains in combination with low and medium fertilizer rates with a 14.3% yield increase on the N<sub>40</sub>P<sub>40</sub>K<sub>40</sub> background and 12.3% on the N<sub>80</sub>P<sub>80</sub>K<sub>80</sub> background. The highest studied rate of mineral fertilizer, N<sub>120</sub>P<sub>120</sub>K<sub>120</sub>, had also promoted the yield increase, but to a much lesser extent, indicating the disadvantage of the chosen fertilizer rate for the efficiency effect of *A. brasilense* inoculation.

Analyzing the yield levels of potatoes grown on mineral backgrounds without inoculation and in combination with

inoculation, it was noted that the effect of *A. brasilense* on the crop productivity was equivalent to the action of a certain number of mineral fertilizers. Thus, the difference in yield means between variants with the highest applied dose ( $N_{120}P_{120}K_{120}$ ) and  $N_{80}P_{80}K_{80}$  without inoculation was 4.2 t/ha. At the same the yield difference between  $N_{120}P_{120}K_{120}$  and  $N_{80}P_{80}K_{80}$  backgrounds combined with the action of crop inoculation was only 1.4 t/ha. In some years, the effect of inoculation on crop yields was equivalent to the action of mineral fertilizers at the lowest studied dose ( $N_{40}P_{40}K_{40}$ ). Overall these findings indicate the possibility of reducing the amount of mineral fertilizers in crop production when combine with the inoculation to achieve the planned result.

Higher efficiency of inoculation used in the cultivation of different crops grown on low rates of nitrogen fertilizers has been reported in numerous publications. According to Freitas and Germida (1990), PGPBs are more effective with the reduced amount of nutrients in the soil. Shaharooma et al. (2008) reported that nitrogen utilization efficiency in response to *Pseudomonas fluorescens* inoculation has increased in response to all levels of wheat fertilization. Depending on the mineral fertilizer rates (25, 50, 75, and 100% to the recommended rate of nitrogen, phosphorus, and potassium), it ensured 115, 52, 26, and 27% yield increase compared to the uninoculated wheat plants, respectively.

Our results have also indicated the optimality of low and medium fertilizer rates for the manifestation of inoculation efficiency. Thus, in particular, potato inoculation on the medium fertilizer background,  $N_{80}P_{80}K_{80}$ , averaged 25.6 t/ha over the 4 years of research, which is close to the figures obtained in the variant with the highest used fertilizer rate,  $N_{120}P_{120}K_{120}$  – 27.0 t/ha. The inoculation effect, in this case, was equivalent to the action of a certain amount of mineral fertilizers. That can be used in estimations of mineral fertilizers as inoculation can reduce the rates without affecting crop yields.

The inoculation efficiency effect was noticeable upon the crop cultivation following the green lupine manure. The increase of the nitrogenase activity in the rhizosphere of potato plants and the crop yield was observed upon the *A. brasilense* introduction. This data has confirmed the results of other researchers. Thus, the maximum diameter of cobs, their raw and dry weight was recorded for lettuce inoculated with *Azospirillum* bacteria (GM1M1Az3) on the background of green manure and mulching (Borthakur et al., 2012). It has also been reported that the interaction between green manure and seed inoculation with *Herbaspirillum seropedicae* has had a positive effect on corn yield, contributing to the higher number of kernels and their weight (Avila et al., 2020).

In contrast to the effect on green manure, background inoculation did not affect the yield of potatoes in the variants where the farmyard manure was applied. The latest research findings on this topic were reviewed by Wani (1990) indicating the high efficiency of the combination of non-symbiotic nitrogen-fixing bacteria with manure. However, freely existing diazotrophs cannot be compared with associative bacteria, given the mechanisms of their interaction with plants. At the same time, there is evidence of the high efficacy of PGPB used following

a pig slurry background. Thus, a study by Lai et al. (2008) showed that lettuce growth in soil fertilized with pig slurry and inoculated with *Azospirillum rugosum* IMMIB AFH-6 was significantly lower compared to the one of the inoculated plants grown on the mineral background. However, the addition of only half of the recommended rate of mineral fertilizer to the pig slurry background had ensured the highest yield gains and an increase of other studied parameters of inoculated plants (Lai et al., 2008).

Studies conducted by Yildirim et al. (2011) have shown that broccoli inoculation with *Bacillus cereus*, *Rhizobium rubi*, and *Brevibacillus reuszeri* following the farmyard manure had contributed to the increase of crop productivity (up to 24.3%), chlorophyll content (up to 14.7) and nutrients uptake compared to control (manure only) values. Combination of farmyard manure with crop inoculation with *Azotobacter* has increased the biological yield of wheat (Esmailpour et al., 2013) and corn (Dutta et al., 2014).

According to Hadi et al. (2015), the maximum productivity of black cumin seeds was recorded for the crops inoculated with *Azotobacter* and *Azospirillum* on farmyard manure, 5 t/ha, background. Used separately, both bacteria and manure applications have a positive effect on crop yields. However, their combination ensured a better outcome. In their review on the importance of PGPB for the absorption of nutrients by inoculated plants from fertilizers, Adesemoye and Kloepper (2009), do not differentiate manure and chemical fertilizers as substrates containing chemical elements and consider promising the use of microbial preparations on the manure background. The result obtained in our experiment, which is quite the opposite of the findings mentions above, may be explained by the conditions of the experiment (crop choice, characteristics of inoculant, manure dose, soil type, etc.). On other hand, it designates the study prospects of the combination of PGPB with farmyard manure. The data also indicates a certain limitation of the use of biological preparations in the organic production systems, as their efficiency can be compromised. At the same time, the effectiveness of biologicals may even advance with their introduction into the soil not with manure, but through vermicomposting, as noted by Song et al. (2015).

The inoculation had ensured the reduction of the nitrates content in potato tubers, especially, when used on mineral fertilizer background. Besides its effect on the output product quality, crop inoculation ensured higher content of starch and ascorbic acid in potato tubers. The increase in vitamin C content can be beneficial for its possible positive effects. Potatoes are an important source of vitamin C, not only because of their relatively high content but also because they can be stored for a prolonged period. Therefore, the improvement in the ascorbic acid content in potatoes will have a beneficial effect on human nutrition (Love and Pavek, 2008). It is also known, that ascorbic acid can neutralize to a great extent the harmfulness of nitrates for warm-blooded organisms (Hirnerth and Classen, 1984; Shehata, 2005). Thus, the increase in its content following the reduction of nitrates content in the variants with *A. brasilense* inoculation promotes the value of the product.

Even so, the lowest dose of mineral fertilizers was shown to be the most optimal in terms of environmental sustainability

of production, higher doses ( $N_{80}P_{80}K_{80}$ ) are a more reasonable compromise between environmental feasibility and crop productivity. Green manure use despite its vital role in regulating processes of biological transformation of nitrogen was practically disadvantageous, while the application of farmyard manure is advisable with some reservations. Crop inoculation with *A. brasilense* was proved to be beneficial influencing the optimization of the ecological conditions of croplands, the increase of potatoes productivity and the improvement of the output quality of potato crops.

## CONCLUSIONS

The study of nitrogenase activity and  $N_2O$  emissions in the rhizosphere of potato plants in dynamics has shown that the application of low ( $N_{40}P_{40}K_{40}$ ) and medium ( $N_{80}P_{80}K_{80}$ ) doses of mineral fertilizers are physiologically and ecologically optimal for cultivation on leached chernozem, especially in combination with inoculation. Crops inoculation with *A. brasilense* on these mineral fertilizer backgrounds has ensured higher nitrogen fixation activity, a significant reduction of denitrification levels, and the highest crop productivity compared to the other fertilizer options. The inoculation effect on the course of nitrogen fixation and denitrification crop cultivation on the background of 40 t/ha of manure was significantly leveled, while the highest manifestation of the efficiency of biological preparation was observed under the green manure background. Taken as a whole,

the use of *A. brasilense* in potato growing technologies has a positive effect on the accumulation of starch and ascorbic acid and helps to reduce the content of nitrates in the output products.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

## AUTHOR CONTRIBUTIONS

VV performed the experimental data analysis and worked on the manuscript discussion session. SD and KV carried out the field experimental data acquisition, quantification of basic physiological groups of microorganisms, and data analysis. KV and VS performed the study of potential nitrogenase activity and potential denitrification activity in rhizosphere soil and data analysis. MV helped with the data interpretation and manuscript preparation. All authors read and approved the final manuscript.

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# Climate Change and Salinity Effects on Crops and Chemical Communication Between Plants and Plant Growth-Promoting Microorganisms Under Stress

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During the last two decades the world has experienced an abrupt change in climate. Both natural and artificial factors are climate change drivers, although the effect of natural factors are lesser than the anthropogenic drivers. These factors have changed the pattern of precipitation resulting in a rise in sea levels, changes in evapotranspiration, occurrence of flood overwintering of pathogens, increased resistance of pests and parasites, and reduced productivity of plants. Although excess CO<sub>2</sub> promotes growth of C<sub>3</sub> plants, high temperatures reduce the yield of important agricultural crops due to high evapotranspiration. These two factors have an impact on soil salinization and agriculture production, leading to the issue of water and food security. Farmers have adopted different strategies to cope with agriculture production in saline and saline sodic soil. Recently the inoculation of halotolerant plant growth promoting rhizobacteria (PGPR) in saline fields is an environmentally friendly and sustainable approach to overcome salinity and promote crop growth and yield in saline and saline sodic soil. These halotolerant bacteria synthesize certain metabolites which help crops in adopting a saline condition and promote their growth without any negative effects. There is a complex interkingdom signaling between host and microbes for mutual interaction, which is also influenced by environmental factors. For mutual survival, nature induces a strong positive relationship between host and microbes in the rhizosphere. Commercialization of such PGPR in the form of biofertilizers, biostimulants, and biopower are needed to build climate resilience in agriculture. The production of phytohormones, particularly auxins, have been demonstrated by PGPR, even the pathogenic bacteria and fungi which also modulate the endogenous level of auxins in plants, subsequently enhancing plant resistance to various stresses. The present review focuses on plant-microbe communication and elaborates on their role in plant tolerance under changing climatic conditions.

**Keywords:** climate change, salinity, halotolerant PGPR, biofertilizers, stress tolerance

## INTRODUCTION

### Drivers of Climate Change

Variations in the atmospheric conditions of a particular region for a long period is known as climate and changes in the atmospheric variables beyond the average value is known as climate change. Different forces are responsible for variations in atmospheric variables. They are broadly categorized into two main groups: anthropogenic and natural drivers. Anthropogenic drivers are well-mixed greenhouse gases (WMGHGs), i.e., CO<sub>2</sub>, CH<sub>4</sub>, and nitrous oxide (N<sub>2</sub>O), whereas solar irradiance and earth orbit, volcanic eruption, and chemical weathering of rocks are examples of natural climate drivers. The greenhouse gasses cause changes in climate by trapping heat, initiating wildfires, and disrupting food supply (Cassia et al., 2018). Volcanic eruptions cause a reduction in the amount of solar radiation, decreasing the temperature in the troposphere and changing atmospheric circulation patterns. Similarly, the chemical weathering of rocks also caused climate cooling by consumption of CO<sub>2</sub> (Huh, 2003). The effects of natural drivers on climate change are relatively less compared to anthropogenic impacts (Nelson et al., 2006; Rosa et al., 2015; Myhre et al., 2017). Synthetic fertilizers used in agriculture are responsible for the production of about 80% growth rate of N<sub>2</sub>O (Ciais et al., 2014). Global rice production is responsible for >10% of total CH<sub>4</sub> emission (Smartt et al., 2016).

### Variables of Climate Change

The bioindustry of agriculture is greatly influenced by temperature, precipitation, and sunlight. These elements are vulnerable to climate change. A significant increase in global mean temperature (GMT) is induced by artificial drivers as compared to natural drivers of climate (Myhre et al., 2017). It influences precipitation patterns which might lead to drought or inundation. An increase in temperature and decrease in precipitation would affect soil properties (Gelybó et al., 2018). It also affects soil organic carbon as a decrease in water ratio lowers the decomposition process and mineralization of different organic compounds (Karmakar et al., 2016; Onwuka and Mang, 2018). Change in soil temperature also influences soil moisture content, water holding capacity, and soil particle size. Increased soil surface temperature also increases the rate of evaporation and restricts the movement of water in soil profile (Onwuka and Mang, 2018). One study has shown that clay size and organic matter, which decrease in response to increased soil temperature, lead to reduced cation exchange capacity (CEC) (Certini, 2005). Reduced productivity of livestock, horticulture, and cultivated crops is directly related to broad scale alterations in climatic conditions (Malla, 2008).

### Negative Impacts of Climate Change on Agricultural Productivity

Climate change and food security are two main issues of the 21st century. The world population is expected to reach 9 billion by the end of 2050, and food requirements are expected to escalate by 85% (FAO, 2017). The agriculture sector is highly threatened by the increased frequency of droughts, heavy rainfall, fluctuations in temperature, salinity, and insect pest attacks

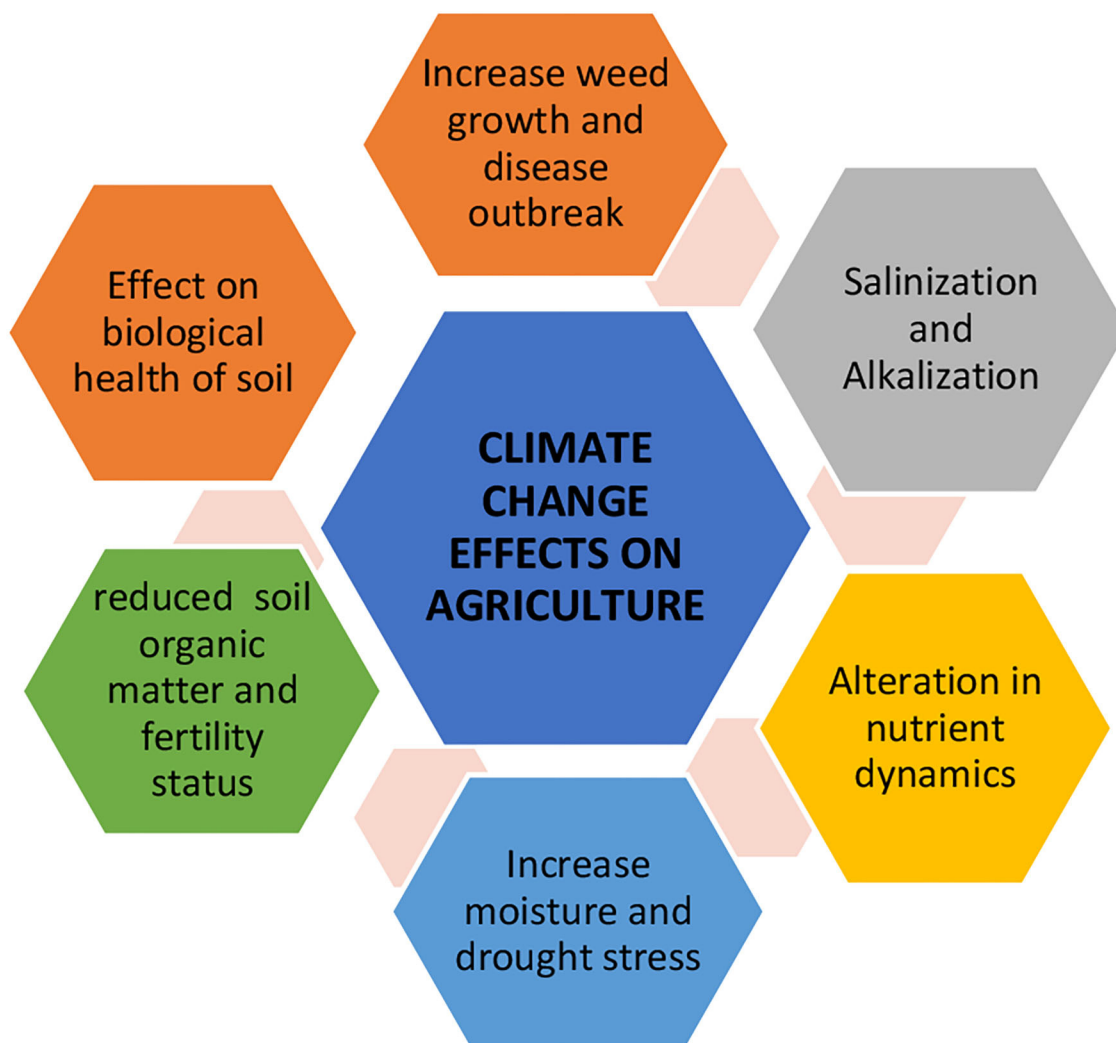
on major food crops (Dhankher and Foyer, 2018; Hussain et al., 2019). The FAO declared that 20% of total emissions of GHGs were from agriculture and land use practices. Changes in precipitation patterns, increased frequency of dry spells and drought, rising temperatures, rising sea levels, and temperature variability have negative impacts on agriculture production (Figure 1). Zhao et al. (2017) constructed different simulation models to investigate the effect of increased temperature on yield of four crops by analyzing yield data from 46 research articles and 48 sites on a global scale. He concluded that for each degree increase in global mean temperature (GMT), the production of wheat has been predicted to reduce by 6.0%, rice by 3.2%, maize by 7.4%, and soybean by 3.1% around the world. Depending on geographical regions, the effect of temperature may be positive or negative. In general, a rise in temperature has caused a shift in the phenological development of crops (Teller, 2016), lower water use and nitrogen use efficiency (Ullah et al., 2019), shortening of vegetative and reproductive growth phases of plants (Wang et al., 2018), and decrease in the number of grains and grain-filling duration (Lin et al., 2017). The proliferation of insects and pests at high rates which attack major crops are indirect effects of climate change (Dhankher and Foyer, 2018). The largest grain producers of the world viz. China, the US, and France are facing major infestations of crop pests and consequent yield losses (Shrestha, 2019). This is because increased temperatures and precipitation promote the growth and spread of many pest species.

### Climate Change and Salinity

Soil salinity is one of the impacts of climate change in coastal agriculture land, as rises in sea levels has increased salinity from 1 to 33% over 25 consecutive years (Rahman et al., 2018). Sea level rise is associated with the increase in global warming and occurs due to the melting of glaciers and ice sheets as well as the thermal expansion of sea water. Bhuiyan and Dutta (2012) demonstrated a comprehensive understanding of the possible effects of sea level rise with the aid of a hydrodynamic model. Sea level rise includes flooding and salinization and has implications for water resources. Rising sea levels increase the salinity of both surface water and ground water through saltwater intrusion.

Salinity is more evident in semi-arid, coastal agriculture lands, and particularly in arid regions of the world (Kasim et al., 2013; Qadir et al., 2014; Hashem et al., 2018). Changes in weather patterns have resulted in the frequency of recurrent drought or rain falling above the average value for more than a decade (Ayanlade et al., 2018). The upward movement of water in areas with shallow water tables and coastal areas with sea water intrusion resulted in root zone salinity. Changes in precipitation and temperature have a greater influence on soil salinity. In one study conducted by Bannari and Al-Ali (2020), it has been reported that the long-term effects of increased temperatures and decreased precipitation for 30 years showed a positive correlation with increased soil salinity in arid landscape due to less over leaching of salt in the soil as determined by Landsat sensor data including Thematic Mapper (TM), Thematic Mapper Plus (ETM+), and Operational Land Imager (OLI). In coastal agriculture lands, the salinity has increased from 1 to 33% in the last 25 years (Rahman et al., 2018).





**FIGURE 1** | Impact of climate change on agriculture.

On a global level, more than 831 Mha of agriculture land is salt-affected (FAO, 2008); salinity affected 397 M ha, while sodicity affected up to 434 M ha land (Martinez-Beltran, 2005; Setia et al., 2013). Out of the total cultivated and irrigated agriculture land, 50% is affected by high salinity on a global level (Zhu, 2001; Gengmao et al., 2015). Irrigation with saline water, low precipitation, and high evapotranspiration are key factors that cause salinization at a rate of 10% annually to agricultural lands. At this rate more than 50% of arable land would be salinized by 2050 (Jamil et al., 2011). Expansion of salt-affected arable lands has emerged as a major threat toward world food security. About 6% of the cultivated area is destroyed by soil salinization with a continued addition of 1–2% every year around the globe, causing significant yield losses of staple grain crops like maize, rice, and wheat (Munns and Tester, 2008). High rates of evapo-transpiration results in accumulation of salt on the soil surface (Ashraf et al., 2002; Munns, 2002). Most of the underground water used for crops in such conditions

become brackish and contains a high content of soluble salt ions, such as  $\text{Na}^+$  and  $\text{Cl}^-$ , and lower quantities of  $\text{K}^+$ ,  $\text{Ca}^{2+}$ , and  $\text{NO}_3^-$  (Abro et al., 2007; Munns and Tester, 2008). The presence of these ions results in hyper ionic salt stress, which induces metabolic impairment and oxidative stress through the generation of reactive oxygen species (ROS), thus adversely impacting the yield of crops (El-Hendawy et al., 2005; Petrov et al., 2015; Caverzan et al., 2016).

### Negative Impacts of Salinity on Crop Physiology

Salinity exerts its detrimental effect on plants by two mechanisms: osmotic stress and ion toxicity. The first effect is short term and occurs due to the uptake of  $\text{Na}^+$  and  $\text{Cl}^-$  which reduce osmotic potential between root and soil solution and infiltrate water availability (Abbasi et al., 2016). Secondly, high concentrations of  $\text{Na}^+$ ,  $\text{Cl}^-$ , or  $\text{SO}_4^{2-}$  induce ion toxicity that affect nutrient uptake (Tavakkoli et al., 2011). Sodium chloride (NaCl) toxicity

is directly related to electrical conductivity (EC), which is an indicator of plant tolerance to salt stress (Isla and Aragüés, 2010). Crop tolerance decreases beyond  $2 \text{ dSm}^{-1}$ , while some plants grow well even at  $8 \text{ dSm}^{-1}$ . Beyond this limit, growth and yield are negatively impacted. The adverse effects of salinity on crop morphology, physiology, and yield are listed in **Figure 2**. Seeds sown in saline conditions are poorly germinated (Ullah and Bano, 2019). Salinity decreases the endogenous level of phytohormones and inhibits seedling establishment (Egamberdieva and Kucharova, 2009). If seeded in the first phase of salinity, shoot growth will be affected in later stages (El Sayed, 2011; Wakeel et al., 2011). Even if seedlings are vigorous and maintain normal growth in the second phase of salinity, yield will be affected at the end. Salinity causes various physiological impairments in plants; due to less stomatal conductivity, C-fixation capacity becomes limited, disturbing the catalytic activities of enzymes that fix C and destroy photosynthetic pigments (Omoto et al., 2012). A significant decrease in shoot and root biomass has been recorded in plants grown in saline soil (Kalhor et al., 2016; Genc et al., 2019).

Studies showed that salinity (10 mM NaCl) reduced the yield of *Triticum aestivum* up to 65% (Khan et al., 2004; Shafi et al., 2010). Elevated levels of salt in the root medium reduced the uptake of K, Ca, and Mg (Keutgen and Pawelzi, 2009; Hussin et al., 2013), leading to disturbances in stomatal conductance and transpiration rate (Sumer, 2004). Iron (Fe) is an important metal activator (co-factor) of different antioxidant enzymes (Kumar et al., 2010; Sharma et al., 2012), which helps in regulating life sustaining processes in plants, i.e., photosynthesis and

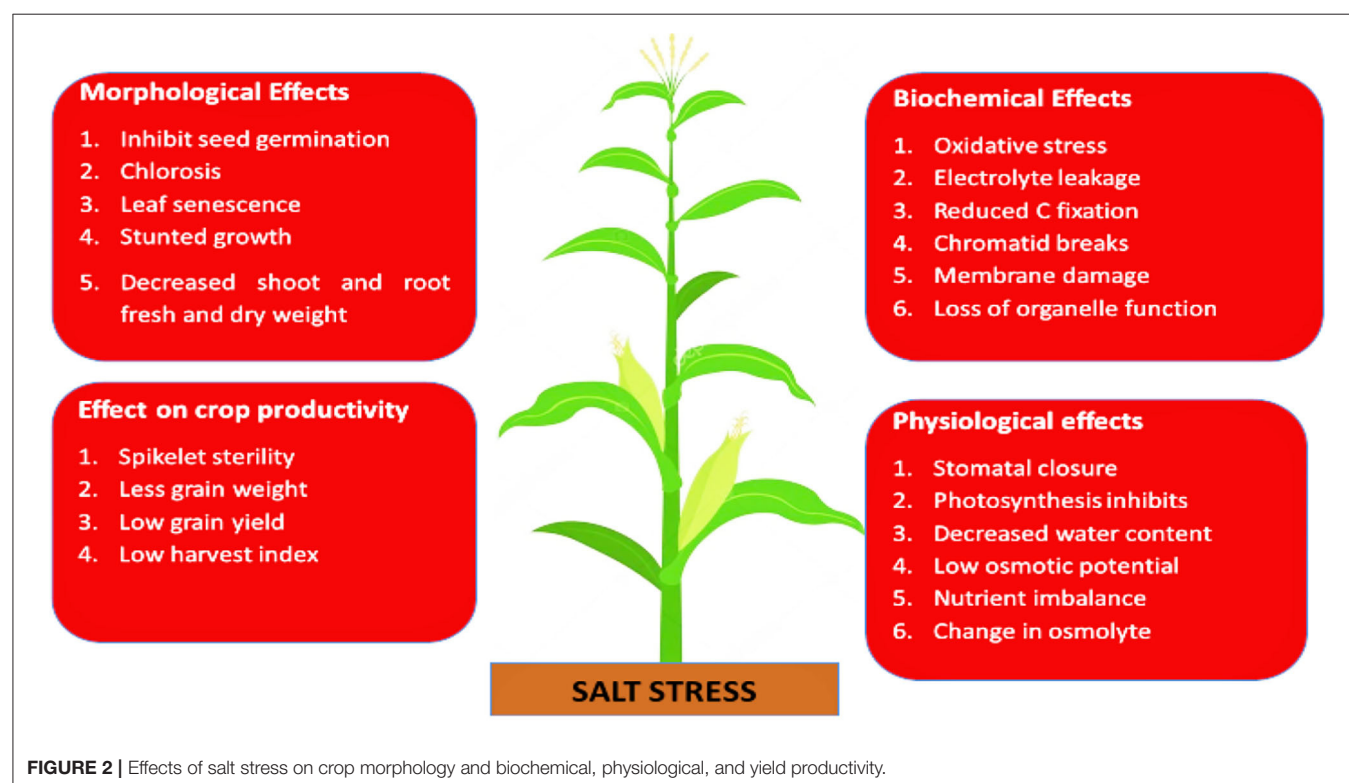
chloroplast biosynthesis. Rabhi et al. (2008) reported that saline sodic soil is deficient in Fe. Plants grown in such soil showed interveinal chlorosis and a declined yield (Lan et al., 2011). Chloride ion ( $\text{Cl}^-$ ) is a predominant ion in saline environments and the uptake of N is indirectly correlated to it. Salinity-induced increased Cl uptake is often associated with decreased uptake of nitrate by crops (Wang et al., 2012; Sadale and Karadge, 2013; Yu et al., 2016; Hasana and Mayake, 2017; Kanagaraj and Desingh, 2017).

Oxidative stress induced by salinity as secondary stress leads to the production of ROS, i.e.,  $\text{O}^{2-}$ , OH, and  $\text{H}_2\text{O}_2$  (Apel and Hirt, 2004; Voothuluru and Sharp, 2012; Chawla et al., 2013). As a toxic byproduct of aerobic metabolism, ROS are primarily found in the chloroplast, mitochondria, and peroxisome. Their enhanced production cause lipid peroxidation that affects photosynthesis and membrane permeability, damages nucleic acids, inhibits enzymes, and activates programmed cell death (Apel and Hirt, 2004; Tanou et al., 2009; Mishra et al., 2011; Srivastava and Dubey, 2011). *Brassica napus* and *Triticum aestivum* grown in saline conditions experienced poor growth and yield due to elevated levels of saline water (Hasanuzzaman et al., 2011a,b).

## STRATEGIES TO COPE WITH SALINITY STRESS UNDER CHANGING CLIMATE

### Physical Methods

Some physical methods to reclaim salt-affected soil include scraping, deep plowing, deep tillage, sub soiling, sanding, horizon mixing, profile inversion, flushing, and leaching. Scraping is



used by farmers to mechanically remove salt from soil surface; however, it is a temporary measure to improve plant growth, and the ultimate disposal of salt remains a major problem. Flushing is used to desalinate soil with surface salt crusts. This method can flush small amounts of salt but has little practical significance. The most common method to reclaim saline soil involves leaching, in which good quality water is supplied to surface soil to leach salt levels (Ghafoor et al., 2012; Yan and Marschner, 2013). The leached salt is then removed with an adequate drainage system.

## Chemical Methods

### Reclamation by Inorganic Amendment

Saline sodic soil can be reclaimed by the application of  $\text{Ca}^{2+}$ -containing chemicals which replace  $\text{Na}^+$  at soil exchangeable sites, followed by leaching with good quality water supply (Gupta and Abrol, 1990). Application of gypsum in soil with low concentrations of carbonate has been extensively studied (Horney et al., 2005; Gharaibeh et al., 2011). It is commonly used to supply  $\text{Ca}^{2+}$ . However, for soil containing high carbonate content, sulphuric acid is recommended (Horney et al., 2005), which increases soil  $\text{Ca}^{2+}$  levels by dissolving  $\text{CaCO}_3$  in soils (Zia et al., 2007; Vance et al., 2008). The use of inorganic amendments are costly and labor intensive, and is an unhealthy practice for beneficial microbes.

### Reclamation by Organic Amendment

The physico-chemical and biological properties of soil can be improved by the application of organic matter amendment, as they accelerate salt leaching and improve aggregate stability and water holding capacity (Walker and Bernal, 2008), thus enabling better plant growth in salt-affected soils. These are cheap and easily organic amendments which contain soil nutrients, organic matter, and enhance both cation exchange capacity and soluble exchangeable  $\text{K}^+$ , which compete with  $\text{Na}^+$  in saline sodic soil, and limits its entry at exchangeable sites (Walker and Bernal, 2008). Examples of organic amendments include farmyard manure, poultry manure, municipal solid waste compost, and olive mill waste compost (Ahmad et al., 2006; Tejada et al., 2006; Lakhdar et al., 2009). Application of organic amendments have significant effects in an area with low rain fall but cause secondary salinization in other areas where rainfall is abundant (Diacono and Montemurro, 2011).

## Biological Methods

### Phyto-Reclamation

Phyto-reclamation is an environment-friendly approach to reclaim saline and saline sodic soils (Rabhi et al., 2008; Ashraf et al., 2010). In this method, salt-tolerant plant species are used to reclaim saline soil. A significant improvement in soil organic matter and water holding capacity and decrease in soluble salts and exchangeable  $\text{Na}^+$  have been recorded (Mishra and Sharma, 2003; Nosetto et al., 2007). Salt-affected soil contains Ca in the form of calcite which is insoluble and unable to displace  $\text{Na}^+$  from cation exchange complexes. However, the higher partial pressure of  $\text{CO}_2$  and enhanced production of carbonic acid in the root zone could assist in the solubilization of calcite which provide soluble  $\text{Ca}^{2+}$  for exchanging  $\text{Na}^+$  (Qadir

et al., 2005). Root exudates of salt-tolerant plant species contain  $\text{H}^+$  which enhances  $\text{Na}^+$  uptake and its subsequent removal from the field. Examples of halophytes include *Atriplex* spp., *Aegiceras corniculatum*, *Bruguiera gymnorrhiza*, *Chenopodium album*, *Plantago media*, *Rhizophora mucronata*, *Suaeda australis*, and *Salsola vermiculata* (Qadir et al., 2007; Hasanuzzaman et al., 2014). Phytoremediation has certain limitations in sustainable agricultural productivity, as it changes the microbial community, and takes several growing seasons to remediate polluted soil.

## Plant Growth-Promoting Rhizobacteria for Reclamation of Saline Soils

Use of PGPR to reclaim saline soil is a far better approach than chemical and organic fertilizers because of its environment-friendly and persistent nature, with PGPR proliferating slowly and gradually in inoculated soil and ensuring their survival for decades. For this purpose, PGPR should be isolated from their native stress habitat (Sandhya et al., 2010), and reinoculated into affected fields in order to improve the soil physicochemical properties to reclaim it and in turn improve growth and yield of crops (Nosheen and Bano, 2014; Khan and Bano, 2016). A collective approach to exploit the potential of PGPR to alleviate salinity and sodicity has recently received the attention of modern agriculturalists (Arora, 2015). In the current climate change scenario, exploitation of PGPR could be an eco-friendly strategy to promote organic farming (Figure 3).

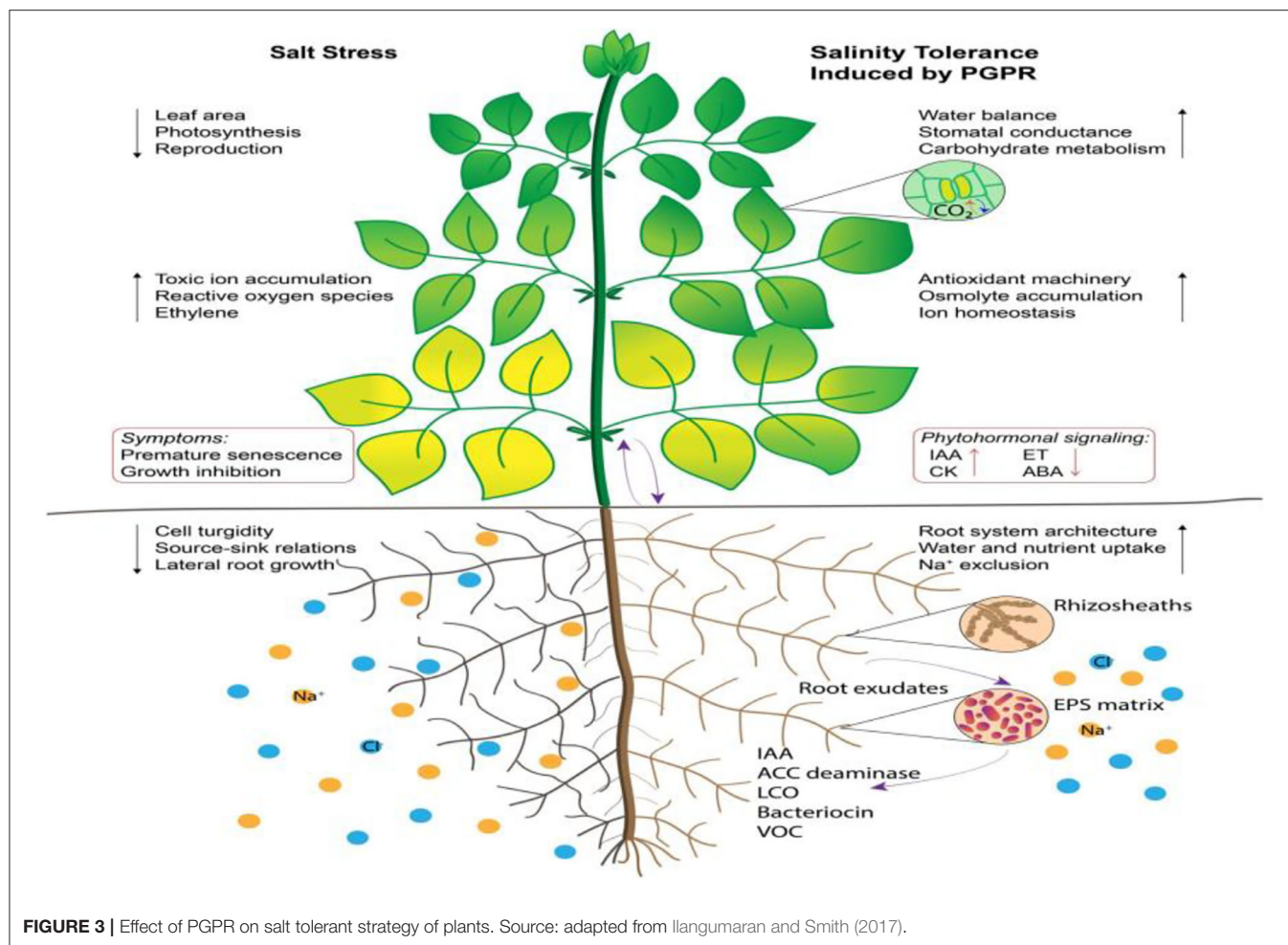
## Salt Tolerance Mechanism of PGPR

Under saline habitats, PGPR have developed two potential mechanisms to cope against salt stress (Etesami and Beattie, 2017). The first one is “salt in cytoplasm” while maintaining the osmotic potential of the cell. It was discovered in halobacteria and extreme halophilic archaea (Kunte, 2009). The PGPR accumulates intracellular  $\text{K}^+$  higher in concentration than  $\text{Na}^+$  in the environment, as  $\text{Na}^+$  are exported out through Na/K pumps (Sleator and Hill, 2002; Grant, 2004). The accumulation of K inside the cytoplasm helps in maintaining pH, acts as an activator of intracellular enzymes, and promotes accumulation of other compatible solutes (Epstein, 2003). The second mechanism is termed as “organic osmolyte” in which PGPR accumulate water soluble compatible solutes that maintain turgidity and electrolyte concentration without altering cellular metabolism and protect proteins and membranes at high salt concentration (Roberts, 2005; Tsuzuki et al., 2011; Schroeter et al., 2013; Chen et al., 2015). The osmolytes include glycerols, glutamic acid, glycine betaine, ectoine, hydroxy ectoine, proline, sugars, and trehalose (Khan et al., 2019). PGPR produces a plethora of substances which maintain the composition of outer cellular envelopes. Examples are cyclic lipopeptides (CLP) excreted by *Bacillus* and *Pseudomonas* cells (Raaijmakers et al., 2006; Banat et al., 2010).

## PGPR, Climate, and Salinity

There are five main reasons why PGPR is an ideal candidate for plants: (i) their participation in C, N, P, and S cycle; (ii) immobilization of toxic ions and solubilization of essential nutrients; (iii) production of plant growth regulators; (iv) use as soil conditioner and nutrition mobilizer (Damodaran et al., 2013); and (v) induction of salinity tolerance in plants by





modulating higher enzymatic activities (Kannan et al., 2015). It is a cheap and eco-friendly approach which consists of living inocula of PGPR already inhabiting the soil. However, selection of the best microbe which proliferates well and improves resistance to abiotic stresses should be the first priority.

Jing et al. (2015) and Delgado-Baquerizo et al. (2016) demonstrated that change in climate (seasonal variation or temperature change) can affect diversity and effectiveness of microorganisms. Indirect effects of changes which affect the functionality and microbial community include plant biodiversity, rate, composition of root exudates of host plants, and plant composition. There are also climate-related modulations of nutrient cycling and changes in the duration of the life cycle of plants which affect microbial functioning and community too.

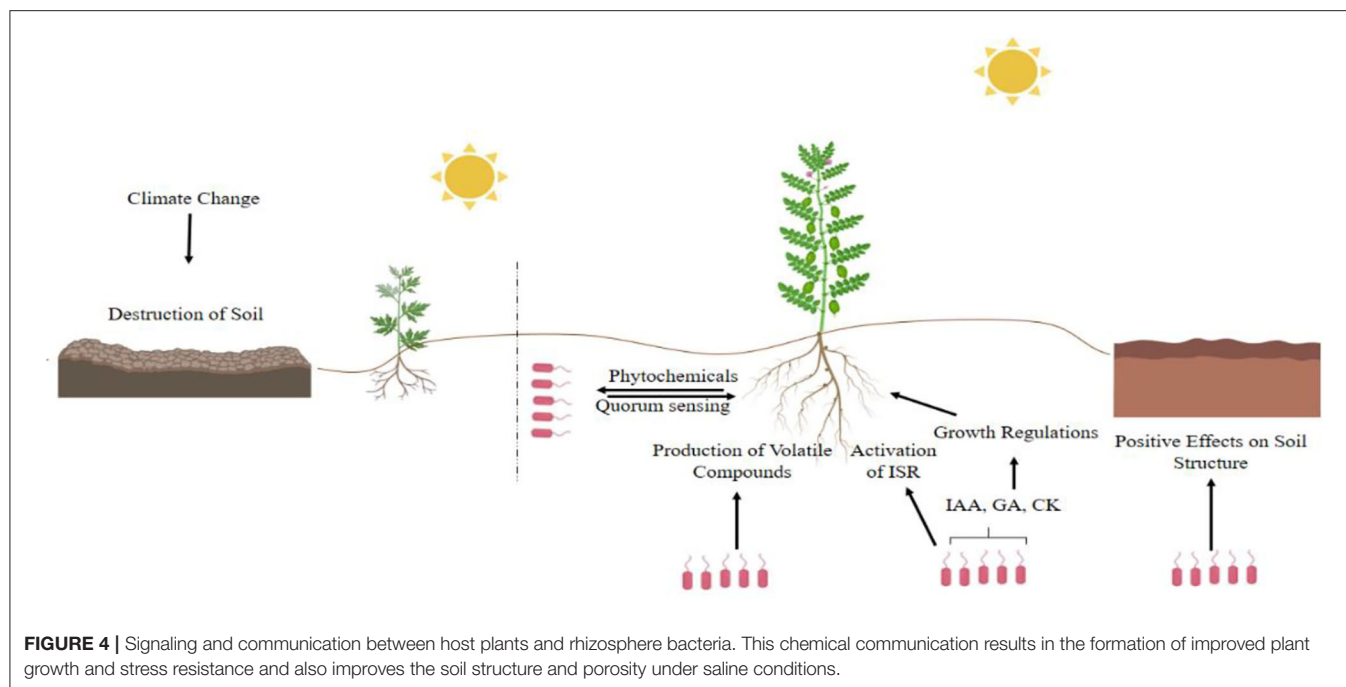
## CHEMICAL COMMUNICATION BETWEEN PLANTS AND MICROORGANISMS

### Signaling Molecules of PGPR Origin

The complex network of interkingdom signaling between plants and PGPR is governed by a number of secondary metabolites and root exudates (Bukhat et al., 2020; Khan et al., 2020a). The

nature of such communication is dynamic and influenced by a number of environmental factors. For survival and proliferation of both plants and PGPR, this cross talk is necessary because it has many advantages (the details of which are given in Figure 4). For interkingdom signaling the first signal that was exchanged between the host plant and its microbial partner was the flavonoid (2-phenyl-1, 4-benzopyrone derivatives) of plant origin (Rossen et al., 1985; AL-Kahtani et al., 2020). This molecule induces bacterial nod gene expression. Upon activation, *nod* gene code LCOs are secreted into bacteroids and act as a signal for the initiation of nodules. Myc-LCOs have a role in AMF symbiotic association with plants. Another interkingdom signaling system recently identified in plant-associated bacteria is based on HEHEAA molecule, which is spontaneously formed from plant-derived ethanolamine. It acts as a signal for LuxR solo, PipR, which is the co-inducer of *pipA* in plant root endophytic bacterium *Pseudomonas* GM79 (Coutinho et al., 2018). Bacteria produce extracellular chemical signals, which control different phenotypes in bacteria and also help in establishing an association with plants. The idea of quorum sensing (QS) signal molecules came from the discovery of autoinduction in marine bacteria *Vibrio fischeri*. In this phenomenon, bacteria produce an autoinducer signal called





homoserine lactones (AHLs) which induces bioluminescence when population density reaches a specific threshold level. So, this phenomenon was named autoinduction. Primary signal molecules generated by bacteria in QS are autoinducers which are either AHLs, quinoline signals, autoinducer-2 (AI-2), auto inducing peptides (AIPs), cyclic dipeptide, indole, short peptides, diffusible signal factors (DSF), or pheromones. AHLs were the first autoinducers identified in gram negative bacteria that regulate the QS phenomenon through LuxI/LuR regulatory proteins. In gram positive bacteria this process is regulated by small peptide autoinducers.

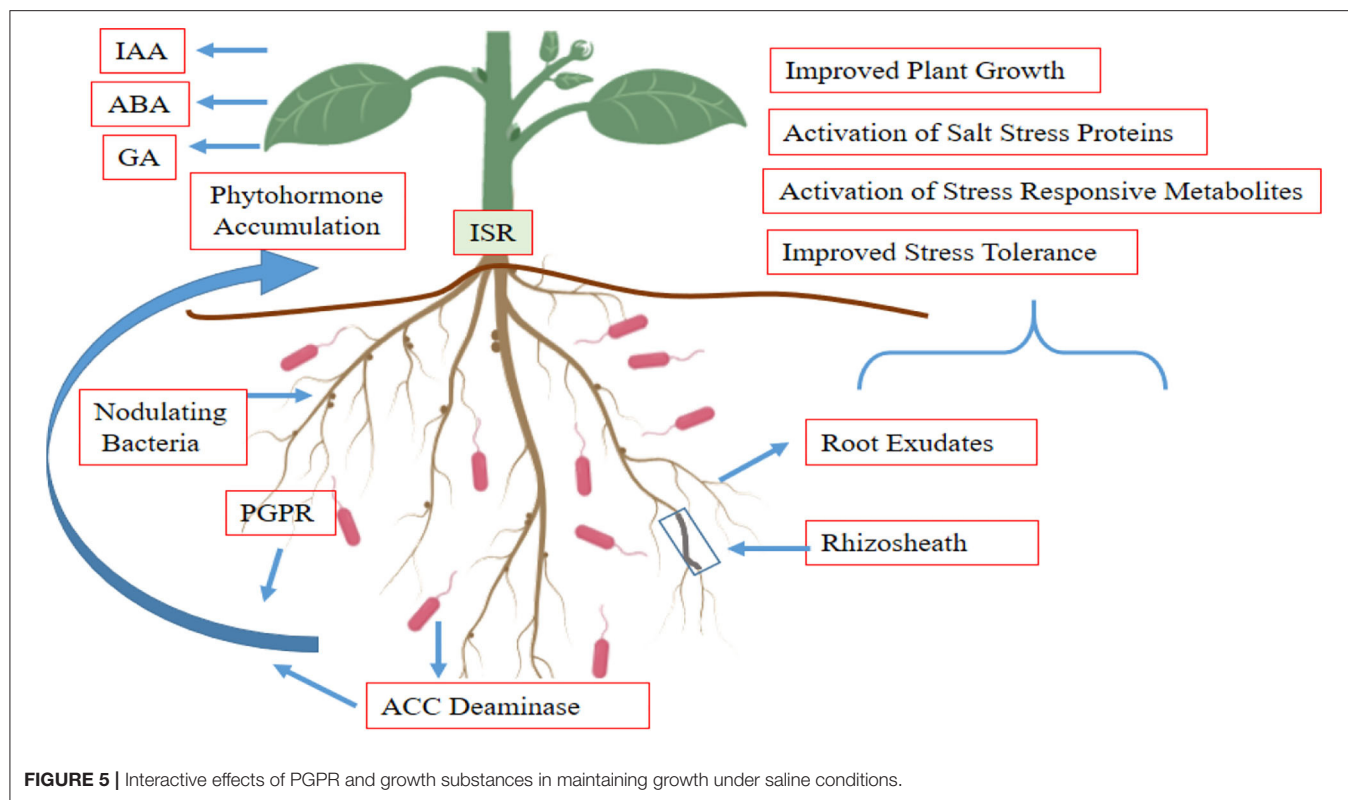
Antibiotics are another potential class of bacterial signaling molecule. Ethanolamine acetamide is an active inducer for the PipR transcription regulator in root endophyte *Pseudomonas* sp. GM79 (Coutinho et al., 2018). It is also present in leaf macerates of *Populus*. Microbes secrete phytohormone that act as growth and defense regulators to plants, i.e., gibberellic acid (GA), cytokinin (CK), abscisic acid (ABA), indole acetic acid (IAA), and jasmonic acid (JA). Volatile compounds (VOCs) are another class of molecules present in extracellular products of microbes that modulate plant growth and maintain soil health (Figure 5). These include terpenes, nitrogen compounds, pyrazines, indole, and volatile sulfur compounds (Pandey et al., 2013; Groenhagen et al., 2014; Song et al., 2015; Tyc et al., 2015). EPS secreting PGPR helps in stabilizing soil physicochemical properties, improving water holding capacity and CEC (Upadhyay et al., 2012). Some secondary metabolites act as signaling molecules, i.e., in response to flavonoids present in root exudates, and PGPR secrete nod factor which initiates nodule formation in host plants (Miransari and Smith, 2009). A thuricin compound extracted from *B. thuringiensis* enhances tolerance of *Arabidopsis thaliana* against

250 mM NaCl (Subramanian, 2013). Polyamine secreting PGPR if inoculated to plants and promotes root architecture and stomatal conductance against stress conditions (Zhou et al., 2016). The above-mentioned examples of signaling molecules along with thousands of others mediate a complex network of signaling in rhizosphere which may be (i) quorum sensing-based microbial intra or inter species signaling, (ii) plant to microbe via plant based signals, or (iii) signaling from microbes to plants.

### Signaling Molecules of Plants Origin

Many compounds of root exudates act as general chemoattractant i.e., arabinogalactan protein exuded from plant root tips into the rhizosphere to attract beneficial microbes and repel pathogens. Sugars and organic acids present in the root exudates serve as growth factors and mediate antifungal activities in certain microbes (Khan et al., 2018a). Citric acid and fumaric acid present in root exudates attract microbes and help in biofilm formation (Neal et al., 2012; Zhang et al., 2014). Flavonoids present in root exudates act as an important signal for nodule formation by inducing bacterial *nod* genes.

Strigolactones (SLs) and cutin monomers are plant-derived signals perceived by both beneficial and pathogenic bacteria (Smith, 2014). SLs belong to plant terpenoid lactones and are involved in plant fungus symbiosis. In Pi-deficient soil, SLs induce the inhibition of shoot branching in host plants and stimulate the growth of arbuscular mycorrhiza fungi (AMF) by increasing hyphal branching toward the host. During AMF infection, Myc factor is also secreted by AMF which stimulates common symbiosis signaling pathway (CSSP) or Sym (Shahid et al., 2020). Myc factors induce lateral root development by increasing root area and by providing starch to fungi to



establish symbiosis. As the CSSP pathway triggers, intracellular  $\text{Ca}^{2+}$  spiking occurs, which activates CCaMK, which in turn activates transcription of certain genes responsible for symbiosis establishment. The next step is the formation of a pre-symbiotic structure known as hyphopodium by mature hyphae. It facilitates the penetration of AMF into the roots. RAM2 gene is responsible for the formation of hyphopodium. This gene encodes glycerol-3-phosphate acyl transferase enzyme which degrades cutin monomers in the plant cell wall. Thus, cutin monomers act as a plant signal for the formation of hyphopodium. At the same time, host root cells form PPA (Pre-penetration apparatus) which direct the path for AMF colonization from hyphopodium to intracellular space, and a new structure is formed called arbuscule. The entering of AMF hyphae into the root cortex and formation of arbuscule is controlled by the Sym pathway.

Another important molecule present in root exudate is lysophosphatidylcholine which regulates the phosphate transporter gene in fungi and ensures the monitoring of Pi available to plants. In the case of ectomycorrhizal fungi (EMF), the signaling molecules that control the association with host plants are of different classes i.e., abietic acid, flavonoid rutin, hesperidin, quercetin, and chrysin compounds in root exudates lead to germination of EMF. These fungi also secrete phytohormones which should influence root morphology before the establishment of a symbiotic structure, as they reduce the growth of lateral roots where EMF establish. EMF also secrete a signaling molecule hypaphorine, an amino acid derivative which induces these morphological changes. Phenolic acids

synthesized by plants are involved in plant-microbe symbiotic association, and AMF symbiosis also plays a role in plant defense. Phytoalexins are secreted by wounded plants to repel or kill microbes. Flavonoids act as chemoattractant to rhizobia and induce the expression of nod gene by synthesis of LCOs. Coumarins are phenolic compounds found across a wide variety of plant species and have a role in iron acquisition and modulating root microbiome composition. Using synthetic community (SynCom) inoculation, it was found that the abundance of *Pseudomonas* was higher in coumarin deficient *Arabidopsis* as compared to wild type, because wild type generated a specific coumarin, sideretin, which had a toxic effect by generating  $\text{H}_2\text{O}_2$  so it caused toxicity to *Pseudomonas* strain (Khan et al., 2019; Voges et al., 2019).

Benzoxazinoids belong to indole-derived metabolites in plants that control belowground and above ground interactions. Correlating LC-MS metabolite profiling with operation taxonomic unit (OUT) sequencing data showed that benzoxazinoid stimulates the abundance of Methylophilaceae bacteria while repressing the abundance of Xanthomonadaceae bacteria. Camalexin is another indole compound which can modulate the functionality of root associated microbes (Khan et al., 2020b). Plant microbe interaction assay showed that this compound plays a key role in modulating the effectiveness of growth promoting bacteria because camalexin deficient *Arabidopsis* mutants were unable to receive growth promoting potentials that were provided by mutualistic strains. As root exudate shape microbe community, at the same time microbes

also influence the root exudate composition. For example, microbe's metabolite 2,4-DAPG and zearelenone alter the amino acid composition of tomato in order to maintain osmoregulation (Weller et al., 2007).

### Secondary Metabolites Nature on the Basis of Function

A diverse group of bioactive metabolites are produced by PGPR which are not important for normal growth and development but play an important role in stress conditions. On the basis of function, they are categorized into growth hormones, chelating compounds, antibiotics, antifungal, antitumor, and pigment compounds (Guo et al., 2013; Olanrewaju et al., 2017; Asadullah et al., 2020). Examples of such bioactive metabolites include 2, 4-diacetylphloroglucinol (DAPG), amphisin, HCN, phenazine, pyoluteorin, tensin, oligomycin A, xanthobaccin, and zwittermicin. One important role of siderophore secreting PGPR is their ability to reduce Fe availability to phytopathogens and render their proliferation (Olanrewaju et al., 2017). *P. putida* secretes Putidacin siderophore which is effective against phytopathogens (Parret et al., 2003). Some metabolites are soluble in nature, i.e., ribosomal peptides, non-ribosomal peptides, and polyketides. Pyoverdine is an example of a non-ribosomal peptide, which acts as an iron chelating compound (Hider and Kong, 2010; Khan et al., 2018b).

### Positive Impacts of Chemical Communication on Agricultural Productivity

Literature has reported on the potential role of PGPR in marinating the growth and yields of crops under different stress conditions (Table 1). GC-MS analysis showed that *Cronobacter*

sp. JZ38 produce indole and sulfur VOCs which possess volatile mediated antagonistic activity against *Phytophthora infestans* in plate assay and also act as growth inducer by promoting root and shoot biomass in *Arabidopsis thaliana* under contact assay (Eida et al., 2020). Due to the difference in AHLs structure, plant response also varies. AHL with short acyl chains increase root growth and primary root elongation in *Arabidopsis thaliana*, while AHL with long acyl chains induce resistance. This phenomenon is known as AHL priming. Rodríguez et al. (2020) evaluated the antagonistic behavior of *Pseudomonas segetis* P6 (isolated from *Salicornia europaea* rhizosphere) against phytopathogens. This bacterium produced an enzyme penicillin acylase which had degraded AHLs extracted from crude extract of bacterial phytopathogens. The strain P6 inhibited the virulence caused by pathogens through secretions of AHLs. When plants were observed, no symptoms of soft rot were found in potato tuber and carrot slice assay. An experiment was conducted on *Trichoderma asperellum* and *B. amyloliquefaciens* following simultaneous and sequential inoculation method (Karuppiah et al., 2019). The expression of defense-related vital genes and secondary metabolites were upregulated in *Trichoderma* and down regulated in *Bacillus*. Thiamine, an important cofactor for the biosynthesis of IAA in plants, was upregulated in *Trichoderma* and increased root length in maize plants' under biotic stress condition. Pipecolic acid was detected under a simultaneous inoculation of plants with beneficial microbes that induced systemic acquired resistance (SAR). Aminoglycoside is a low molecular weight antibiotic and pyocyanin is a pigment producing molecule secreted by *P. aeruginosa*; both act as signaling molecules and induce the development of biofilm by *rhizobia* species. Cis-2-dodecenoic acid is a type of diffusive

**TABLE 1 |** Alleviation of different stresses in crops by PGPR.

PGPR	Crop	Stress	Mechanism	References
<i>B. amyloliquefaciens</i>	Rice	Salinity	Increase the plant height, leaf size, root length and dry matter.	Nautiyal et al., 2013
<i>B. cereus</i> , <i>P. moraviensis</i>	<i>Triticum aestivum</i>	Saline sodic	Alleviate the effect Cu, Cr, Co, Cd, Ni, Mn, Pb and improve growth.	Hassan et al., 2017
<i>B. subtilis</i> BMB26	<i>Cucumis melo</i>	Disease	Control sclerotium rot disease caused by <i>Sclerotium rolfsii</i> .	Darma et al., 2016
<i>B. subtilis</i> , <i>B. atrophaeus</i> , <i>B. sphaericus</i>	Strawberry	Salinity	Increase biomass of strawberry.	Karlıdag et al., 2013
<i>B. subtilis</i> and <i>Arthobacter</i> sp	Wheat	Salinity	Impact on growth oxidative status.	Upadhyay et al., 2012
<i>P. fluorescens</i> , <i>Variovorax</i> sp.	<i>Brassica napus</i>	Heavy metal	Improve the phytoextraction of Cd, Cu, Pb, Zn by <i>Brassica napus</i> .	Dabrowska et al., 2017
<i>Burkholderia cepacia</i> MPC-7	Pepper	Disease	Secrete organic acids that stop the growth of <i>Phytophthora capsici</i> .	Sophaeareth et al., 2013
<i>Microbacterium</i> , <i>Curtobacterium</i>	Brassica nigra	Heavy metal	Alleviate the adverse effect of Zn, Pb, Cu, As. Improve germination.	Roman-Ponce et al., 2017
<i>P. putida</i> , <i>B. pumilus</i> , <i>L. sphaericus</i> , <i>Ex. aurantiacum</i>	Maize	Saline sodic and heavy metals stress	Improved soil physico-chemical properties reduced the uptake of Ni, Cr and Pb, enhanced yield.	Ullah and Bano, 2019
<i>P. fluorescens</i> strain ALEB 7B	<i>Atractylodes lancea</i>	Pest pathogen attack	Secrete 2-Piperidonone, inhibits the growth of phytopathogenic <i>A. rolfsii</i> .	Zhou et al., 2014
<i>Stenotrophomonas maltophilia</i> SBP-9	Wheat	Salinity	Defense response against <i>Fusarium graminearum</i> and improve growth.	Singh and Jha, 2017

signal factor (DSF) secreted by *Burkholderia cenocepacia*. It inhibits the growth of fungal pathogens such as *Candida albicans*. Luteolin is a strigolactone present in root exudates of *Medicago truncatula* and acts as a chemoattractant for *rhizobia* to induce nod gene expression. Wild type DIMBOA maize produces more benzoxazinoid and attracts more *P. putida* than DIMBOA deficient mutant maize. It was demonstrated in a study that the spraying of leaf spot pathogen of tomato (*P. syringae*) on *Arabidopsis thaliana* resulted in a higher proportion of malic acid in root exudates. The presence of aromatic acid in root exudates recruited a large number of plant growth-promoting *B. subtilis*. Root exudates of *Ocimum basilicum* contain rosmarinic acid which showed antagonistic activity against pathogenic *Pythium*. Hao et al. (2016) reported that volatiles released from *B. amyloliquefaciens* FZB42 promoted biomass of *Arabidopsis thaliana*, and a study conducted by Asari et al. (2016) revealed that seedlings of *Arabidopsis thaliana* exhibited a 2-fold increase in fresh and dry weight after 18 days of exposition to volatiles emitted from *B. amyloliquefaciens*.

PGPR secrete and modulate the concentration of auxin (IAA), cytokinin, gibberellins (GA), ethylene, and abscisic acids (ABA) in plants which regulate various metabolic activities (Egamberdieva and Kucharova, 2009; Waadt et al., 2015; Wani et al., 2016; Khan et al., 2018a). IAA, a physiologically active hormone produced by almost 80% soil microflora, utilizes tryptophan as precursor molecule (Li et al., 2018; Khan et al., 2020a). This hormone is primarily involved in cell division, morphogenesis, elongation of plants, and ultimately improved growth of shoots and yield (Asim et al., 2013; Ahemad and Kibret, 2014; Bhardwaj et al., 2014). *Pseudomonas* sp. are well-known IAA producers (Reetha et al., 2014; Kumar et al., 2015; Khan et al., 2020b), along with the secretion of organic acids like gluconic acid which solubilize insoluble phosphate and ease their availability to plants (Otieno et al., 2015; Mishra et al., 2018; Sarkar et al., 2018). Spaepen et al. (2008) reported morpho-physiological changes in root architecture upon inoculation with *Azospirillum* strain which was tested *in vitro* for phytohormone (IAA, GA, and Cytokinin) production potential. A similar outcome on wheat root morphology was demonstrated by Sadeghi et al. (2012) after being treated with *Streptomyces*. Another study to strengthen PGPR's role in plant growth promotion by signaling and regulating phytohormone level was presented by Arkhipova et al. (2007), who found remarkable signaling of cytokinin from root to shoot after root inoculation with cytokinin synthesizing bacteria *B. subtilis*.

GA facilitates germination and elongation of stem, flower, and fruit setting (Cassán et al., 2001; Hayat et al., 2010; Pandya and Desai, 2014). PGPR also secrete abscisic acid, which stimulates growth activities under stress. It is secreted by many PGPR such as *Azospirillum brasilense*, *Bacillus subtilis*, *Bacillus licheniformis*, and *Pseudomonas putida* (Cohen et al., 2008; Dos Santos et al., 2020; Kousar et al., 2020). Ethylene is a gaseous hormone which controls fruit ripening (Masood et al., 2012; Nazar et al., 2014). However, under salt stress elevated levels of ethylene arrest different physiological activities of plants. PGPR modulate ethylene level by hydrolyzing ACC into  $\text{NH}_3$  and  $\alpha$ -ketobutyrate (Siddikee et al., 2010; Gamalero and Glick, 2015; Ali and Kim,

2018) and reduce the deleterious effect of ethylene under salinity stress (Bal et al., 2013; Han et al., 2015). Under salinity stress, rice growth is significantly increased when treated with ABA producing PGPR over control (Shahzad et al., 2017; Billah et al., 2020). It was confirmed by Zhou et al. (2017) when he inoculated *Chrysanthemum* with ABA producing *B. licheniformis* under saline condition; a significant increase in seedling establishment, biomass, and photosynthesis was recorded over control plants.

One of the most important mechanisms to combat salinity stress is the production of compatible solutes. These are low molecular weight compounds that have a key role in mitigating the adverse effect of salt stress. Proline is the most studied osmolyte under salt stress, which has been reported in plants inoculated with PGPR. It has a synergetic effect on the production of other computable solutes under induced salt stress. For example, proline is involved in the synthesis of trehalose, another osmolyte in plants when inoculated with PGPR (Chen et al., 2007). Similarly, an increased concentration of total soluble sugar modulated by leaf protein content has been recorded in plants treated with PGPR (Upadhyay and Singh, 2015). Another class of osmoprotectant is glycine betaine which maintains higher leaf water potential during stress conditions (Hoque et al., 2007; Peng et al., 2008). Inoculation of maize with rhizobacteria significantly improved proline, total soluble sugar, and phenolic contents under salt-affected field condition (Iqbal et al., 2016). *De novo* synthesis of proline in *Azospirillum* can boost the wheat plant's endogenous proline accumulation (Han and Lee, 2005). Paul et al. (2005) found that *P. pseudoalcaligenes* combat high salt concentration via the production of Ala, Gly, Glu, Ser, Thr, and Asp amino acids, which help in osmotic adjustments as well as in the correct folding of polypeptide chains for maintaining structurally stable proteins under adverse environmental conditions (Streets et al., 2006). The strategy adapted for stress alleviation by these microbes includes modification of chemical constituents through deposition or alteration in capsular compounds to enhance water retention and carbon diffusion by providing micro-environment as reported in *P. fluorescens* (Ali et al., 2009). Moreover, salt-responsive genes' expression in a halophilic PGPR, such as *P. pseudoalcaligenes* and *B. subtilis*, encode a wide range of stress proteins which behave as chaperones for cellular macromolecules (Volker et al., 1994; Paul et al., 2005).

Climate change has adversely affected soil nutrient and fertility status (Pareek, 2017). This problem can be overcome by the application of PGPR which improve soil nutrient status and their uptake into plants through the release of nitrogenase, phosphatase, dehydrogenase, siderophores, and exopolysaccharides (Arshad et al., 2008; Cohen et al., 2009). Decline in  $\text{CO}_2$  fixation due to hyper osmosis cause the production of reactive oxygen species (ROS), which alter the physiology and histology of plants through changes in normal metabolic activities, damage to the phospholipid bilayer, and ultimately cell apoptosis (Johnson et al., 2003; Hichem et al., 2009; Miller et al., 2010; Halo et al., 2015). PGPR activates antioxidants and defense-related enzymes in plants and help in the mitigation of ROS (Chakraborty et al., 2013). To protect plants against oxidative stress and mimic the harmful effect



of H<sub>2</sub>O<sub>2</sub>, plants have been inoculated with PGPR (Kim et al., 2005; Upadhyay et al., 2012). The inoculation has significantly enhanced the antioxidant enzymatic system and mitigated the adverse effect of salt stress as compared to control (Damodaran et al., 2013; Jha and Subramanian, 2013). *B. megaterium* and *Enterobacter* sp. modulate ROS scavenging enzyme (SOD, CAT, APX) in *Abelmoschus esculentus* when grown under saline condition (Habib et al., 2016; Khan et al., 2018b).

## CONCLUSION AND FUTURE PERSPECTIVES

Climate change has negatively impacted the duration and intensity of seasonal changes e.g., a reduced winter season with less snow fall followed by abrupt run off in early spring, subsequently resulting in flood inundation in different areas of the world. The increased temperature during daytime in summer resulted in high evapo-transpiration that resulted in the accumulation of salt on the soil surface. It has adversely impacted physicochemical properties of agricultural land. PGPR had stimulatory effects on the growth and physiology of the plant under induced salt stress. They increased the availability of essential nutrients K and Ca, enhanced Fe and Zn in the rhizosphere soil, and enhanced the uptake by roots and its translocation to leaves and grain. The PGPR induced

salt tolerances is mediated by the production of secondary metabolites which have antimicrobial and anti-larvicidal and act as signaling molecules. It is mediated by the presence of ACC deaminase and IAA production. PGPR induces tolerance against salinity by enhancing root proliferation and root biomass and improving the water use efficiency of wheat. This minimizes the oxidative stresses but improves osmoregulation. PGPR, the natural microflora, have adopted different strategies to cope with the changing climate. Under salt stress they produce different kinds of bioactive metabolites which help in their persistence as well as providing a synergetic effect to plant growth and yield. Therefore, ideal candidates of PGPR should be selected for better agriculture productivity in salt-affected areas.

## AUTHOR CONTRIBUTIONS

AU: formal analysis, investigation, methodology, software, validation, visualization, writing-original draft, writing-review, and editing. AB: conceptualization, data curation, formal analysis, investigation, project administration, resources, supervision, writing-original draft, writing-review, and editing. NK: conceptualization, data curation, formal analysis, project administration, resources, software, supervision, validation, writing-original draft, writing-review, and editing. All authors contributed to the article and approved the submitted version.

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# Culture Media Based on Leaf Strips/Root Segments Create Compatible Host/Organ Setup for *in vitro* Cultivation of Plant Microbiota

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Plant microbiota have co-evolved with their associated plants in the entire holobiont, and their assemblages support diversity and productivity on our planet. Of importance is *in vitro* cultivation and identification of their hub taxa for possible core microbiome modification. Recently, we introduced the *in situ-similis* culturing strategy, based on the use of plant leaves as a platform for *in vitro* growth of plant microbiota. Here, the strategy is further extended by exploring plant organ compatible cultivation of plant microbiota when grown on corresponding leaf/root-based culture media. Pooling the advantages of MPN enrichment methodology together with natural plant-only-based culture media, the introduced method efficiently constructed a nutritional milieu governed by vegan nutrients of plant origin, i.e., leaf strips/root segments, immersed in plain semi-solid water agar. MPN estimates exceeded log 7.0 and 4.0 g<sup>-1</sup> of endo-rhizosphere and endo-phyllosphere, respectively, of maize and sunflower; being proportionate to those obtained for standard culture media. With sunflower, PCR-DGGE analyses indicated divergence in community composition of cultivable endophytes primarily attributed to culture media, signaling a certain degree of plant organ affinity/compatibility. Based on 16S rRNA gene sequencing of bacterial isolates, 20 genera comprising 32 potential species were enriched; belonged to Bacteroidetes, Firmicutes, and Alpha-/Gammaproteobacteria. The described cultivation strategy furnished diversified nutritive platform in terms of homologous/heterologous plant organ-based medium and ambient/limited oxygenic cultivation procedure. Duly, cultivability extended to > 8 genera: *Bosea*, *Brevundimonas*, *Chitinophaga*, *Pseudoxanthomonas*, *Sphingobacterium*, *Caulobacter*, *Scandinavium*, and *Starkeya*; the latter three genera were not yet reported for Sunflower, and possible unknown species or even one new putative genus. Thus, both potential members of the

major microbiome and rare isolates of satellite microbiomes can be isolated using the presented method. It is a feasible addition to traditional cultivation methods to explore new potential resources of PGPB for future biotechnological applications.

**Keywords:** Plant microbiota, *In situ similis* cultivation, leaf strips/root segments culture media, plant organ compatible cultivation, MPN enrichment of plant microbiota, PCR-DGGE, endo-phylosphere/endo-rhizosphere of sunflower plants

## INTRODUCTION

The plant microbiome comprises highly associated keystone microbial taxa, the core microbiome, being essential for the fitness of the plant holobiont (Toju et al., 2018; Compant et al., 2019). Similar to other environmental microbiomes, the majority of its members fails to grow under *in vitro* laboratory conditions and still under shadow (Libby and Silver, 2019). They are, therefore, often considered as the “microbial dark matter” (Lok, 2015). Especially, the low abundant taxa, satellite/rare taxa, that are increasingly recognized as contributors to community stability by acting as a reservoir that can rapidly respond to environmental changes, are often missed in isolation studies (Shade et al., 2014).

A major breakthrough in exploring environmental microbiota has been achieved with the development of DNA sequencing technologies, i.e., culture-independent techniques, and big data handling and analysis that have revolutionized our capacity to realize the complexity of microbial diversity and function. However, the absence of cultured representatives of many lineages hinders the ability to discover the roles of numerous lineages of Bacteria and Archaea present in different environments and to study how they interact with each other (Nichols et al., 2008; Stewart, 2012; Cross et al., 2019). Therefore, recent studies have revived extensive isolation efforts combined with genome sequencing and phenotypic characterization (Bai et al., 2015; Mauchline et al., 2015; Levy et al., 2018). And, several techniques have been proposed for bacterial cultivation by employing genomic data to understand either microbial interactions (network-directed targeted bacterial isolation) or ecosystem engineering [reverse genomics] (Salam et al., 2020). Such reverse-genomics, genome-informed antibody engineering, enabled cultivation of target specific groups of as yet-uncultured microbes, especially those present at low abundance or have no selectable phenotypes (Cross et al., 2019).

The reasons behind microbial “uncultivability” are numerous, and have been associated with a requirement for factors produced by other microbes, strict interspecies interactions, slow growth, competition/inhibition and dormancy (Cross et al., 2019). Here, extensive efforts are devoted to improve the cultivability of different environmental microbiota based on simulating the *in situ* environments to include less abundant but functional members. This included the optimization of substrate compositions and concentrations, gelling agents, incubation time, cell density, trace additives of syntrophic growth factors, as well as signaling molecules. Successfully, such efforts improved cultivation of rarely isolated bacterial phyla (Nichols et al., 2008, 2010). Further, high-throughput methods were innovated to

improve cultivation capabilities and recover not yet cultivated microorganisms; e.g., diffusion chamber (Kaeberlein et al., 2002; Bollmann et al., 2007), gel microdroplet-based microfluidic systems (Manome et al., 2001; Zengler et al., 2002), microfluidic streak plates (Jiang et al., 2016), combined with a number of micro-devices (Ingham et al., 2005, 2007). These tools allowed the recovery and characterization of new ecologically important isolates (Morris et al., 2002; Zengler et al., 2002; Ferrari et al., 2005; Nichols et al., 2008).

The paradigm shift in the study of the human gut microbiome happened through the application of multiple culture conditions (microbial culturomics). Such strategy involves the use of multiple isolation culture media, a range of culture conditions, prolonged incubation and rapid identification of developed colonies with MALDI-TOF MS and/or 16S rRNA gene sequencing (Lagier et al., 2016). Taking into consideration that the combinations of all culture media used throughout such strategy are basically “compatible to the humans/animals” in respect of composition, being bovine-based broth cultures together with various supplements of blood, rumen fluid, stool extract. Along that path, the plant-only-based culture media, based on juices, saps, homogenates and/or dehydrated powders of tested host plants were developed to be more compliant with the plant microbiome culturability (Nour et al., 2012; Sarhan et al., 2016; Youssef et al., 2016). In fact, such plant-only-based culture media proved to be competitive to replace several synthetic culture media, and to increase the cultivability of the plant microbiota. With the aid of culture-dependent and independent techniques, we were able to enrich previously not yet cultured bacteria (Sarhan et al., 2016, 2018, 2020; Hegazi et al., 2017; Nemr et al., 2020). Further, we later introduced the intact leaf-based culturing strategy that allowed the direct inoculation and cultivation of microorganisms on the leaf surfaces in the form of culture pads, following the rationale of “natural environments envelop multiplex of nutrients necessary for the growth of their inherent microbiota” (Nemr et al., 2020). Here, majority of plant nutrients are supplied to the growing microorganisms in their natural/proportionate concentrations and gradients, (Nemr et al., 2020). Exemplarily for sunflower (*Helianthus annuus* L.) such strategy, named “leaf *in situ similis* cultivation,” allowed the recovery of microorganisms reluctant for cultivation and extended the cultivable diversity from the endo-phylosphere and the endo-rhizosphere to members of Firmicutes, Proteobacteria and Actinobacteria. As well, culturing strains of genera not commonly reported for sunflower, e.g., *Aureimonas*, *Sphingomonas*, *Paracoccus*, *Kosakonia*, and *Erwinia*, was achieved using such *in situ similis* cultivation technique (Nemr et al.,



2020). However, there are still numerous genera (158), which already were detected using 16S rRNA metagenomic sequencing techniques in sunflower, but were not yet cultured *in vitro* (Tamošiune et al., 2020).

The “Most Probable Number, MPN” method, based on the use of liquid growth medium, is commonly used and recommended to enrich and estimate the concentration of viable microorganisms in environmental samples (Sutton, 2010; [http://www.microbiologynetwork.com/content/jgxp\\_v14n4\\_most-probable-number-method-use-in-qc-microbiology.pdf](http://www.microbiologynetwork.com/content/jgxp_v14n4_most-probable-number-method-use-in-qc-microbiology.pdf); <https://microbeonline.com/probable-number-mpn-test-principle-procedure-results>). Although it is laborious and requires longer times for incubation, the method is effective in enrichment and detection of low abundant organisms, especially those present in highly turbid/particulate samples, e.g., sediments, sludge, mud, etc., that cannot be analyzed by plate count and/or membrane filtration. In principle, the replicated dilution series of samples in selective enrichment liquid broth are prepared, and then growth is confirmed by conventional phenotypic and biochemical assays. It is reported and recommended in literature (Sutton, 2010; Russo et al., 2014) that MPN enrichment can be followed by plating microorganisms from positive tubes on selective agar plates and subsequent biochemical and molecular biology assays for isolates genera/species identification. Often the procedure is subjected to improvement in respect of developing new methods of growth measurements as well as novel selective media suitable for target organisms. Lately, MPN was successfully integrated with qPCR, in order to considerably reduce the time required for growth confirmation and detection of pathogens, e.g., *L. monocytogenes* and *E. coli* O157:H7 with lower concentration of as low as 1 CFU g<sup>-1</sup> in the corresponding selective media (Russo et al., 2014). As well to improve cultivability and performance of the method, variety of liquid media were developed for specific environments, where source material is included in the enumeration/isolation culture media, e.g., celluloses, activated sludge, marine sediments, and samples from the oil drilling industry, clarified rumen, soil extract and bacterial extracts (Mah and Smith, 1981; Wais, 1988; Tanner, 1989; Wirth and Wolf, 1990; Vester and Ingvorsen, 1998). Extracts from blood, serum, animal tissues, fecal material, sewage sludge supernatant, and clay particles were used as supplements to promote growth of microorganisms with fastidious and/or unknown requirements (Laanbroek and Geerligs, 1983). The principle of using natural media is expected to improve MPN enumeration of microorganisms in diverse ecosystems, especially those physio types which can be detected by chemical and molecular methods. So far, and to our knowledge, plant materials have not yet been used for MPN cultivation and enrichment of plant endophytic bacteria.

The major objective of the present study is to take advantage of the merits of the MPN method to apply our approach of “*in situ similis* cultivation” and to enrich the cultivable community of endophytic bacteria residing in the endo-phyllosphere and endo-rhizosphere compartments of sunflower plants. The developed MPN culturing strategy is based on leaf strips and/or root segments of tested host plants, as sole sources of nutrients,

immersed in semi-solid water agar tubes. It is postulated that such semi-solid set up is creating compatible gradients of natural nutrients and gas phases in close proximity and along the immersed plant tissues to satisfy growth and enrich a wide array of plant microbiota. Furthermore, we aim to explore affinity of homologous/heterologous cultivation of plant endophytes on respective plant organs, i.e., preference of development of endophytes of endo-phyllosphere on homologous leaf strips-based culture media and of endo-rhizosphere on homologous root segments-based culture media. MPN enrichment was followed by subjecting positive tubes to: (a) derive MPN estimates, (b) analyze composition/diversity of the culturable bacterial community by Denaturing Gradient Gel Electrophoresis (DGGE), (c) isolate on agar plates/semi-solid tubes of respective plant/organ culture media representative isolates, followed by subsequent 16S rRNA gene sequencing for identification.

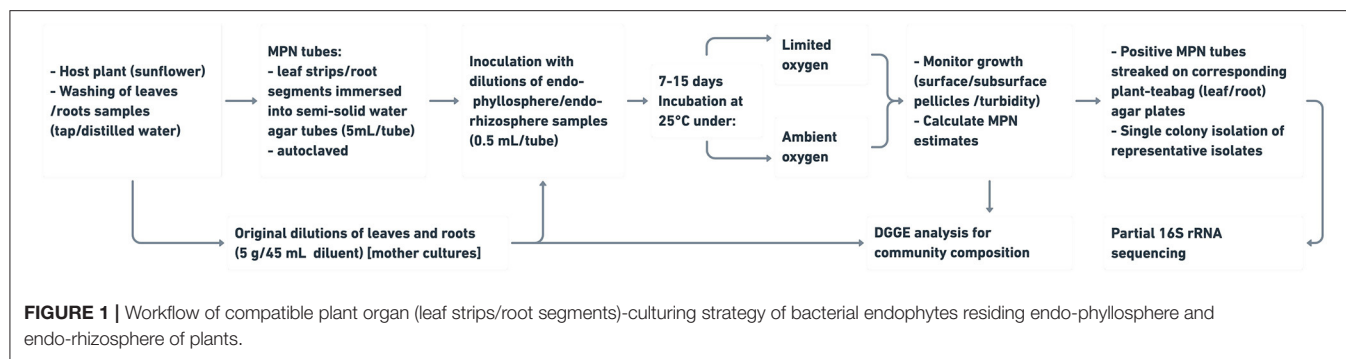
## MATERIALS AND METHODS

### Experimental Approach and Design

We postulated that plant leaves and root segments immersed in plain semi-solid water agar deliver compatible plant nutrients in their factual diversity, complexity and concentrations, and create a natural milieu for enrichment of tested bacterial plant endophytes. For this purpose, a number of preliminary experiments were carried out to initially assess the suitability of leaf strips of a number of host plants in the constructed semi-solid MPN tubes to support *in vitro* growth of corresponding plant endophytes. The developed macroscopic and microscopic growth was monitored and evaluated. Followed, two main experiments were conducted to test: (a) MPN enrichment and estimation of endophytes of maize plants on the expense of diffused nutrients of plant leaves compared to the standard culture media of R2A and CCM, and (b) the compatibility of leaf strips compared to root segments to support cross cultivation of homologous/heterologous endo-phyllosphere and endo-rhizosphere bacterial communities of sunflower plants: growth was measured in terms of population densities, MPN estimates, and community composition of the cultivable sunflower microbiome as measured by PCR-DGGE analysis and by 16S rRNA gene sequencing of representative isolates (Figure 1).

### Plant Materials and Sampling

Preliminary experiments tested the host plants of sunflower, maize, berseem clover, faba bean, lupine and mango. Extended experiments focused on maize (*Zea mays* L., cv. Cairo-1) and sunflower (*Helianthus annuus* L., Giza landrace 13). All tested host plants were grown in the experimental fields of the Faculty of Agriculture, Cairo University, Giza, Egypt (30.0131°N, 31.2089°E), and sampled at the stage of 50% flowering. For sampling, three healthy plants were randomly-sampled, where the vegetative parts were first collected, and then the root systems (intact roots with closely adhering soil) were carefully uprooted. Samples were transferred to the lab and stored at



4°C until microbiological analysis was conducted within 24 h after sampling.

## Culture Media

### Plant-Teabag Culture Medium

The plant powder teabags were prepared according to Sarhan et al. (2016), with concentration of 0.5 g L<sup>-1</sup> dehydrated powder prepared from the tested plant leaves/roots. For convenience, such plant teabag culture media were particularly used in the form of: a) agar plates (2% agar-agar, w/v) for single colony isolation of representative isolates enriched in positive MPN culture tubes, and b) semi-solid agar tubes (1.8 g agar-agar L<sup>-1</sup>) for further subculturing of secured isolates.

### Standard Chemically-Synthetic Culture Media

The standard culture media of R2A (Reasoner and Geldreich, 1985), and N-deficient combined carbon sources medium, CCM (Hegazi et al., 1998), were prepared as previously described by Elsayey et al. (2020). Agar was added to prepare semi-solid agar tubes (1.8 g agar-agar L<sup>-1</sup>) and agar culture media (2% w/v). The pH of both culture media was adjusted to 7.2, and then diluted to 1:10 (w/v) and half strength, respectively.

### Preparation of Semi-solid Culture Media Based on Leaf Strips and Root Segments

Fresh plant leaves/roots were carefully washed with tap and distilled water; then leaves were cut into strips (1 cm × 6 cm), while intact tap roots with adhering secondary roots were cut into segments of 6 cm length. Leaf strips (one piece) or root segments (2–3 segments) were transferred into each of test tubes containing 5 mL semi-solid (1.8 g agar-agar L<sup>-1</sup>) water agar. Then, tubes after being sterilized were inoculated with the prepared endo-rhizosphere and endo-phylosphere samples (s. below Endo-Rhizosphere/Endo-Phyllosphere Sample Preparation and MPN-Culturing for the Recovery of Bacterial Endophytes). The present culturing method depends on the sole use of strips/segments of leaves/roots to prepare natural plant semi-solid culture media to enrich and enumerate endophytes using the MPN method (Figure 1).

All prepared culture media were autoclaved for 20 min at 121°C, and then kept overnight at 25°C for sterility check before use.

### Incubation Conditions at Ambient and Limited Oxygen Conditions

For MPN estimation, two sets of MPN culture tubes were prepared for incubation at 25°C, under either ambient oxygen or limited oxygen conditions. Under ambient oxygen conditions, MPN tubes with conventional cotton plugs were incubated in traditional incubators, with ambient O<sub>2</sub> concentration, ca. 20.95%. While for limited/deficient oxygen conditions, under aseptic conditions, cotton plugs of MPN tubes were replaced by sterilized suba-seal rubber plugs after inoculation, and then the overhead gas phase inside the tubes was replaced with nitrogen gas. Next, MPN tubes were kept in tight glass jar, with a diameter 9.5 cm and height 22.5 cm, fitted with two glass valves at the top to allow in/out gas exchange. Once a week, the gas phase inside the jars was flushed/replaced with nitrogen gas to maintain limited oxygen conditions within the jar. A candle was placed in the jar to ensure that upon flame extinction, oxygen is exhausted.

### Endo-Rhizosphere/Endo-Phyllosphere Sample Preparation and MPN-Culturing for the Recovery of Bacterial Endophytes

Samples of plant leaves and roots, of maize and sunflower plants, were surface sterilized. Aliquots of 5 g of fresh, full grown and healthy leaves were carefully washed with tap and distilled water, surface-sterilized with ethanol (70% for 1 min), sodium hypochlorite (3% for 5 min) and ethanol (70% for 1 min) and then carefully washed with sterilized distilled water (de Oliveira Costa et al., 2012; Jackson et al., 2013). For roots, 5 g of almost intact roots (taproot with adhering secondary roots) were surface-sterilized with 95% ethanol for 5–10 s; followed by 3% sodium hypochlorite for 30 min and then carefully washed with sterilized distilled water (Youssef et al., 2004).

Original suspensions, named mother culture, of both endo-phylosphere and endo-rhizosphere, were prepared under sterile conditions by crushing ca. 5 g surface-sterilized leaves or roots in a Waring blender with ca. 45 mL of basal salts of CCM as diluent. Further, 10-fold serial dilutions were prepared in the same diluent, then dry weights (at 70°C) were determined for suspended leaves and roots (Youssef et al., 2016). Aliquots of

0.5 mL of each of suitable dilutions ( $10^{-2}$ : $10^{-5}$ ) of plant endo-rhizosphere and ( $10^{-1}$ : $10^{-4}$ ) of endo-phyllosphere samples were used to inoculate the above prepared semi-solid MPN tubes of leaf strips and root segments. Two sets of MPN tubes of either plant spheres were prepared, and incubated under ambient oxygen and limited oxygen conditions. Macroscopic growth, in terms of bacterial surface/subsurface pellicles and/or developed turbidity, and microscopic observation of growing cells, was monitored throughout 7–15 days of incubation at 25°C. MPN estimates were derived using Cochran's Table (Cochran, 1950). To assure sterility of culture media used as well as to obtain appropriate growth index required for MPN estimation, it is imperative to obtain no-growth in a number of MPN culture tubes of the inoculated higher dilutions.

Positive MPN culture tubes of sunflower plants were subjected to further plating on corresponding plant agar plates for the purpose of single colony isolation of representative isolates. After 7–5 days of incubation under ambient and limited oxygen conditions, respectively, aliquots (20  $\mu$ L) from homogenized growth in representative positive MPN tubes were surface-plated on agar culture media prepared from corresponding sunflower leaves or roots-based culture media. Then, agar plates were incubated for 7–15 days under corresponding incubation conditions. For limited oxygen conditions, agar plates were kept in tight plastic box, 25  $\times$  37  $\times$  18 cm (16.65 L capacity), fitted with two valves at the top/bottom to allow in/out gas exchange. Every 2 days, the gas phase inside the box was flushed/replaced with nitrogen gas to create limited oxygen conditions within the box. A candle was placed in the box to ensure that upon flame extinction, oxygen is exhausted. More than 1,068 colonies were randomly picked to represent all CFUs proportional to their abundance for further sub-culturing on corresponding plant agar plates, and isolates recovered were subjected to 16S rRNA gene analysis (Supplementary Table 1).

## DNA Extraction and 16S rRNA Gene Analysis of Bacterial Isolates

The genomic DNA of successively-subcultured isolates (Supplementary Table 1) was extracted using the QIAGEN DNeasy Plant Mini Kit (Qiagen Inc., Hilden, Germany) according to the manufacturer's instructions (Sarhan et al., 2016). The 16S rRNA genes were amplified with the forward primer "9bfm" (GAGTTTGATYHTGGCTCAG) and reverse primer "1512R" (ACGGHTACCTTGTTACGACTT) (Mühling et al., 2008). The PCR setup in a 25  $\mu$ L volume was as follows: QIAGEN TopTaq Master Mix Kit 12.5  $\mu$ L, PCR water 5.5  $\mu$ L, primer 9 bfm (3.1 pmol  $\mu$ L $^{-1}$ ) 2.5  $\mu$ L, primer 1512R (3.1 pmol  $\mu$ L $^{-1}$ ) 2.5  $\mu$ L, and target DNA (ca. 15 ng  $\mu$ L $^{-1}$ ) 2.0  $\mu$ L. The amplification of DNA was performed according to the thermal amplification cycling program: 4 min initial denaturing at 96°C, 30 thermal cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min, and extension at 74°C for 1.5 min. The PCR products were purified using PCR purification kit (Qiagen Inc.), concentration adjusted to 10 ng

$\mu$ L $^{-1}$  and submitted for sequencing to Eurofins MWG Operon (Ebersberg, Germany).

## DGGE Analysis

### DNA Extraction From MPN Tubes and Root/Shoot Mother Cultures

After 7–15 days of respective ambient and limited oxygen incubation, all surface/sub-surface pellicles developed in 3 MPN positive tubes, as replicates, representing the lowest dilution of endo-phyllosphere ( $10^{-1}$ ) and endo-rhizosphere ( $10^{-2}$ ) of all tested culture media, were individually harvested in 2.0 mL Eppendorf tubes. The pellicles' harvest, as well as samples of previously prepared original leaves/root suspensions (mother cultures; in three replicates), were centrifuged at 13,000 rpm for 10 min. DNA was extracted from the resulting pellets using the genomic DNA Extraction Mini Kit (iNtRON Biotechnology, Kyungki-Do, Korea). DNA quality was checked using the BioDrop  $\mu$ LITE spectrophotometer (Biochrom, Holliston, MA, USA).

### Amplification of the 16S rRNA Gene and DGGE Fingerprinting

The protocols for 16S rRNA gene amplification and nested PCR of the V3-16S rRNA gene by using the 341f-GC (CGCCGCGCGCGCGGCGGGCGGGGCGGGGCACGGGG-CCTACGGGAGGCAGCAG) and 518r (ATTACCGCGGCTGCTGG) primers (Muyzer et al., 1993; Mühling et al., 2008), adopted by Sarhan et al. (2016), were used. 16S rRNA gene amplification from DNA extracted, either from the pellicles' harvest or original leaves/root suspensions, mother cultures, was performed with the Hightech Thermocycler (SensoQuest, Göttingen, Germany).

DGGE was performed using the VS20WAVE-DGGE (Clever Scientific Ltd, Warwickshire, UK). Aliquots of 10  $\mu$ L of each sample were mixed with 3  $\mu$ L of a 6X loading dye (glycerol, xylene cyanol, bromophenol blue), then heated at 95°C for 5 min, and stored at 65°C until loading. Amplicons were electrophoresed on an 8% acrylamide gel containing a 30 to 70% denaturing gradient of formamide and urea with 1  $\times$  TAE buffer. After 3 min of initial migration at 200 V to push the sample into the gel, DGGE was conducted at 60°C for 20 h at 50 V. The gel was stained for 30 min with the 6X ethidium bromide, photographed, and analyzed for DGGE band profiles with the MicroDOC System with UV Transilluminator (Clever Scientific Ltd, Warwickshire, UK). A self-created standard of mixed PCR products from 4 pure bacterial strains (*Arthrobacter globiformis* DSM 20124 [GC content 62%], *Bifidobacterium breve* DSM 20213 [GC content 58.8%], *Pectobacterium carotovorum* DSM 30168 [GC content 49%], *Lactobacillus plantarum* DSM 20174 [GC content 44.3%]) was included in every DGGE run (Supplementary Figure 4). All of these strains were obtained from the "Deutsche Sammlung von Mikroorganismen und Zellkulturen" (DSMZ, dsMZ.de) and revived according to the provider's instructions.



## Phylogenetic Analysis

For phylogenetic analyses, the 16S rRNA gene sequences were taxonomically assigned by comparison with those available in GenBank using BlastN (Altschul et al., 1990). The 16S rRNA gene sequences of the isolates and their closely/nearest related strains were aligned together with respective type strain (T) sequences by Clustal Omega version 1.2.4 (Madeira et al., 2019). The alignment was trimmed with trimAl version 1.4.rev22 (-gt 0.8/0.7 -st 0.001 -cons 70) (Capella-Gutiérrez et al., 2009). Phylogenetic trees were constructed by using the Maximum Likelihood method under the GTRCAT model, implemented in RAXML (Stamatakis, 2014). Bootstrapping was performed on 1,000 replicates, and the inferred tree was saved in Newick format and visualized with iTol (itol.embl.de) (Letunic and Bork, 2007).

## Statistical and Numerical Analyses

The analysis of variance (ANOVA, Tukey-test) and the honestly significant differences (HSD) were calculated using MSTAT-C software (Michigan State University, MI, United States). The R-package “ggplot2” was used for the construction of barplots.

The band profiling of DGGE gels was analyzed using CLIQS (TotalLab, Newcastle upon Tyne, UK) and binary data were exported and further used for  $\alpha$ -diversity indices calculations using PAST v.3.22 (Hammer et al., 2001).

## RESULTS

### Semi-solid Culture Media Based on Leaf Strips of Various Host Plants Equipped *in vitro* Enrichment of Plant Endophytes

Preliminary experiments demonstrated that nutrients available in leaf strips of a variety of host plants immersed in plain semi-solid water agar are sufficient enough to support and enrich *in vitro* growth of plant endophytes. The semi solid tubes prepared from leaf strips of berseem-clover, faba bean; lupine and mango created rich nutritional matrix that supported profuse growth of plant endophytes. Microscopic examination of developed surface/subsurface pellicles resolved diverse cell morphologies, e.g., short rods, cocci cells, long rods, spore-forming bacilli, free spores and yeast cells (Supplementary Figure 1).

### Comparable to Standard R2A and CCM, Leaf Strips-Based Culture Media Supported Good Growth of Bacterial Endophytes of Maize Endo-Phyllosphere

Growth/enrichment of bacterial endophytes of maize endophyllosphere was assayed in semi-solid agar tubes prepared from standard culture media, CCM and R2A, and strips of both young and old leaves of maize plants. Upon incubation, the visual/macrosopic and confirmed microscopic observation of bacterial growth was monitored in the prepared semi-solid culture tubes. It appeared that leaf strips furnished a rich nutritional milieu that supported dense MPN estimates of bacterial endophytes of maize endo-phyllosphere, being  $> \log 7.0 \text{ g}^{-1}$  leaf and very much proportionate to

**TABLE 1 |** MPN estimates of bacterial endophytes of endo-phyllosphere of maize as developed on semi-solid culture media of leaf-strip-based culture media and diluted standard CCM and R2A culture media: One-way ANOVA analysis for culture media effect.

Treatments (MPN semi-solid culture media)	log MPN $\text{g}^{-1}$ leaf
1/10 strength R2A	8.685 <sup>a</sup>
½ strength CCM	8.454 <sup>b</sup>
Maize-old leaves	7.184 <sup>d</sup>
Maize-young leaves	8.168 <sup>c</sup>

Statistically significant differences are indicated by different letters ( $P \leq 0.05$ ).

those derived for standard R2A and CCM culture media (Table 1; Supplementary Figure 2). Strips of young leaves were relatively more nutritive, and significantly supported higher MPN estimates compared to old leaves.

### Not Only Leaf Strips but Also Root Segments Promoted *in vitro* Cultivation of Bacterial Endophytes of Sunflower Plant Compartments

With sunflower plants, preliminary tests indicated that not only leaf strips but also root segments supported good growth of bacterial endophytes of endo-phyllosphere and endo-rhizosphere. Microscopic examination confirmed the presence of dense and diverse cell morphologies, e.g., short rods, long rods, spore-forming bacilli, free spores and cocci cells (Supplementary Figure 3). Therefore, experimentation was enlarged to include semi-solid culture media based on both leaf strips and root segments, as well as incubation under ambient and limited oxygen conditions.

The most probable number (MPN) method was used to estimate the population densities of bacteria in both compartments of the sunflower endo-rhizosphere and endo-phyllosphere. MPN estimates were based on visual and microscopic observation/confirmation of bacterial growth developed in semi-solid agar culture media based on leaf strips and root segments under ambient or limited oxygen conditions. In general, bacterial populations of either plant compartments amply enriched in MPN culture tubes based on both plant leaf strips and root segments.

Under ambient oxygen atmosphere, significant differences were attributed to the single effects of plant spheres and culture media. Higher MPN estimates were reported for the endo-rhizosphere ( $6.9 \log \text{ MPN estimates g}^{-1}$ ) compared to the endo-phyllosphere (only  $3.8 \log \text{ MPN estimates g}^{-1}$ ) (Table 2). Notably, the root segments-based culture medium supported somewhat lower bacterial growth ( $5.3 \log \text{ MPN estimates g}^{-1}$ ) in comparison to the leaf strips-based culture medium ( $5.4 \log \text{ MPN estimates g}^{-1}$ ). Of interest is that higher MPN estimates were achieved for homologous vs. heterologous culturing; i.e., endo-rhizosphere bacteria favored culture media based on root segments with  $7.2 \log \text{ MPN estimates per g root}$  related to only  $6.7 \log \text{ MPN estimates g}^{-1}$  when grown on leaf strips-based culture medium. Likewise, endo-phyllosphere bacteria preferred



**TABLE 2 |** MPN estimates of endo-rhizosphere and endo-phyllosphere bacteria of sunflower plants as developed on leaf strips/root segments-based semi-solid culture media, under ambient oxygen conditions: two-way ANOVA analysis.

Treatments	Log MPN estimate g <sup>-1</sup> leaf/root
<b>Factor (A) Plant spheres</b>	
Endo-rhizosphere	6.936 <sup>a</sup>
Endo-phyllosphere	3.828 <sup>b</sup>
<b>Factor (B) culture media based on leaf strips and root segments</b>	
Root segments	5.353 <sup>b</sup>
Leaf strip	5.411 <sup>a</sup>
<b>Two-way interactions (A X B)</b>	
Endo-rhizosphere/root segments culture media (RR)	7.209 <sup>a</sup>
Endo-rhizosphere/leaf strip culture media (RL)	6.663 <sup>b</sup>
Endo-phyllosphere/leaf strip culture media (PL)	4.159 <sup>c</sup>
Endo-phyllosphere/root segments culture media (PR)	3.497 <sup>d</sup>

Statistically significant differences are indicated by different letters ( $P \leq 0.05$ ).

**TABLE 3 |** MPN estimates of bacteria of endo-rhizosphere/endo-phyllosphere of sunflower plants as developed on leaf-strips/root segments- based semi-solid culture media, under limited oxygen conditions: two-way ANOVA analysis.

Treatments	Log MPN estimate g <sup>-1</sup> leaf/root
<b>Factor (A) Plant Spheres</b>	
Endo-rhizosphere	5.873 <sup>a</sup>
Endo-phyllosphere	5.233 <sup>b</sup>
<b>Factor (B) culture media based on leaf strips and root segments</b>	
Root segments	5.463 <sup>a</sup>
Leaf strip	5.643 <sup>a</sup>
<b>Two-way interactions (A X B)</b>	
Endo-rhizosphere/root segments culture media (RR)	5.908 <sup>a</sup>
Endo-rhizosphere/leaf strip culture media (RL)	5.837 <sup>a</sup>
Endo-phyllosphere/leaf strip culture media (PL)	5.449 <sup>b</sup>
Endo-phyllosphere/root segments culture media (PR)	5.017 <sup>bc</sup>

Statistically significant differences are indicated by different letters ( $P \leq 0.05$ ).

the culture medium based on leaf strips with 4.1 log MPN estimates g<sup>-1</sup> contrast to 3.5 log MPN estimates g<sup>-1</sup> on root segments-based culture medium (Table 2).

Under limited oxygen conditions, the effects of plant spheres and culture media were not as distinctive as under ambient oxygen conditions. But, likewise, the endo-rhizosphere MPN estimates were higher compared to those of the endo-phyllosphere (5.9 and 5.2 log MPN estimates g<sup>-1</sup>, respectively). The culture medium effect was not significant (Table 3). Compared to heterologous cultivation, the homologous cultivation on corresponding leaf strips/root segments culture media were more advantageous; however, differences did not approach significant levels.

## DGGE Analysis Indicated Divergence in Community Composition of Cultivable Bacterial Endophytes of Sunflower Attributed to Homologous/Heterologous Cultivation

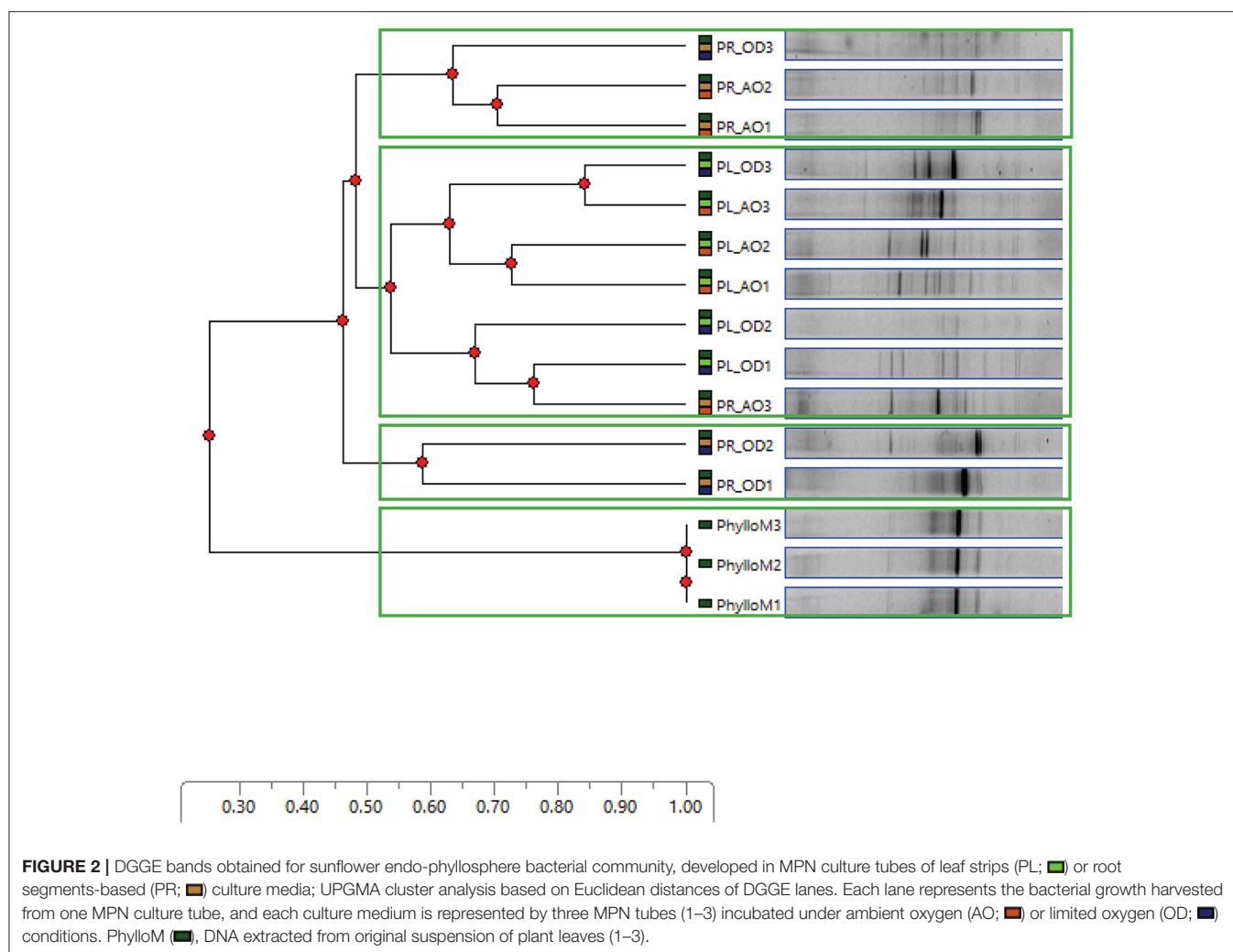
PCR-DGGE fingerprinting of the 16S rRNA gene segment recovered from *in vitro* growth developed in MPN culture tubes, representing all combinations of tested culture media and growth conditions, was used to compare the composition of enriched cultivable endophytic communities, in both endo-rhizosphere and endo-phyllosphere of sunflower. We primarily aimed at checking possible differential enrichment of endophytic bacteria of endo-phyllosphere when developed in homologous leaf strips-based culture media compared to the heterologous root strips-based culture media, and vice versa. Included in the analysis as well were the genomic 16S rRNA samples that were extracted from the initial root and leaf suspensions, originally prepared for MPN culturing. DGGE analysis resulted in clear banding patterns (Supplementary Figure 4), and cluster analyses of produced DGGE bands of bacterial endophytes

of both plant compartments are illustrated in Figures 2 and 3, respectively.

Based on the analysis of distance scores by Phoretix 1D pro software, the constructed dendrogram of endo-phyllosphere is shown in Figure 2. Setting a Cluster Cutoff Value of 0.55 resulted in four different clusters. The original suspension of the leaf material clearly clustered apart from all other samples at earlier similarity level of 0.20. The remaining three clusters divided the MPN culture tubes in regard to the culture medium used. One independent cluster, at similarity level 0.52, contained endo-phyllosphere bacteria developed in the homologous MPN leaf strips-based culture media. While the separated endo-phyllosphere bacteria grown on the heterologous root segments-based culture medium split into two subgroups/clusters. No obvious trend/effect was related to the oxygen status during incubation. These results show a distinct enrichment of endo-phyllospheric bacteria when grown on leaf strips-based compared to root segments-based media.

As to the cultivable bacterial communities of the endo-rhizosphere (Figure 3), and at the same Cluster Cutoff Value of 0.55, two independent clusters were detectable. The first cluster separated endo-rhizosphere communities developed on the MPN culture tubes of homologous root segments away from those of the heterologous leaf strips-based MPN culture tubes. The second cluster contained sub-clusters of the community of DNA extracted from the original suspension of plant roots and of the culturable endo-rhizosphere community grown on leaf strips-based culture medium. Again, no specific separation or pattern was specifically acclaimed to oxygen availability during incubation.

Comparing the band pattern composition and density of the original plant materials to those developed in leaf strips/root segments-based MPN culture tubes, an enrichment of minor



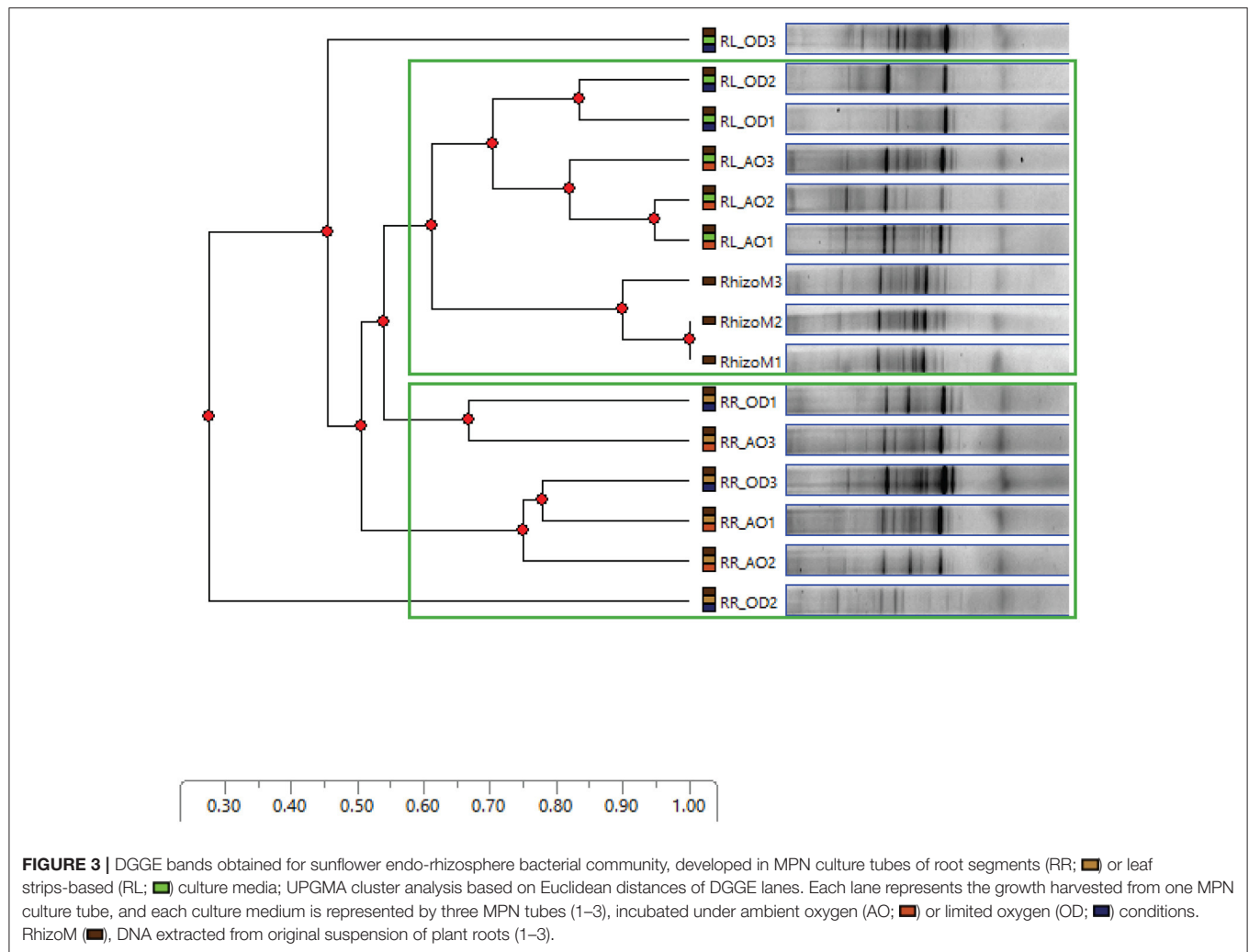
abundant groups was visible in the plant-based medium. Bacterial endophytes with higher GC content seem to be notably enriched on the plant-based semi-solid MPN culture tubes prepared for either endo-phylosphere or the endo-rhizosphere samples.

### Leaf Strips/Root Segments Setup Significantly Enriched/Extended Diversity of Bacterial Isolates Representing Culturable Bacterial Endophytes of Sunflower Plants

Due to problems known to be related to domestication of isolates (Nichols et al., 2010), out of 1,068 CFUs randomly selected on all tested culture media and under various incubation conditions, a number of 566 isolates were progressively sub-cultured. And, 163 of which produced good quality sequences (Supplementary Table 1). These isolates represented the cultivable microbiota of endo-rhizosphere (69) and endo-phylosphere (94) of sunflower plants, being developed under ambient (116) or oxygen limited conditions (47),

and enriched on leaf strips and root segments-based culture media (Figures 4, 5; Supplementary Table 2). Over all, the leaf strips/root segments cultivation strategy in terms of combinations of plant compartments, tested culture media and growth conditions create opportunities for the isolation of multiple and diverse bacterial genera of sunflower microbiota (Supplementary Figure 5). In total, such combinations facilitated the cultivability of 20 genera, belonging to the three phyla Bacteroidetes, Firmicutes and Proteobacteria, and comprising 32 potential species (Supplementary Table 2).

Of common presence and prevalence was the genus *Bacillus* with 76 isolates (46.6% of total isolates), with potential species of *Bacillus* sp. (46 isolates), *B. cereus* (10), *B. anthracis* (7), *B. subtilis* (4), *B. safensis* (4), *B. altitudinis* (2), *B. thuringiensis* (1), *B. megaterium* (1), and *B. pumilus* (1). The following genera were *Paenibacillus* (19 isolates, 11.7%); *Rhizobium* (18 isolates, 11%); *Pseudomonas* (15 isolates, 9%) and *Enterobacter* (8 isolates, 5%) (Supplementary Figure 5). The remaining genera, representing 53.4% of the isolates, were of low incidence, represented with  $\leq 4$  isolates each, such as *Novosphingobium* (4), *Agrobacterium* (3), *Pseudoxanthomonas* (3), *Scandinavium*. (3), *Starkeya* (3),



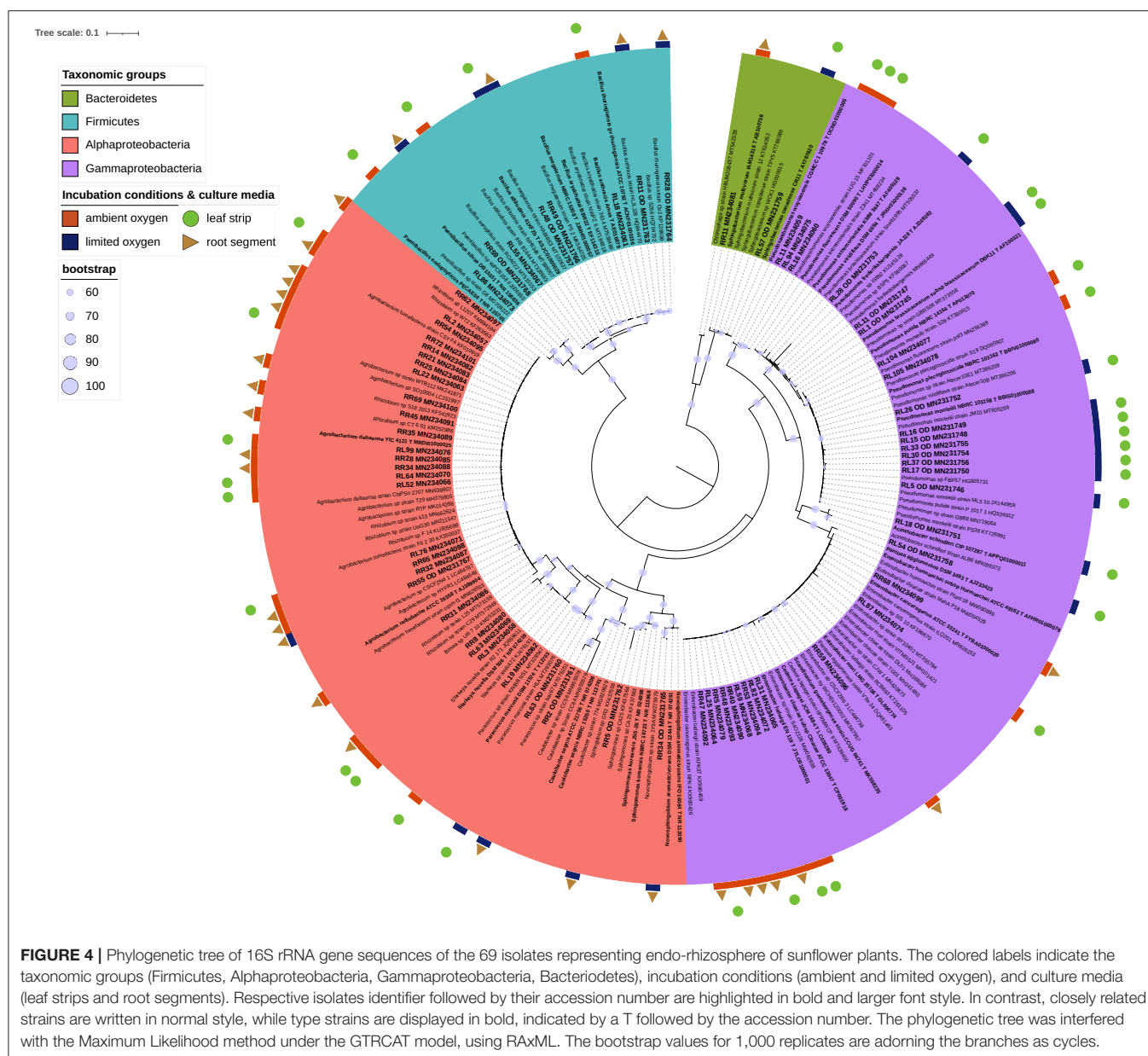
*Sphingobium* (2), and with single isolate of *Acinetobacter*, *Bosea*, *Brevundimonas*, *Caulobacter*, *Chitinophaga*, *Paracoccus*, *Pantoea*, *Sphingobacterium*, and *Sphingomonas*. Interestingly, the taxonomic and phylogenetic analysis revealed significantly lower identity values for the isolates PR85 (MN232172, *Sphingobium* sp.) and PR21\_OD (MN231735, *Bacillus* sp.) of 98.85 and 98.49% identity for their partial 16S rRNA gene fragments of 435 and 797 nt length, respectively (Supplementary Table 2). Moreover, isolate PR21\_OD showed a unique split that resulted into a long branch, indicating an earlier divergence from the *Sphingobium* isolates (Figure 5).

### Homologous/Heterologous Culturing on Leaf Strips/Root Segments-Based Culture Media of Significant Impact and Signaling Potential Host Organ Compatible Cultivation

Irrespective of culture media and growth conditions, the endo-rhizosphere isolates were of higher diversity and belonged to 18 genera, associated to three different phyla, dominated by

Proteobacteria (Alphaproteobacteria), rather than by Firmicutes and Bacteroidetes (Figure 6). In contrast, only 6 genera were recovered from the endo-phylosphere, which are assigned to two phyla, dominated by Firmicutes more than Proteobacteria (mostly Alphaproteobacteria).

In detail, 50 of the *Bacillus* isolates from the endo-phylosphere (in total 70 of 76 isolates) were cultivated on the root segments based medium and among them the majority of the isolates grew under ambient oxygen condition (38/50). And, 17 out of 19 *Paenibacillus* isolates were obtained from the endo-phylosphere, being more abundant (16/17) on leaf strips-based medium under ambient oxygen level. Endo-rhizospheric isolates of the genus *Rhizobium* prevailed under ambient oxygen level in both plant compartment sources with higher presence on root specific media (12/18). Noteworthy, all 15 *Pseudomonas* isolates, mostly isolated from the endo-rhizosphere (14/15), exhibited preferences for cultivation on leaf strip media only, mostly under limited oxygen conditions (13/15). *Enterobacter* isolates, all of which isolated from the endo-rhizosphere, obtained exclusively under ambient oxygen and on both leaf strips/root segments culture media.



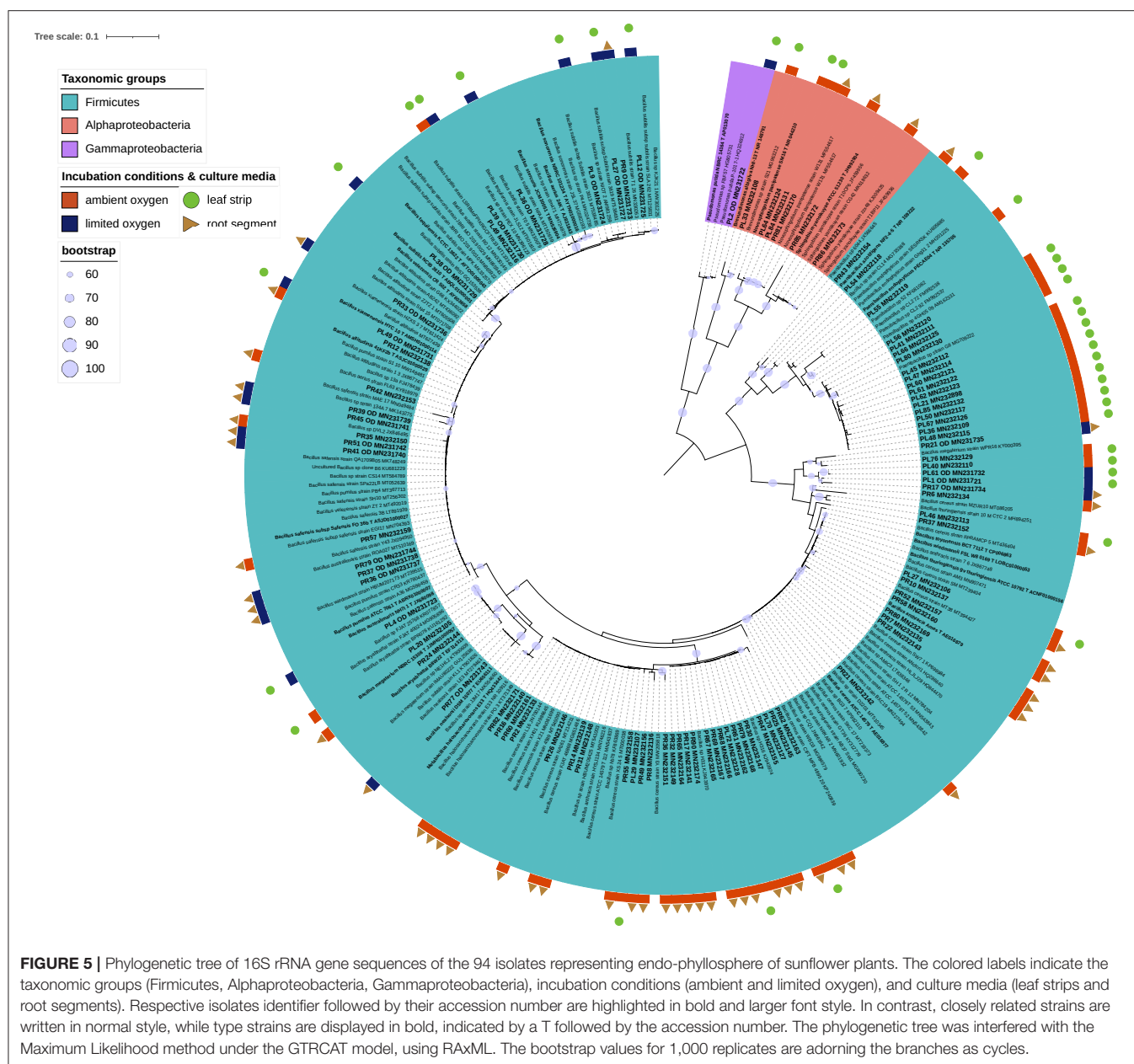
Regarding the plant spheres, 2 genera of *Brevundimonas* and *Sphingobium* were solely isolated from the endo-phylosphere, while 14 genera were obtained uniquely from the endo-rhizosphere (e.g., *Rhizobium*, *Enterobacter*, *Agrobacterium*, *Starkeya*, *Caulobacter* and *Scandinavium*). Interestingly an overlap of only 4 genera, *Bacillus*, *Paenibacillus*, *Novosphinobium*, and *Pseudomonas*, was detected for both plant compartments (**Figure 7A**).

Comparing the cultivability on both culture media, a number of bacterial genera were confined to either culture media, where 7 genera were recovered on leaf strips-based culture medium (e. g. *Brevundimonas*, *Pseudoxanthomonas*, *Starkeya*, *Sphingobacterium*) and 7 genera exclusively on root segments-based medium

(e. g. *Agrobacterium*, *Sphingobium*, *Bosea*, *Caulobacter*, *Chitinophaga*). The remaining 6 genera, *Bacillus*, *Paenibacillus*, *Rhizobium*, *Enterobacter*, *Novosphingobium*, and *Scandinavium*, exhibited good growth in both culture media (**Figure 7B**).

Representatives of the cultivable genera varied in their oxygen requirements, where 10 genera favored ambient oxygen (e.g., *Rhizobium*, *Enterobacter*, *Scandinavium*, *Starkeya*, *Pseudoxanthomonas*, *Bosea*, *Chitinophaga*, *Brevundimonas*) and 5 genera dominated the limited oxygen conditions, such as *Sphingobacterium* and *Caulobacter* among others (**Figure 7C**). The representative isolates of *Bacillus*, *Paenibacillus*, *Novosphingobium*, *Pseudomonas*, and *Agrobacterium* were able to grow and develop under both ambient and limited oxygen conditions.

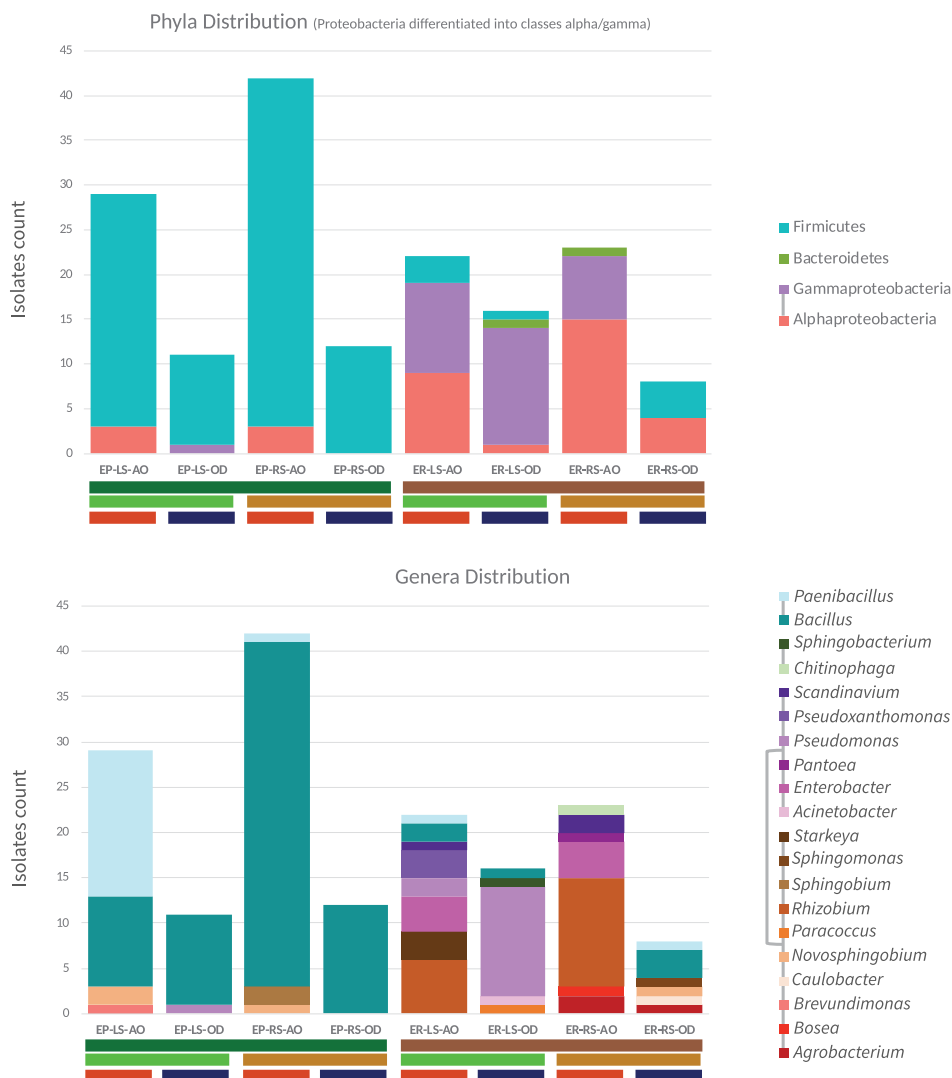




Characteristically and under ambient oxygen, a number of genera were exclusively developed in the homologous combinations of endo-rhizosphere/root segments (*Bosea*, *Pantoea*, and *Chitinophaga*) and of endo-phylosphere/leaf strips (*Brevundimonas*) culture media. Heterologous combinations allowed the growth of representative of a few numbers of genera, e.g., endo-rhizosphere/leaf strips (*Starkeya*, *Pseudoxanthomonas*), endo-phylosphere/root segments (*Sphingobium*). Under limited oxygen conditions, isolates representing *Caulobacter* and *Sphingomonas* were particularly enriched under homologous conditions of endo-rhizosphere/root segments. Among other examples of heterologous combinations are *Acinetobacter*, *Sphingobacterium*,

and *Paracoccus* that were enriched from the endo-rhizosphere on leaf strips media.

However, we must bear in mind, that representative isolates of the other genera were commonly grown under a variety of combinations of tested growth conditions (**Supplementary Figure 6**). Exemplarily, under limited oxygen growth conditions, and common to the ambient oxygen conditions, representatives of *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Agrobacterium*, and *Novosphingobium* were enriched. Hereby, under ambient conditions, mostly all *Paenibacillus* isolates were enriched from endo-phylosphere on leaf strips-media, while phylospheric *Bacillus* isolates were more often found on root segments- media

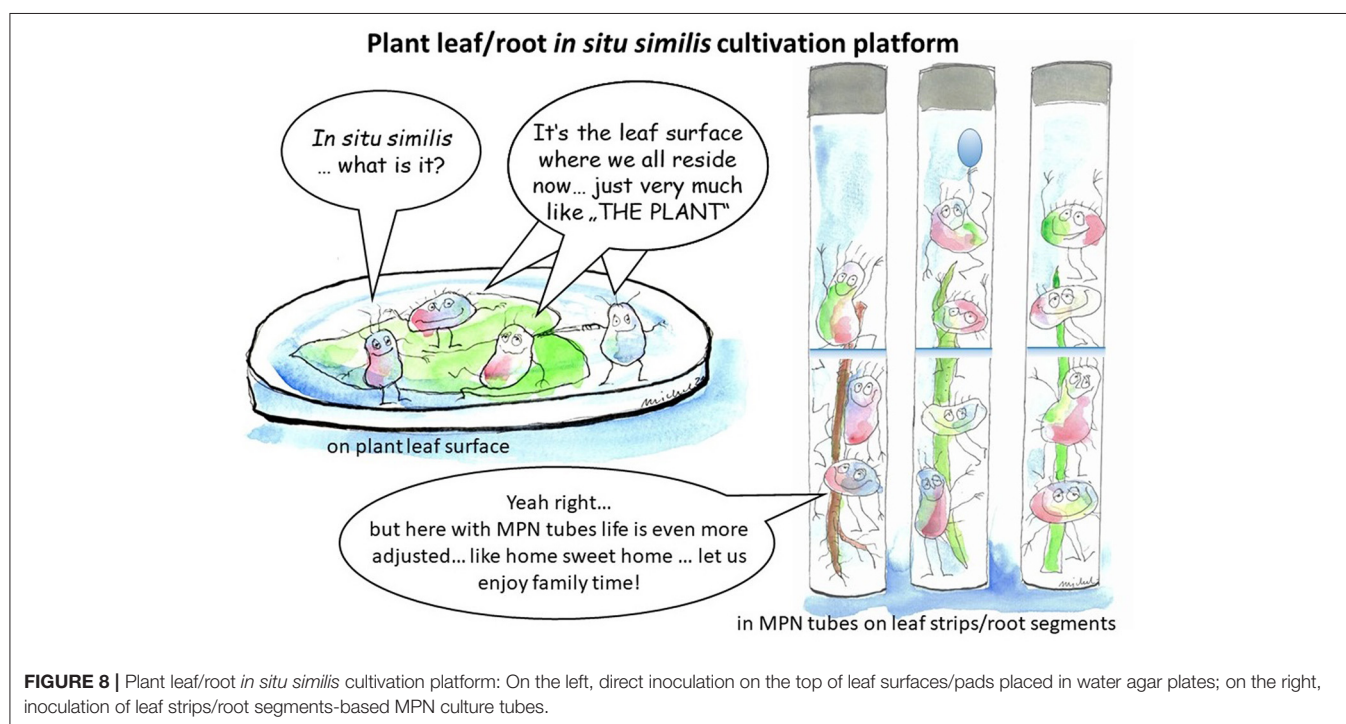
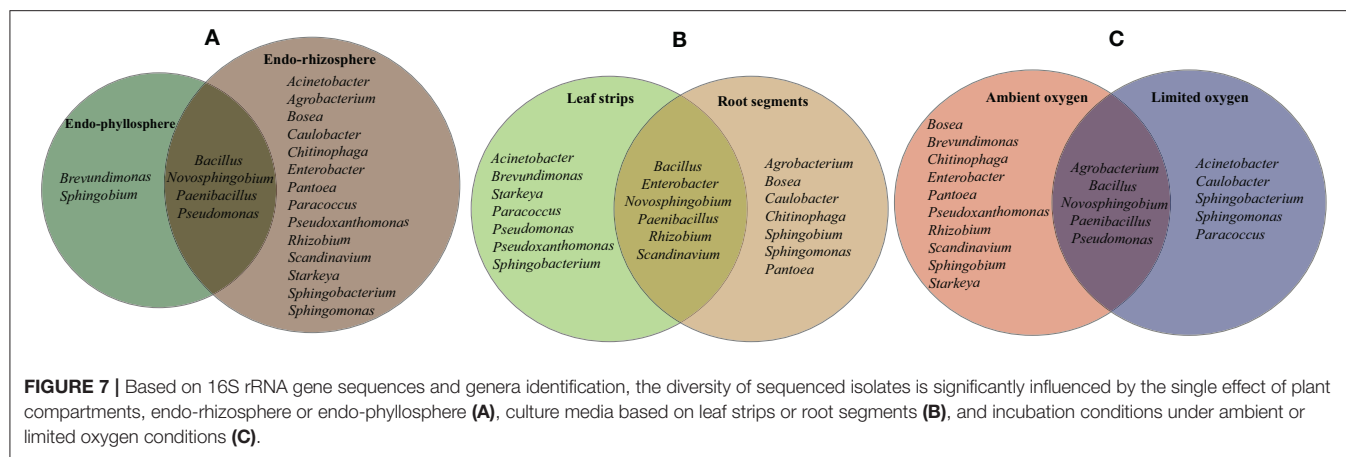


**FIGURE 6 |** Based on 16S rRNA gene sequences, the taxonomic status of endo-phylosphere (EP) and endo-rhizosphere (ER) bacterial isolates enriched on leaf strips (LS) and root segments (RS) based culture media under ambient (AO) and limited oxygen (OD) conditions. Respective isolate counts are shown on phylum and genus levels. The phyla affiliation of each genus is indicated by the phyla-specific coloring and connected lines in the legend.

(Supplementary Figure 6B). Regarding the Proteobacteria isolates, among 18 *Rhizobium* isolates that were enriched from endo-rhizosphere under ambient oxygenic conditions, 6 were preferably grown on leaf strips; and out of the 8 rhizobacterial *Enterobacter* recovered under oxygen availability, 4 isolates were enriched on leaf strips-based media (Supplementary Figure 6A). Notwithstanding these commonalities, the cultivation and recovery of other multiple genera were found to be allied to one or more of the specific cultivation conditions provided, in terms of plant compartments, plant-organ-culture media and/or oxygen availability.

## DISCUSSION

Cultivation-dependent approaches are becoming a forefront of microbiological research to explore the potential of environmental microbiomes, and to shed light on their ecology, physiology and biotechnological application. In fact, traditional methods for *in vitro* cultivation recover <1% of bacteria from all sorts of natural environments. Here, better understanding of the bacterial environment allowed the development of new culture media and new culture conditions required by the hidden fastidious bacteria. The subject was broadly treated and reviewed by quite a number of publications (e.g., Burmølle et al., 2009;



Sarhan et al., 2019; Bonnet et al., 2020; Elsayey et al., 2020; Nemr et al., 2020).

As a breakthrough, we provided sufficient evidences that plant materials “as such” without any supplement, are compatible vegan substrates, very competitively support *in vitro* cultivability and explore diversity of plant microbiota (Sarhan et al., 2019). Further, we successfully advanced the intact plant leaf-based culturing method as “*in situ similis* strategy” for exploring the plant microbiota (Nemr et al., 2020). The direct inoculation/cultivation of microorganisms on the top of leaf surfaces/pads (Figure 8) furnish *in vitro* growing microorganisms with nutrients in their natural composition, concentrations and gradients (Watve et al., 2000; Nemr et al., 2020). In the present study we extend the concept of such *in situ-similis* cultivation by exploring plant organ compatible

cultivation of bacterial community of endo-phylospheric/endo-rhizosphere when grown on corresponding leaf strips/root segments-based culture media (see Figure 8 illustrating the two different methods). Efforts were made to pooling the advantages of MPN enrichment methodology together with the natural plant-only-based culture media. Where the MPN culture tubes efficiently constructed a nutritional milieu governed solely by vegan nutrients of plant origin, i.e., leaf strips/root segments, immersed in plain semi-solid water agar. The profuse growth of endophytic bacteria developed in the form of distinguishable surface and sub surface pellicles and/or diffused turbidity of viable cells of diverse morphologies. As to population's density of maize endophytes, the derived MPN estimates of bacterial endophytes in the endo-phylosphere exceeded  $\log 7.0 \text{ g}^{-1}$  root, and were proportionate to those reported with standard R2A

and CCM culture media. Further nutrients' compatibility was distinguished for sunflower endophytes, where a certain degree of plant organ specificity/preference was reported for culture media based on homologous plant organ, i.e., endo-rhizosphere bacteria favored culture media based on root segments while those of endo-phylosphere preferred culture media based on leaf strips (Table 2). With 16S rRNA gene sequences of representative isolates, it appeared that homologous cultivation of endo-rhizosphere samples in root segments-based culture medium favored species of the genera of *Bosea*, *Chitinophaga*, *Pantoea*, *Caulobacter*, *Scandinavium*, and *Agrobacterium*, where *Scandinavium* and *Caulobacter* were isolated for the first time among sunflower microbiota. With leaf strips-based culture media, a number of potential rare species of the genera of *Bacillus* and *Sphingobium* were enriched from the endo-phylosphere community. Surprisingly isolates of the genus *Starkeya* were isolated for the first time from the sunflower endo-rhizosphere when cultivated on leaf strips-based culture media under ambient oxygen conditions. Under restricted oxygen concentrations, preference of better growth of the endo-phylosphere community was reported in comparison to that of endo-rhizosphere.

A number of arguments have been put forward to justify the exceptional power of bacterial enrichment and selection provided in the set-up of MPN-semi solid culture tubes. It is reported that the environmental conditions at the solid (e.g., leaf/root)-liquid (e.g., semi-solid water agar) interface, that is constructed in the semi-solid MPN tubes, differ from those in conventional culture media in the form of the bulk aqueous phase of liquid culture media and/or solid phase of agar plates (Fletcher, 1984). Accordingly, the growth and physiological activity of bacteria attached to leaf/root surfaces may differ from that of free-living cells in respect of: (a) nutrient concentration and/or accessibility may be different at the interface because of adsorption/absorption/desorption of low molecular weight or macromolecular substrates; (b) direct contact that allows the surfaces of the natural plant substratum to provide a site for colonization and the development of a bacterial biofilm. This particular microenvironment of growth allows normal/natural interactions between multiple residents of various functions in the plant compartments.

In spite of the preference reported for culturability of *Bacillus* sp., the applied MPN method provided a unique nutritional platform and culturing conditions for the growth of not-yet-cultured genera of sunflower microbiota. As we obtained 163 representative isolates, belonging to 20 genera, expanding the diversity of cultivable sunflower microbiota to include multiple genera representing Alphaproteobacteria (*Bosea* sp.; *Brevundomonas* sp.; *Caulobacter* sp.; *Starkeya* sp.); Gammaproteobacteria (*Pseudoxanthomonas* sp.; *Scandinavium* sp.) and Bacteroidetes (*Chitinophaga* sp.; *Sphingobacterium* sp.) (Table 4; Supplementary Figure 5). Recently, Tamošiune et al. (2020) reported the occurrence of 158 different genera in sunflower phyllosphere and rhizosphere using 16S rRNA metagenomics sequencing method. Adding up all genera described for sunflower plants, including those of our study, a total of 176 different genera are until now *in vitro* recaptured out of the sunflower microbiota (Supplementary Table 3).

**TABLE 4 |** Genera of cultivable endophytic bacteria of sunflower isolated by *in situ similis* culturomic strategies compared to other conventional culturing methods reported in literature.

Bacterial genera	<i>In situ similis</i> MPN culture method <sup>a</sup>	<i>In situ similis</i> leaf surface method <sup>b</sup>	Conventional culturing methods reported in literature <sup>c</sup>
<i>Achromobacter</i> (or <i>Alcaligenes</i> )	–	–	Yes <sup>3,6</sup>
<i>Acinetobacter</i>	Yes	–	Yes <sup>6</sup>
<i>Azotobacter</i>	–	–	Yes <sup>5</sup>
<i>Aureimonas</i>	–	Yes	–
<i>Agrobacterium</i>	Yes	–	Yes <sup>6</sup>
<i>Asticcacaulis</i>	–	–	Yes <sup>6</sup>
<i>Azospirillum</i>	–	–	Yes <sup>6</sup>
<i>Bacillus</i>	Yes	Yes	Yes <sup>1,2,3,4,5</sup>
<b><i>Bosea</i></b>	Yes	–	–
<b><i>Brevundimonas</i></b>	Yes	–	–
<i>Burkholderia</i>	–	–	Yes <sup>6</sup>
<b><i>Caulobacter</i></b>	Yes	–	–
<b><i>Chitinophaga</i></b>	Yes	–	–
<i>Curtobacterium</i>	–	Yes	–
<i>Chryseobacterium</i>	–	–	Yes <sup>6</sup>
<i>Enterobacter</i>	Yes	–	Yes <sup>4,6</sup>
<i>Erwinia</i>	–	Yes	–
<i>Grimontella</i>	–	–	Yes <sup>6</sup>
<i>Herbaspirillum</i>	–	–	Yes <sup>6</sup>
<i>Klebsiella</i>	–	–	Yes <sup>4,6</sup>
<i>Kocuria</i>	–	Yes	–
<i>Kosakonia</i>	–	Yes	–
<i>Methylobacterium</i>	–	–	Yes <sup>2</sup>
<i>Microbacterium</i>	–	Yes	Yes <sup>6</sup>
<i>Mitsuaria</i>	–	–	Yes <sup>6</sup>
<i>Moraxella</i>	–	–	Yes <sup>6</sup>
<i>Novosphingobium</i>	Yes	–	Yes <sup>6</sup>
<i>Paenibacillus</i>	Yes	Yes	Yes <sup>1</sup>
<i>Pantoea</i>	Yes	Yes	Yes <sup>6</sup>
<i>Paracoccus</i>	Yes	Yes	–
<i>Pseudomonas</i>	Yes	–	Yes <sup>5,6</sup>
<b><i>Pseudoxanthomonas</i></b>	Yes	–	–
<i>Rhizobium</i>	Yes	Yes	Yes <sup>6</sup>
<b><i>Scandinavium</i></b>	Yes	–	–
<i>Serratia</i>	–	–	Yes <sup>6</sup>
<i>Shinella</i>	–	–	Yes <sup>6</sup>
<b><i>Sphingobacterium</i></b>	Yes	–	–
<i>Sphingobium</i>	Yes	–	Yes <sup>6</sup>
<i>Sphingomonas</i>	Yes	Yes	–
<b><i>Starkeya</i></b>	Yes	–	–
<i>Stenotrophomonas</i>	–	Yes	Yes <sup>6</sup>
<i>Variovorax</i>	–	–	Yes <sup>6</sup>
<i>Xanthomonas</i>	–	–	Yes <sup>6</sup>
<b>Total genera</b>	<b>20</b>	<b>13</b>	<b>28</b>

Yes, isolated; –, not isolated; in bold are genera that only isolated by *in situ similis* MPN culture method.

<sup>a</sup>Current manuscript.

<sup>b</sup>Nemr et al. (2020).

<sup>c</sup>Conventional culturing methods reported in literature.

<sup>1</sup>Ambrosini et al. (2016).

<sup>2</sup>Campos et al. (2012).

<sup>3</sup>Forchetti et al. (2007).

<sup>4</sup>Liu et al. (2017).

<sup>5</sup>Raval and Desai (2012).

<sup>6</sup>Ambrosini et al. (2012).



Interestingly, the difference of 18 genera comprises cultivated isolates that did not show up in the previous molecular 16S rRNA metagenomics study. Furthermore, out of the above-mentioned 8 genera, that were genuinely isolated on our MPN-leaf/strips and root/segments-based media (**Table 4; Supplementary Table 3**), isolates of 3 genera were brought into cultivation, *Caulobacter*, *Scandinavium*, and *Starkeya*, that describe novel members of the sunflower microbiome, as they were not reported before (**Supplementary Table 3**, marked in bold letters). Additionally, our results strongly suggest the novel plant organ specific cultivation of at least two isolates, namely PR21\_OD (MN231735, *Bacillus* sp.) and PR85 (MN232172, *Sphingobium* sp.). Very likely they are putative novel species, and even the latter as a putative novel genus, due to their significantly lower identity value and early long branching from other closely related *Sphingobium* isolates currently found in the NCBI nr database. Unfortunately, the 16S rRNA gene fragment size is quite short for both isolates, that might interfere with the phylogenetic tree building algorithm and, thus, needs further proof in future.

Generally, the tested endo-rhizosphere isolates were of higher diversity, represented by 18 genera (belonged to Gammaproteobacteria, Alphaproteobacteria, Firmicutes, and Bacteroidetes) compared to those of the endo-phyllosphere (represented by only 7 genera) of the phyla Firmicutes and Proteobacteria (Alphaproteobacteria and Gammaproteobacteria). This is in accordance to what was reported in literature about the greater microbial diversity in belowground (roots, root zone, and bulk soil) than aboveground (leaves, flowers, and grapes) samples (Zarraonaindia et al., 2015). It is established that among other factors, organ (leaf vs. root) and habitat (epiphytes vs. endophytes) define the community of plant microbiota (Bodenhausen et al., 2013). Our results indicated some overlap at the rank of bacterial genera, namely *Novosphingobium*, *Pseudomonas*, *Bacillus*, and *Paenibacillus*, between leaf- and root-derived bacteria (4 genera, **Figure 7A**). This is in consistence with previous reports on different host plants, which further advance that soil may serve as a common bacterial reservoir for belowground and aboveground (Zarraonaindia et al., 2015; Wagner et al., 2016). Although the communities associated of both plant compartments share some bacterial species, they still differ in structure/composition (Bodenhausen et al., 2013). In the present study 15 genera (75%) were detected in either the endo-rhizosphere or the endo-phyllosphere.

Cross cultivation of rhizobacteria on homologous/heterologous host plan-based culture media, not plant organ specific media, was investigated by Mourad et al. (2018). They indicated that despite the promiscuous nature of the plant materials tested to culture rhizobacteria of different host plants, results indicated that plant materials of a homologous nature to the tested host plant, at least at the family level, and/or of the same environment were more likely to be selected. The present study further highlights cross cultivation of plant microbiota, of endo-rhizosphere and endo-phyllosphere, on homologous/heterologous leaf and root organ specific culture media of the same host plant, sunflower. In other words, to check if the leaf strips-based culture

medium will enrich the homologous endo-phyllosphere specific community different to that developed on the heterologous root segment based medium, and vice versa with the endo-rhizosphere populations? Results of CFUs counts reflected such a plant organ compatible culturability. In addition, the DGGE profiles obtained for endo-phyllosphere showed that bacterial communities developed in MPN culture tubes of homologous leaf strips clustered away from those of MPN culture tubes of heterologous root segments. Comparably, with endophytes of the endo-rhizosphere, endophytes developed on the homologous root segments separated away from those of the heterologous leaf strips-based MPN culture tubes.

Analyzing the 16S rRNA based community composition of representative isolates underlined as well such plant organ affinity/compatibility. In general, the leaf strips-based culture medium enriched a distinct bacterial community compared to the root segments-based culture medium for the tested samples of both endo-phyllosphere and endo-rhizosphere of sunflower plants (**Figure 6; Genera Distribution**). An additional impact was demonstrated for incubation under different oxygen availability that further increased the richness of cultivable organisms (**Supplementary Figure 6**). As to the endo-phyllosphere community, the homologous leaf strips-based culture medium generally enriched *Novosphingobium* sp. and *Brevundimonas* sp. under ambient oxygen conditions, while the heterologous root segments-based medium favored the growth of *Sphingobium* sp. (**Figure 6; Supplementary Figure 6B**). On both culture media and under ambient or limited conditions common were isolates of different *Bacillus* species. *Bacillus safensis* and *Bacillus pumilus* were only recovered from endo-phyllosphere samples when they were grown on root segments-based culture medium. For endo-rhizosphere community, common genera that developed on both leaf strips/root segments-based medium were *Rhizobium*, *Enterobacter*, and *Scandinavium*. The root segments-based medium additionally enriched *Agrobacterium*, *Pantoea*, *Chitinophaga*, and *Boesa*. While the leaf strips-based medium enriched *Pseudomonas* growth from endo-rhizosphere sunflower samples, particularly under limited conditions (**Figure 6; Supplementary Figure 6A**). Additionally, isolates of *Starkeya* sp. and *Pseudoxanthomonas* sp. were detected from the endo-rhizosphere on leaf strips-based culture medium.

Currently, thorough assessment of genetic diversity of microbial communities is gained by high-throughput techniques such as NGS; however, PCR-DGGE (Muyzer et al., 1993) is a routine and commonly used technique in plant-soil ecosystem studies. Admitting that the technique has some limitations (Orlewska et al., 2018; Vischetti et al., 2020), recently a number of protocols are optimized for use with plant and soil DNA. They are confirmed to be reliable, do not require complex bioinformatics for the analyses of results, and present an overall picture on the main differences in microbial community composition when coupled with culture-dependent microbiological approaches (Sarhan et al., 2016; Vischetti et al., 2020). And, common to the NGS methods, different primer sets can be used in PCR-DGGE, to addressing microbial communities at the phylogenetic level (e.g., 16S primers for bacteria and archaea),

or at the functional level, depending on functional selected genes (Valášková and Baldrian, 2009). The obtained DGGE profiles during this study were indicative to reflect differences in community composition attributed to the type of culture media, not the oxygen status during incubation. For endo-phyllosphere, bacterial communities developed in MPN culture tubes of homologous leaf strips clustered away from those of MPN culture tubes of heterologous root segments. As well, endophytes of the endo-rhizosphere produced two independent clusters separating endophytes developed on the homologous root segments distant from those of the heterologous MPN leaf strip culture tubes. Such differences in DGGE patterns points to a certain degree of plant-organ affinity and compatibility, that architect the community composition of culturable bacterial endophytes of endo-phyllosphere/endo-rhizosphere in response to growth in leaf strips/root-based culture media. An effect that is directly allied to the nutritional make-up of the plant leaves or roots used in the preparation of the plant-only-based culture media. In this respect, it is reported that the mean C, N, and P concentrations and C/N, C/P, and N/P ratios differed among leaf, stem and root of plants, and that stoichiometric patterns in different plant organs had different responses to environmental variables (He et al., 2015).

## CONCLUSION

The presented strategy of *in vitro* cultivation based on strips/segments of plant leaves/roots construct an appropriate semi-solid interface together with a continuum of varying concentrations of nutrients and gas phases. Such conditions credibly meet complex nutritional requirements of the diverse taxa of plant endophytes. The strategy enhanced cultivation of plant microbiota, and significantly extended diversity of cultivable populations. As well, it shows plant organ compatibility for enrichment of plant microbiota when cultivated on the respective homologous plant organ-based culture media. This strategy is a feasible supplement to traditional cultivation to probe deeper for the yet-uncultured taxa and fill the many cultivation gaps of plant microbiota. It is presented as a significant tool to bringing additional potential resources of plant growth promoting bacteria into cultivation for further biotechnological applications.

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## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

## AUTHOR CONTRIBUTIONS

NH, SR, KE-S, and RN: conceptualization. RN, SA, MK, AB, MRA, and HG: methodology. SP, RN, MH, HY, and MRA: software. NH, SR, SP, KE-S, MF, and HG: validation. RN, MH, HY, HG, MRA, AM, and MA: formal analysis. RN, SA, MK, AB, and MRA: investigation. SR, NH, and MF: resources. SR, NH, SP, MF, and KE-S: data curation. RN, NH, MF, KE-S, HG, and MA: writing—original draft preparation. NH, SR, SP, MF, and MB: writing—review and editing. RN, MB, SP, AM, and MH: visualization. NH, SR, MF, and KE-S: supervision. NH, SR, MH, and MA: project administration. SR and NH: funding acquisition. All authors have read and agreed to this version of the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.660790/full#supplementary-material>

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# Isolation of Endophytic Salt-Tolerant Plant Growth-Promoting Rhizobacteria From *Oryza sativa* and Evaluation of Their Plant Growth-Promoting Traits Under Salinity Stress Condition

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The application of plant growth-promoting rhizobacteria (PGPR) as vital components for plant growth promotion against biotic and abiotic stresses could be a promising strategy to improve crop production in areas vulnerable to increasing salinity. Here, we isolated Seventy-five endophytic bacteria from roots of healthy *Oryza sativa* grown in a saline environment of the southern coastal region of Bangladesh. The endophytes in a culture of  $\sim 10^8$  CFU/ml showed arrays of plant growth-promoting (PGP) activities: phytohormone (Indole acetic acid) production (1.20–60.13  $\mu\text{g/ml}$ ), nutrient (phosphate) solubilization (0.02–1.81  $\mu\text{g/ml}$ ) and nitrogen fixation (70.24–198.70  $\mu\text{g/ml}$ ). Four genomically diverse groups were identified namely, *Enterobacter*, *Achromobacter*, *Bacillus*, and *Stenotrophomonas* using amplified ribosomal DNA restriction analysis followed by their respective 16S rDNA sequence analyses with that of the data available in NCBI GenBank. These four specific isolates showed tolerance to NaCl ranging from 1.37 to 2.57 mol/L in the nutrient agar medium. Under a 200 mmol/L salt stress *in vitro*, the bacteria in a culture of  $10^8$  CFU/ml exhibited competitive exopolysaccharide (EPS) production: *Stenotrophomonas* (65  $\mu\text{g/ml}$ ) and *Bacillus* (28  $\mu\text{g/ml}$ ), when compared to the positive control, *Pseudomonas* spp. (23.65  $\mu\text{g/ml}$ ), a phenomenon ably supported by their strong biofilm-producing abilities both in a microtiter plate assay, and *in soil* condition; and demonstrated by images of the scanning electron microscope (SEM). Overall, the isolated endophytic microorganisms revealed potential PGP activities that could be supported by their biofilm-forming ability under salinity stress, thereby building up a sustainable solution for ensuring food security in coastal agriculture under changing climate conditions.

**Keywords:** biofilm, endophytes, plant growth-promoting rhizobacteria, salt-tolerant, sustainable agricultural production

## INTRODUCTION

Soil salinity, one of the most vicious abiotic stresses of global magnitude is predominantly generated as a result of sea-level rise owing to climate change, and accountable for the loss of agricultural production and sustainability (Shahid et al., 2018). Constant salt deposition from intruding saltwater in agricultural fields interferes with soil physicochemical properties that led to permanent depletion in plant water absorption followed by dehydration and osmotic stress in plants (Kaushal and Wani, 2016). The accumulation of ions ( $\text{Na}^+$  and  $\text{Cl}^-$ ) beyond threshold limits badly affects plant metabolic machinery, transpiration system, photosynthesis, and most importantly, ionic balance and nutrient (N, Ca, K, P, Fe, Zn) uptake; thereby retarding overall growth, seed germination, reproductive development, and yield of crops (Gupta and Huang, 2014; Islam et al., 2015; Hashem et al., 2016; Egamberdieva et al., 2019; Albdaiwi et al., 2020). Scientific communities across the world are earnestly involved in finding novel solutions of plant stress tolerance strategies without compromising proper growth to fulfill food demands under limited resource availability. One such environment-friendly scheme includes the strengthening of plants' defense systems by employing beneficial microbes (Glick, 2014; Kasim et al., 2016; Sharma et al., 2016).

The plant kingdom is inhabited by a diverse group of endophytic bacteria which grow symptomless within plants as an integral part of host metabolism and function, bearing commensalism or mutualism relationship with plants (Haidar et al., 2018; Vinayarani and Prakash, 2018). These endophytic bacteria employ different mechanisms to stimulate plant growth, ensure nutrient availability of mineral assets such as phosphorus, iron; produce phytohormones, various secondary metabolites, volatile compounds, siderophores, lytic enzymes, and antibiotics to counter phytopathogens; and enhance stress tolerance in plants (Boddey and Dobereiner, 1988; Hallmann et al., 1997; Lazarovits and Nowak, 1997; Wakelin et al., 2004; Waqas et al., 2012). Some endophytes are well-reported to produce exopolysaccharide (EPS) outside of cell surfaces under inhospitable conditions which secure water holding capacity along with nutrient uptake around the plant roots, mitigating the effects of salinity and desiccation under salinity stress (Balsanelli et al., 2014; Ilangumaran and Smith, 2017; Ansari and Ahmad, 2018; Etesami and Beattie, 2018).

In the present study, an attempt was made to isolate endophytic plant growth-promoting rhizobacteria (PGPR) associated with *Oryza sativa* grown in saline soils in the southern coastal region of Bangladesh. The isolates were tested *in vitro* for their plant growth-promoting (PGP) abilities and exopolysaccharide production under different levels of salinity. In line with coastal agricultural development prioritizing the crop cultivation, this study can be considered as a climate-smart solution that harnessed an eco-friendly sustainable approach to enhance crop productivity as well as soil fertility with an insight of possible stress tolerance mechanism of endophytic PGPR providing to plants (Rima et al., 2018; Egamberdieva et al., 2019).

## MATERIALS AND METHODS

### Sample Collection

During May 2017, roots of *Oryza sativa* samples were collected from six cultivated fields of three sub-districts of Patuakhali district namely Dumky ( $22^{\circ}26'N$ ,  $90^{\circ}22'E$ ), Bauphal ( $22^{\circ}25.8'N$ ,  $90^{\circ}30.8'E$ ), and Kalapara ( $21^{\circ}59.2'N$ ,  $90^{\circ}14.5'E$ ) (Figure 1). Earlier, electrical conductivity (EC) of the soil samples from these regions were recorded as  $7.88 \pm 0.53$  dS/m (Sultana et al., 2020), hence, the regions were considered saline since yields of many crops are restricted beyond the salinity threshold limit, 4.1–8.0 dS/m (Munns, 2005; Soil Resource Development Institute., 2010). Root samples were carried in a portable ice-box and transported back to the Fermentation and Enzyme Biotechnology laboratory, Department of Microbiology, University of Dhaka, Bangladesh and were stored at  $-20^{\circ}\text{C}$  until further processing.

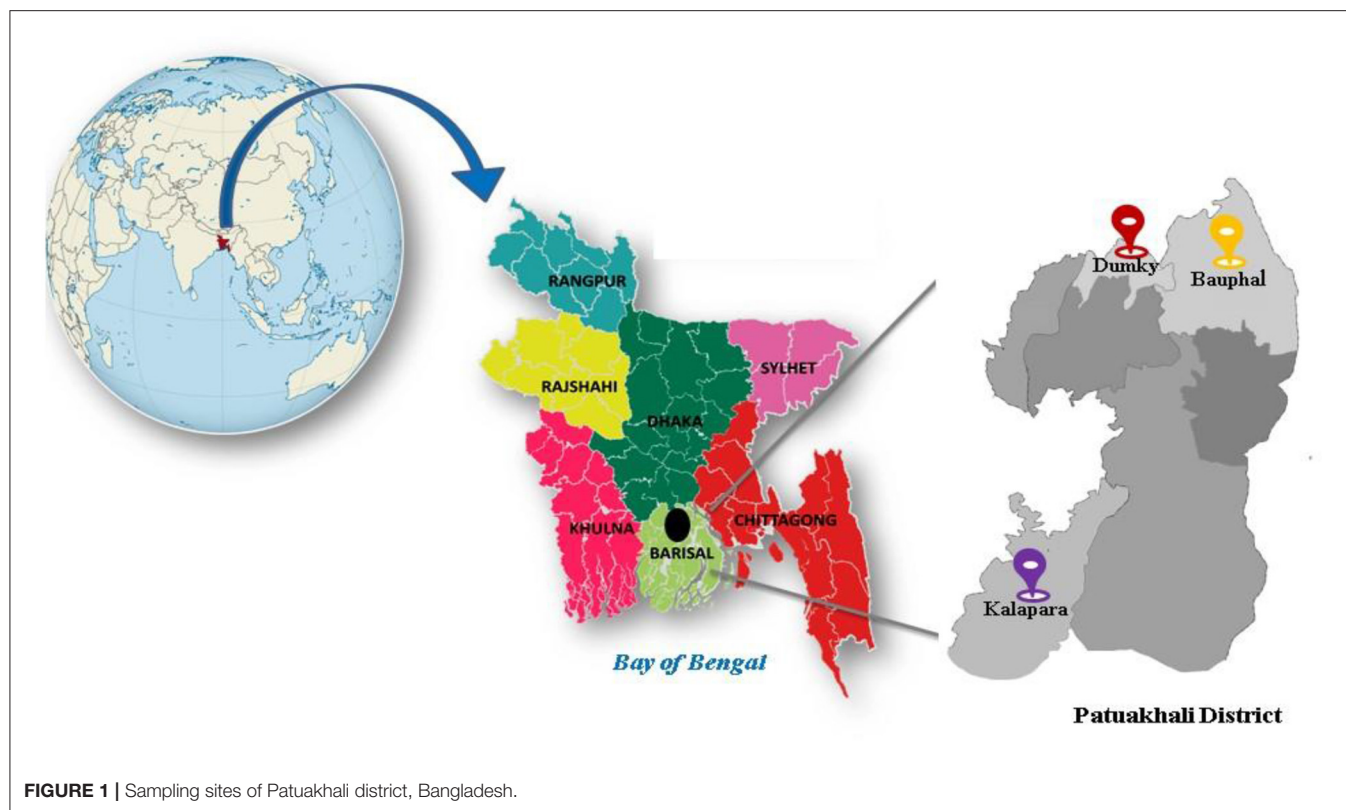
### Isolation of Endophytic Bacteria

The roots of the collected samples were washed under running tap water for 5 min to remove adhering soil particles followed by washing serially in 75% ethanol, 99% ethanol, and finally, 10%  $\text{H}_2\text{O}_2$  (5 min in each) to remove any kind of adhering microbes or epiphytes that were present on the root surfaces. Thereafter, surface-sterilized root tissues, resuspended at 10% in physiological saline (0.85% NaCl) were macerated with a sterilized mortar and pestle. The homogenized root samples were then used to screen endophytic PGPR through the spread plate technique. The processed root samples were not diluted to ensure not to lose a single root endophytic bacterium. Samples were spread plated onto two nitrogen-free media namely Jensen's agar media (10 g sucrose, 10 g glucose, 0.20 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.15 g  $\text{K}_2\text{HPO}_4$ , 0.10 g  $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.05 g  $\text{KH}_2\text{PO}_4$ , 0.02 g  $\text{CaCl}_2$ , 2 mg  $\text{Na}_2\text{MoO}_4$ , 1 mg  $\text{FeCl}_3$ , 1 mg  $\text{Na}_2\text{Mo}_2\text{H}_2\text{O}$ , 15 g agar, pH  $7.2 \pm 0.2$ , in a 1 lit volume at  $25^{\circ}\text{C}$ ) and Ashby's agar media (5 g sucrose, 5 g  $\text{CaCO}_3$ , 0.2 g  $\text{MgSO}_4 \cdot 2\text{H}_2\text{O}$ , 0.2 g NaCl, 0.2 g  $\text{KH}_2\text{PO}_4$ , 5 g benzoate, 10 mg  $\text{FeSO}_4$ , 15 g agar, pH  $7.2 \pm 0.2$ , in a 1 lit volume at  $25^{\circ}\text{C}$ ) working in the Class II Biosafety cabinet (ESCO, Singapore). Plates were incubated for 5–7 days at  $30^{\circ}\text{C}$  incubator (ESCO, Singapore) until the colonies were detected. Colonies with distinct morphological characteristics were selected and purified by sub-culturing two times on respective media before they were stored in a 20% glycerol solution at  $-80^{\circ}\text{C}$ . Since the bacteria were collected exclusively from the root origin, the letter "R" was used as the source of collection, followed by either "J" or "A" representing "Jensen" and "Ashby" agar, respectively, while naming the isolates. For example, R<sub>2</sub>J<sub>6</sub> would represent a bacterium recovered after homogenization of the second root sample, and isolated as the sixth isolate after growth in Jensen agar.

### *In vitro* Assessment of Plant Growth Promoting (PGP) Traits

#### Indole Acetic Acid Production Assay

The amount of IAA produced by the isolated endophytes was determined by following the method described by Gordan and Weber (1950). Initially, the bacterial isolates were cultured in nutrient broth (NB) and 100  $\mu\text{l}$  of bacterial inoculum ( $\sim 10^8$



CFU/ml) from each culture was taken afterward to continue the assay as described by Sultana et al. (2020).

### Phosphate Solubilization Assay

#### Qualitative Assay

The inorganic phosphate solubilization ability of endophytes was observed by spot inoculation of bacterial isolates on Pikovskaya agar and National Botanical Research Institute's Phosphate (NBRIP) growth medium (Pikovskaya, 1948; Nautiyal, 1999). The formation of transparent halo zones around the bacterial colonies after 5–7 days of incubation at 30°C was considered as an indication of phosphate solubilizing activity.

#### Quantitative Assay

Phosphate solubilization by 100 µl of endophytic bacteria ( $\sim 10^8$  CFU/ml) was quantitated by the Molybdenum blue method in NBRIP broth, as described earlier (Sultana et al., 2020).

### Nitrogen Fixation Assay

To determine the nitrogen-fixing capability of isolates, 100 µl of bacterial inoculum ( $\sim 10^8$  CFU/ml) for each selected isolates was inoculated into a test tube containing 5 ml nitrogen-free Jensen's broth and incubated for 5 days at 30°C at 120 r/min on a shaker. The amount of fixed atmospheric nitrogen was determined by the Kjeldahl method (Kjeldahl, 1883).

### Genetic Diversity of Endophytic PGPR

#### Extraction of Total Genomic DNA

Genomic DNA of the endophytes was isolated by boiling method (Queipo-Ortuño et al., 2008). Isolated colonies were grown overnight in 5 ml nutrient broth in an orbital incubator (NEW BRUNSWICK™ 94 EXCELLA® E25/E25R, Germany) at 30°C. After 16 h incubation, 1 ml culture broth from each respective culture was transferred into an eppendorf tube and centrifuged at 15,000 ×g for 5 min. The supernatant was eliminated and the pellet was resuspended in 200 µl nuclease-free water, subjected to boiling at 100°C in a water bath for 10 min, cooled on ice, and centrifuged at 15,000 ×g for 10 min. The supernatant was taken into another fresh eppendorf tube and stored at –20°C. The DNA concentration was measured using a NanoDrop™ 8000 spectrophotometer (THERMO SCIENTIFIC, CALIFORNIA, USA).

#### ARDRA Analysis of 16S rDNA

In order to phylogenetically characterize the bacterial isolates prior to sequencing, Amplified Ribosomal DNA Restriction Analysis (ARDRA) was used to segregate them into clusters using 16S rDNA from the respective bacterial genome as a template. For the amplification of the 16S rDNA region, PCR was performed by 16S rDNA bacterial universal primers: forward (5'-AGAGTTTGATCMTGGCTCAG-3') and reverse (5'-GGTACCTTGTTACGACTT-3') (Stevens and Van Elsas, 2010). PCR was carried out by using a thermal cycler (MODEL AERIS™, 96 WELLS, ESCO MICRO PTE. LTD., SINGAPORE)

with the following amplification conditions: 94°C for 5 min for initial DNA denaturation, 35 cycles at 94°C for 1.5 min (denaturation), 56°C for 1 min (annealing) and 72°C for 1.5 min (extension), and a final elongation step at 72°C for 10 min. The amplified products were analyzed by gel electrophoresis in a 1.5% agarose gel (SIGMA, USA) with a 100 bp DNA ladder (PROMEGA, USA) and visualized by using a gel documentation system (ALPHAMAGER, USA).

In order to find the ARDRA patterns, the purified 16S rDNAs from bacterial isolates were subjected to restriction digestion with *Bsu*RI (*Hae*III) (THERMO SCIENTIFIC) restriction enzyme following the manufacturer's instructions. Ten units of restriction enzyme were added to 10 µl of the amplified DNA and incubated for 4 h at 37°C in a total volume of 30 µl. The digests were resolved by gel electrophoresis on a 1.5% (w/v) agarose gel (SIGMA, USA) and compared to a 100 bp DNA ladder (PROMEGA, USA) for size estimation. Using data from a freely available open-source website (<http://insilico.ehu.es>), a dendrogram was constructed by evaluating the restriction pattern of 16S rDNAs using Dice and UPGMA (Unweighted Pair Group Method with Arithmetic mean) analysis. The data were analyzed as discrete binary variables by “1” to represent the presence of bands and “0” for the absence.

## 16S rDNA Sequencing and Phylogenetic Analysis

16S rDNA PCR products were purified by FavorPrep™ GEL/PCR Purification Kit (FAVORGEN, TAIWAN) following the manufacturer's instructions. Based on PGP activities (*in vitro*) along with ARDRA analysis, four bacterial isolates were sent for automated DNA sequencing (1st Base, Malaysia). The sequences were further analyzed by using BLAST tools on the National Center of Biotechnology Information (NCBI) website. The 16S rDNA gene along with their closest homology sequences were aligned using multiple sequence alignment program CLUSAL W implemented in MEGA X software by using default parameters (Kumar et al., 2018). The phylogenetic tree was constructed by the neighbor-joining (NJ) method using MEGA X software and evolutionary distances were computed with the help of Kimura's 2 parameter models (Kimura, 1980; Saitou and Nei, 1987). The 16S rDNA gene sequences obtained in the present study were submitted to the GenBank nucleotide sequence database.

## Halotolerance Assay

Followed by identification, the four selected endophytic isolates were then screened for salt-tolerance properties using nutrient agar (NA) media supplemented with various levels of NaCl (w/v) such as, 0.63% (107 mM), 1.25% (213 mM), 2.50% (427 mM), 5% (854 mM), 7.50% (1.30 M), 10% (1.71 M), and 15% (2.60 M). Control plate was maintained with 0.05% (8.54 mM) NaCl (w/v). The plates were inoculated with fresh culture following the streak plate method and incubated for 48 h at 30°C and the growth on the NaCl-supplemented plates was compared with the control plate (Albdaiwi et al., 2020).

## Biofilm Detection Assay

Biofilm formation under varying salt concentration by selected bacterial isolates was determined using microtiter plate assay (Auger and Gohar, 2006; Qurashi and Sabri, 2012b). Bacterial cultures were inoculated into LB broth with varying salt concentrations (0, 100, and 200 mM NaCl) in a culture flask and incubated for 24 h at 30°C. After incubation, 200 µl cultures (~10<sup>8</sup> CFU/ml) were transferred to each of the 96 wells polyvinylchloride microtiter plate (Greiner BIO-ONE, Germany) for further incubation at 30°C for 48 h. Plates were washed twice with distilled water, left at room temperature for 30 min. Surface-bound cells were stained with 0.01% (w/v) crystal violet solution at room temperature for 20 min. The wells were then washed with phosphate-buffered saline three times and the dye was solubilized with 200 µl ethanol. The absorbance at 570 nm of the solubilized dye was subsequently determined.

## Biofilm Detection in Soil

To evaluate the biofilm-forming ability of the isolates at different NaCl concentrations in soil, the method described in Qurashi and Sabri (2012b) was followed with some modifications. Briefly, garden soil was autoclaved at 121°C for 40 min and the sterile plastic pots were filled with sterile soil where a sterilized glass slide was placed into each pot. In control pots, neither salt nor an inoculum was used while in test pots; salt concentrations of 0, 100, and 200 mM NaCl in 20 ml solution with an initial bacterial inoculum of ~10<sup>8</sup> CFU/ml in 120 g soil were applied in each pot. In the control pot, the inoculum and salt volume were replaced with sterile distilled water. The pot was sealed and kept in the incubator (ESCO, Singapore) at 37°C for 15 days. After the incubation, the glass slide was removed from the pot and washed carefully with phosphate buffer saline (PBS) to remove the soil particles. The glass slide was put in a flask containing PBS placed on a shaker at 150 rpm for 30 min at 37°C. The bacterial count was estimated in CFU/ml by culturing them in nutrient agar plates through the drop plate method after serial dilution which revealed the biofilm-forming capability of the isolates in soil condition.

## Quantitative Analysis of EPS Production

Production of EPS by the selected bacterial isolates was determined during the growth of a batch culture in particular salt concentrations (0, 100, and 200 mM NaCl) (Qurashi and Sabri, 2012b). Briefly, 250 ml flasks containing 100 ml of a medium (Verhoef et al., 2003) was inoculated with freshly grown bacterial culture (~10<sup>8</sup> CFU/ml) and incubated on a shaker at 160 rpm at 30°C for 48 h. Following centrifugation, the supernatant was collected and added with three volumes of pre-chilled absolute ethanol and placed at 4°C for 24 h to precipitate the EPS fraction. Following centrifugation at 15,000 rpm for 20 min, the pellet containing bacterial EPS was separated and dried at 58°C for 24 h. The dry weight of the collected EPS was measured afterward. All the experiments were performed in triplicate.



## Glucose Quantification in the Produced EPS

Total carbohydrate content was further determined according to the phenol sulfuric acid method from the dried EPS as described earlier (Dubois et al., 1956).

## Biofilm Qualitative Analysis by Scanning Electron Microscope (SEM)

Biofilm formation by bacterial isolates was observed by SEM as described earlier (Sultana et al., 2020). Briefly, isolates were grown in nutrient agar with 0, 100, and 200 mmol/L NaCl overnight, and a single colony was taken and dispersed in absolute ethanol (99.5%) dropped into a glass slide, and dried out under a UV lamp. The sample was observed after platinum coating at different magnification and resolution and the images were captured for the SEM study.

## RESULTS

### Isolation of Endophytic Bacteria

Plating the homogenate of collected root samples of *Oryza sativa* on nitrogen-free media, Jensen's and Ashby's agar gave a selective advantage of isolating nitrogen-fixing bacteria from non-fixers. Seventy-five endophytic bacteria were isolated and were ID'd based on their origin and respective isolation media.

### *In vitro* Assessment of Plant Growth Promoting (PGP) Traits

#### Indole Acetic Acid Production Assay

The bacterial isolates in their respective culture of  $\sim 10^8$  CFU/ml were screened for their ability to produce IAA. The IAA production ranged from 1.20 to 60.13  $\mu\text{g/ml}$  with  $R_4A_3$  producing the maximum (60.13  $\mu\text{g/ml}$ ) (Supplementary Table 1).

#### Phosphate Solubilization Assay

The isolates were tested for their ability to solubilize inorganic phosphate from the media. In Pikovskaya's medium, satisfactory halo zones were not observed (data not shown) but in the NBRIP medium, 57 out of 75 (76%) isolates were able to solubilize phosphate and formed clear zones. Estimated solubilization was recorded as 0.02–1.81  $\mu\text{g/ml}$  (Supplementary Table 1).

#### Nitrogen Fixation Assay

The isolated endophytes were supposed to fix atmospheric  $N_2$  as they could grow in nitrogen-free Jensen's and Ashby's medium. The amount of nitrogen fixation varied from 70.24 to 198.70  $\mu\text{g/ml}$ .  $R_4A_6$  was found as the most efficient one fixing atmospheric nitrogen (198.70  $\mu\text{g/ml}$ ) (Supplementary Table 1).

Thirty isolates out of 75 were short-listed for further study (Supplementary Table 1), thanks to their superior *in vitro* plant growth-promoting assays, their respective quantitative values are illustrated (Figure 2).

## ARDRA Analysis and Identification of the Isolates

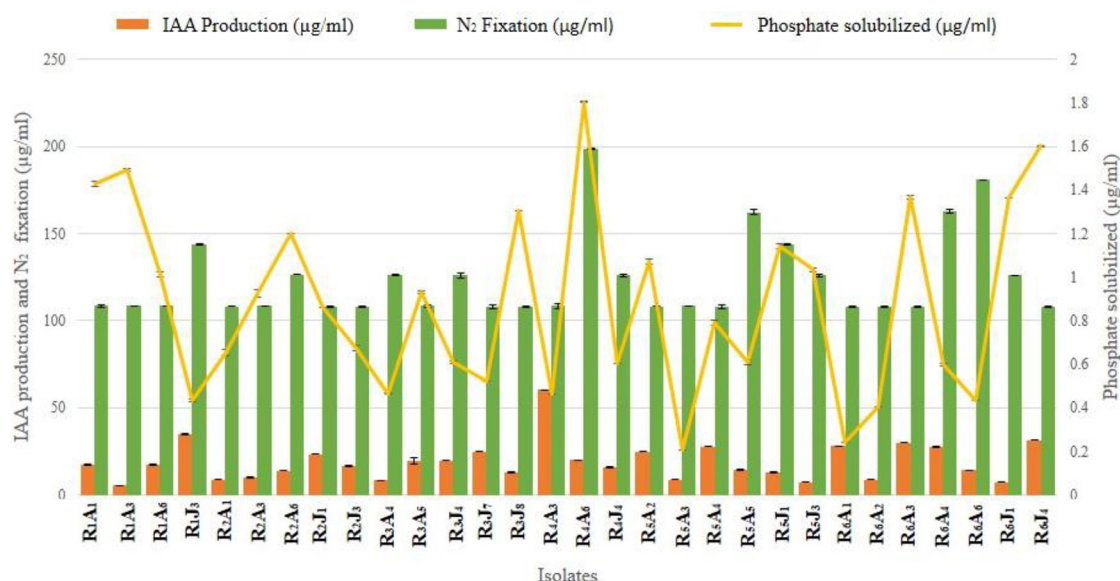
For the restriction analysis of the amplified rDNA procedures, the 16S rDNAs were isolated from the short-listed thirty isolates by PCR using the universal primers that produced  $\sim 1,465$  bp amplification products (Figure 3A) following the method described earlier (Sultana et al., 2020). Upon digestion by restriction enzyme, the 16S rDNAs amplicon of these isolates produced a pattern (Figure 3B) that could be grouped in four clusters, named A, B, C and D in the resulting dendrogram that included 5 (16.67%), 16 (53.33%), 4 (13.33%), and 5 (16.67%) isolates, respectively (Figure 3C). Based on overall PGP attributes (IAA production, Nitrogen fixation and Phosphate solubilization), one finest isolate from each cluster, named  $R_5A_4$ ,  $R_4A_6$ ,  $R_6J_4$ , and  $R_6A_1$  representing clusters A, B, C, and D were chosen, respectively, with a view to sequencing their respective 16S rDNA genes after PCR amplification. The raw tracer files found from the sequencing were manually corrected and assembled by SeqMan for a homology search using the BLAST tool of NCBI and a phylogenetic tree was constructed (Figure 3D) where the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches (Felsenstein, 1985). Sequence analysis of the 16S rDNA gene revealed that isolates  $R_4A_6$ ,  $R_5A_4$ ,  $R_6A_1$ , and  $R_6J_4$  were identified as *Enterobacter cloacae* GRF2, *Stenotrophomonas pavanii* BRF2, *Achromobacter xylosoxidans* DRF2, and *Bacillus aryabhattai* DRF1, respectively. These sequences were then submitted to GenBank [https://www.ncbi.nlm.nih.gov/nucleotide/?term=MT768046:MT768049accn] that assigned the isolates  $R_4A_6$ ,  $R_5A_4$ ,  $R_6A_1$ , and  $R_6J_4$  with accession numbers (Table 1) after necessary verification.

### Halotolerance Assay

The four identified endophytes were tested for their salt tolerance abilities. All of them showed a great level of tolerance. The tolerance ranged from 1.37 mol/L, exhibited by *A. xylosoxidans* DRF2, to 2.05 mol/L by *E. cloacae* GRF2 and *S. pavanii* BRF2, up to 2.57 mol/L NaCl concentration, demonstrated by *B. aryabhattai* DRF1.

### Biofilm Formation Assay

The biofilm formation of the isolates at different salt stress (0, 100, and 200 mM NaCl) was estimated in microtiter plates along with a positive control for biofilm formation, *Pseudomonas* spp, collected from the departmental repository. The test isolates exhibited a different pattern of biofilm formation with increasing salt concentrations. At non-saline conditions, although *E. cloacae* GRF2 showed the highest potential, *S. pavanii* BRF2 outcompeted the rest at 200 mM salt stress ( $P < 0.001$ ). The other strains produced comparable biofilm formation as efficiently as the positive control under saline conditions ( $P = 0.11$ ) (Figure 4A). Under soil condition, which is happened to be a natural medium, the efficacy of biofilm formation was evaluated by measuring changes of growth of the isolates (in CFU/ml) at different salt concentrations (Figure 4B). Isolate *S. pavanii* BRF2 was observed to produce prolific biofilm as compared to



**FIGURE 2** | A representative illustration of *in vitro* plant growth-promoting traits of 30 endophytic PGPR isolates shortlisted from seventy-five, that showed an elevated level of productivity [Nitrogen fixation (108–198.7 µg/ml), Phosphate solubilization (0.2–1.8 µg/ml), and IAA production (5.4–60.1 µg/ml)]. Error bars represent the mean  $\pm$  SD ( $n = 3$ ).

others ( $p < 0.001$ ) under different stress conditions. It produced the highest amount of biofilm at 200 mM NaCl concentration which is 33.93% greater than in 100 mM NaCl concentration. The estimate was also found increased for *B. aryabhattai* DRF1 measured 26.32 and 57.89% in CFU/ml at 100 and 200 mM NaCl concentrations, respectively, compared to that of no added salt condition.

The biofilms are mainly composed of EPS, a high molecular weight carbohydrate compound. We attempted to quantitate its amount in the biofilm materials of the isolates by taking the dry weight of EPS and measuring the glucose content of the EPS. As expected, *S. pavanii* BRF2 produced the highest amount (65 µg/ml) followed by *B. aryabhattai* DRF1 (28 µg/ml) at 200 mM NaCl concentration, and this production was found significant when compared to the positive isolate ( $p < 0.05$ ). The other strains produced a comparable amount of EPS with the positive control (Figure 4C). The total carbohydrates in EPS showed an increase of 73.17% (71.06 mg 100 ml<sup>-1</sup> culture) and 64.6% (59.77 mg 100 ml<sup>-1</sup> culture) for *S. pavanii* BRF2 and *B. aryabhattai* DRF1, respectively, at 200 mM NaCl concentration compared to that of non-saline condition ( $p < 0.05$  for both). On the contrary, the isolate *E. cloacae* GRF2 had a 52.9% (20.90 mg 100 ml<sup>-1</sup> culture) decrease of EPS accumulation (Figure 4D).

## Biofilm Qualitative Analysis by Scanning Electron Microscope (SEM)

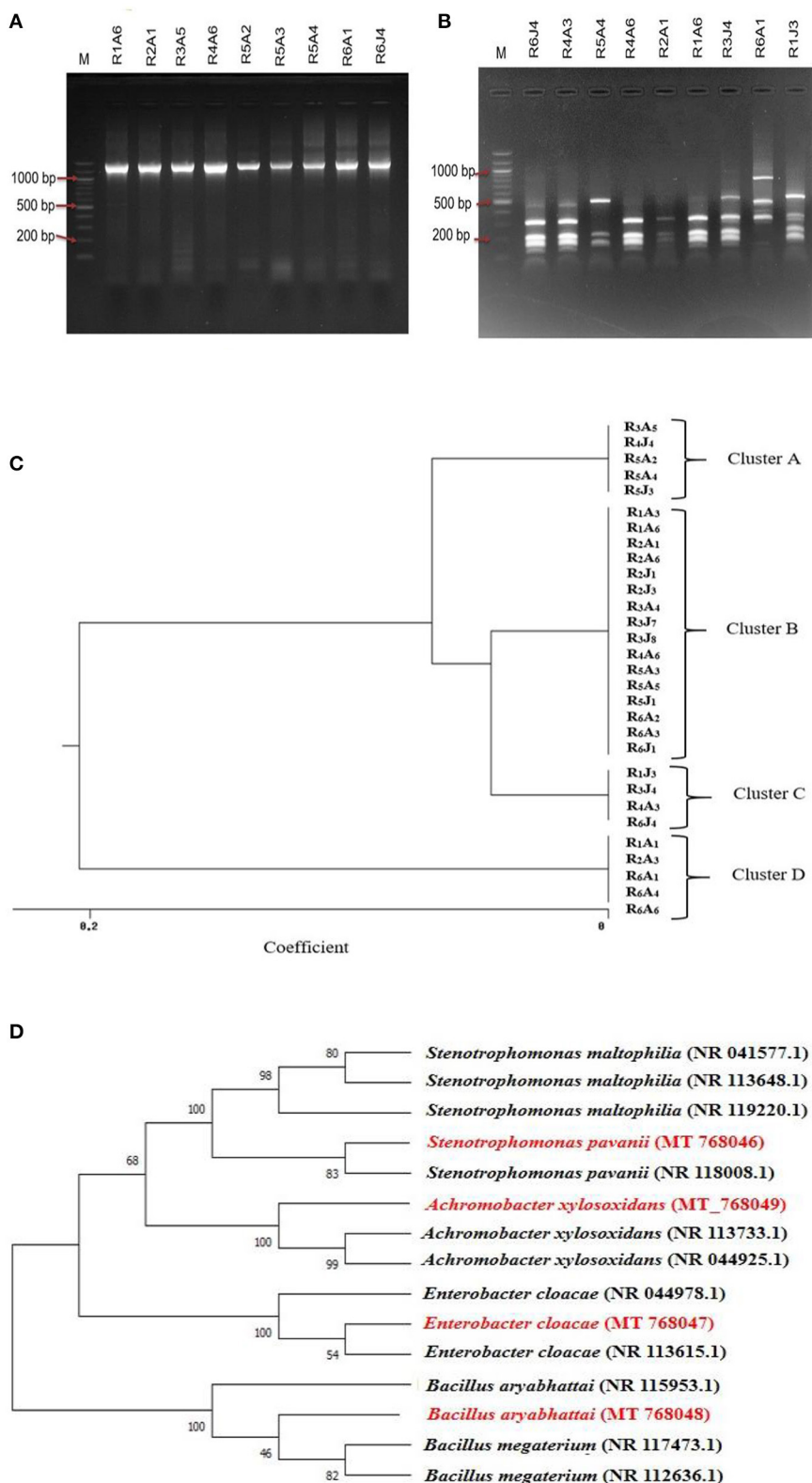
An SEM for observation of bacterial biofilm formation revealed a clear capsule-like layer outside the cell surface, which was more evident when the salt concentration was gradually increased from 0 to 200 mM indicating the formation of biofilm was

stress-driven. Here, the SEM of *S. pavanii* BRF2 is shown as a representative salt-tolerant isolate (Figure 5).

## DISCUSSION

Today's agriculture sector is being stroked by drastic climate changing conditions and the consequent salt intrusion has shrunk coastal agricultural lands ending up creating food insecurity and unsustainability for the ever-increasing population worldwide (Nabti et al., 2015; Shrivastava and Kumar, 2015; Szabo et al., 2016; Ansari et al., 2019). Current approaches of different irrigation methods, traditional breeding, and genetic engineering of salt-tolerant transgenic plants are highly technical and labor -intensive, and thus difficult to implement in practice (Singh et al., 2015; Niu et al., 2018). In order to counter salinity stress with a view to improving crop yields in salinity-prone coastal agricultural lands, the application of PGPR in the form of bioinoculants/ biofertilizer has emerged as a part of climate -smart agricultural practice under the changing climate conditions (Nabti et al., 2015; Sharma et al., 2016; Ansari and Ahmad, 2018).

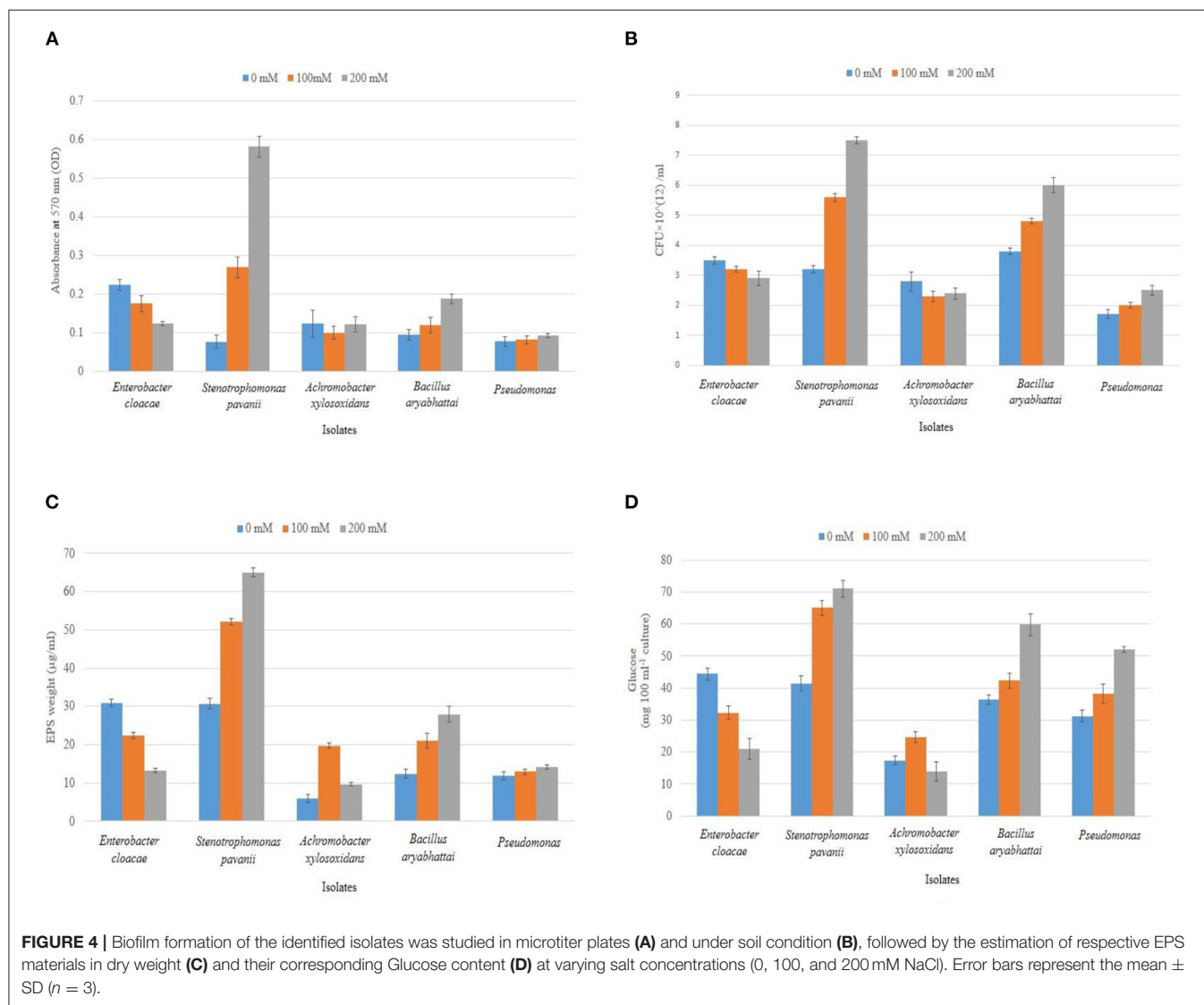
Many endophytes have been reported to be endowed with the inherent capability to cope with salt stress and stimulate stress resilience in plant growth under saline conditions when inoculated with plants (Egamberdieva et al., 2019; Kearl et al., 2019). With a view to creating such bio-bank, here we isolated 75 salt-tolerant endophytes associated with roots of *Oryza sativa* from the low-lying southern coastal region (pH > 7, EC > 7dS/m) of Bangladesh close to the Bay of Bengal, where periodic flushing from seawater has been reported to increase soil salinity. To mention, soil samples having EC > 4 dS/m can be referred to



**FIGURE 3 |** A representative illustration of 16S rDNA products amplified from the respective bacterial genome by PCR and electrophoresed on a 1.5% agarose gel (**A**) and ARDRA analysis of the 16S rDNA amplicon digested with the *BsuRI* (*HaeIII*) enzyme in a 1.5% (w/v) agarose gel; M: 100 bp ladder (**B**), and the resulting restriction pattern was used to produce a Dendrogram (**C**). The phylogenetic tree shows evolutionary distance among isolates based on 16S rDNA gene sequence using MEGA X. Bootstrap values are represented by numbers at the nodes based on 1,000 replications. The scale is the evolutionary distance value (**D**).

**TABLE 1** | Identity profile of 16S rDNA gene partial sequence of four isolates according to BLAST identification.

Isolate ID	GenBank accession no.	Base pair length	Closest comparison isolate (accession number)	Identity (%)
R <sub>4</sub> A <sub>6</sub>	MT_768047	1,411	<i>Enterobacter cloacae</i> (NR_044978)	99.72
R <sub>5</sub> A <sub>4</sub>	MT_768046	1,442	<i>Stenotrophomonas pavanii</i> (NR_118008)	99.51
R <sub>6</sub> A <sub>1</sub>	MT_768049	1,432	<i>Achromobacter xylosoxidans</i> (NR_113733)	98.81
R <sub>6</sub> J <sub>4</sub>	MT_768048	1,443	<i>Bacillus aryabhatai</i> B8W22 (NR_115953)	99.72

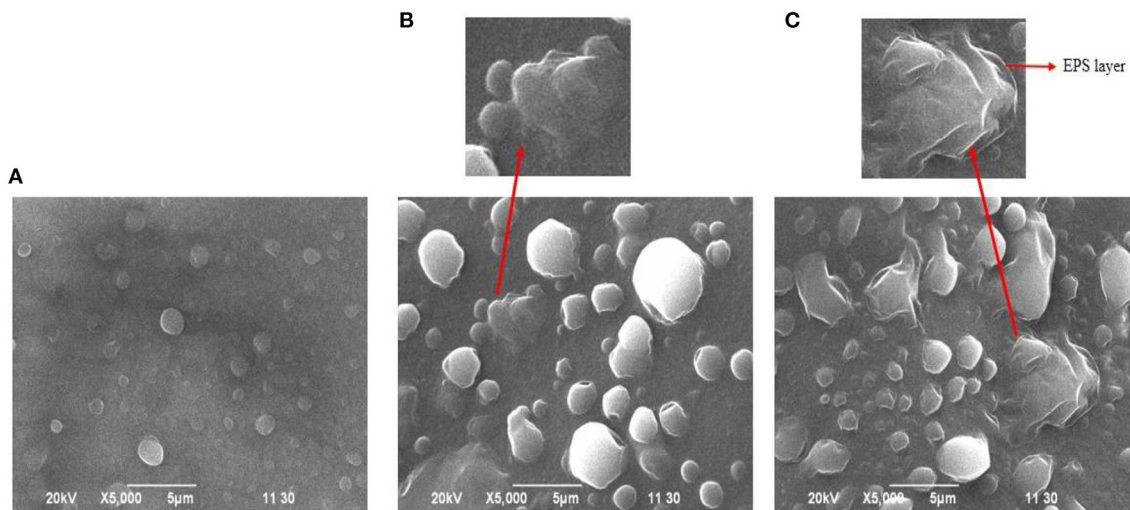


as saline soil (Bhat et al., 2020). One of the significant findings of this study is the screening of IAA producing, phosphate solubilizing, and nitrogen-fixing potential endophytes (analyzed *in vitro*) with an anticipation that they can stimulate the plant growth in the fields under saline condition if provided in the form of biofertilizer; a similar was experienced in our earlier study (Sultana et al., 2020).

IAA is the major auxin in plants, that maintains the growth and developmental stages of plants such as tissue elongation

and cell division, responses to light, gravity, and pathogens, and so on (Glick, 2012). Experiments showed that elevated salt stress affects the concentration of IAA in both xylem and phloem in plants (Junghans et al., 2006). Furthermore, IAA plays a major role in plant-microbe interactions which could be destabilized during different abiotic stresses (e.g., salt stress) (Spaepen and Vanderleyden, 2011). Therefore, the higher IAA producing salt-tolerant endophytes could help the growth-cornered plants by providing additional IAA under





**FIGURE 5 |** Biofilm formation by *S. pavanii* BRF2 at 0 (A), 100 (B), and 200 mM (C) NaCl concentrations under a scanning electron microscope.

salinity conditions as well as furnishing the rhizosphere. In our study, 11 isolates out of 75 were able to produce IAA in a range of 20–55  $\mu\text{g/ml}$  (**Supplementary Table 1**), which is similar or even higher than other reported PGPR that promoted growth of many crops (Sharma et al., 2016). On the other hand, the poor level of phosphorus in saline-prone cultivable lands leaves no other option for the farmers but to use excess conventional phosphorus-containing fertilizers to improve agricultural productivity, which otherwise causes potential surface water pollution, eutrophication, and fertility depletion in soil. The Phosphate Solubilizing Microorganisms (PSM) can improve the growth and yield of crops by mineralizing insoluble soil phosphate to release soluble phosphorus and making it available to plants. Thus, inoculating crops with PSM which can play the same role under saline conditions is a promising strategy to improve world food production without causing any environmental hazard (Alori et al., 2017). In our study, 57 isolates out of 75 were able to solubilize insoluble phosphate in NBRIP media indicating their potentiality to promote the growth of plants under phosphate limited conditions. In quantitative analysis, 12 isolates could solubilize phosphate in a range of 1–2  $\mu\text{g/ml}$  which is lower than the previously reported study on epiphytes (Albdaiwi et al., 2020; Sultana et al., 2020). Concurrently, intervention between salinity and nitrogen availability in soil is a very complex network disturbing almost all processes in plant metabolism and development. Many reports demonstrate that salt-tolerant bacteria associated with the rhizosphere have been found to show better survival, nodulation, nitrogen fixation, and profound nitrogen metabolism under saline conditions (Bala et al., 1990). Salinity adversely affects root nodulation and induces premature senescence of already formed nodules (Swaraj and Bishnoi, 1999). Thus, salt-tolerant endophytes with nitrogen-fixing ability are considered a potential resource for saline soil-based agriculture (Etesami and Beattie, 2018). Thirty-nine isolates found in our

study were able to fix a significant amount of nitrogen (108–198.7  $\mu\text{g/ml}$ ), hence can be considered as a potential weapon for biological nitrogen fixation if supplied during cultivation.

Biofilm development by different bacterial species is an elicited adaptation mechanism of survival in a harsh environment. The role of biofilm developed by endophytic PGPR is now considered as an important trait for effectively surviving in soil and root surface through colonization which is even more significant under salt stress (Santoyo et al., 2016). It was previously reported that biofilm protected bacterial cells at elevated salt stress by forming exopolysaccharide (EPS), a high molecular weight carbohydrate compound attached to the outer surface of bacteria allowing them to attach with each other and to surfaces of plant roots and soil particles (Qurashi and Sabri, 2012a). Bacterial EPS were reported to form a water-absorbing sheath around the roots to support the root system in excessive  $\text{Na}^+$  contained soils (Ashraf and Harris, 2004; Rolli et al., 2015). Moreover, EPS synthesized by endophytic bacteria might bind cations including  $\text{Na}^+$ , decreasing  $\text{Na}^+$  availability for plant uptake and consequently improving the salinity tolerance of the plant (Ashraf and Harris, 2004; Siddique et al., 2011). Inoculation of EPS producing PGPR showed ameliorative effects on the uptake of  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  in plants which resulted in stabilization of soil aggregates and regulation of nutrients and water flow across plant roots through biofilm formation (Grover et al., 2011; Kasim et al., 2016; Ilangumaran and Smith, 2017; Abbas et al., 2019; Egamberdieva et al., 2019). In this study, we report that the intrinsic ability of screened isolates to form biofilm *in vitro* and ‘in soil’ condition was enhanced with increasing NaCl concentrations which is in accordance with previous reports (Qurashi and Sabri, 2012a,b; Kasim et al., 2016). The isolate, *S. pavanii* BRF2 demonstrated strong biofilm formation under saline conditions (**Figure 4**), and this was well-supported in SEM (**Figure 5**) images. Both the isolates *S. pavanii* BRF2 and

*B. aryabhattai* DRF1 outcompeted others in forming biofilm and EPS under the additional saline concentrations (200 mM NaCl). Since the isolates were not challenged beyond 200 mM NaCl, the peak for the *S. pavanii* BRF2 and *B. aryabhattai* DRF1 could not be measured, however, it was well-understood for *A. xylosoxidans* DRF2 (100 mM NaCl).

Our previous study provided a documentary regarding salt-tolerant epiphytes and their demonstrated proven role to support the yield and overall growth of crops under saline conditions (Sultana et al., 2020). In addition to the reported IAA producing, phosphate solubilizing, and nitrogen fixing potentials, the PGPR isolate, *B. aryabhattai* MS9 was shown in another study to reveal its siderophore producing ability in an iron-poor condition under salinity stress (Sultana et al., 2021), making it a suitable candidate for composing biofertilizer. It is also notable that *B. aryabhattai* has been found as a common PGPR member (both epi- and endophytes) with profound PGP activities (Park et al., 2017; Shen et al., 2019). Here, we isolated some potential endophytes, collected from the roots of rice plants grown in saline soil with significant PGP attributes: IAA production, phosphate solubilization, and N<sub>2</sub> fixation (Figure 2). Being the endophytes, these were already acclimatized in the plants' system biology, and that too under the stress condition, where they established a symbiotic relationship with plants, a phenomenon well-supported by other reports (Nautiyal et al., 2013; Khalifa et al., 2016; Abdel-Rahman et al., 2017; Singh and Jha, 2017). Furthermore, their salt-tolerance properties associated with the significant amount of EPS production suggest the mechanism behind their survivability under stress conditions. As endophytic microorganisms from plants of different ecosystems do not involve host specificity (Nair and Padmavathy, 2014), this report supports the notion of other reports about endophytes being a substantial alternative strategy to plants for alleviating abiotic stresses arising from changeable environmental conditions (Khalifa et al., 2016; Haidar et al., 2018; Niu et al., 2018). Overall, the findings from this report support the development of sustainable biotechnological approaches toward the application of endophytes in the improvement of crop yield under stressful conditions.

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## CONCLUSION

The present study demonstrated that biofilm-forming salt-tolerant endophytes with multifarious PGP attributes could be utilized as promising bioinoculants for salinity stress management. The positive effects exhibited by the four isolates need to be evaluated *in vivo* experiments under saline conditions for further practical exploitation in crop production. Utilization of this green biotechnology will have multi-faceted positive impacts and could be a savior for saline-prone areas. In the future, these salt-tolerant endophytic PGPR could improve crop production in an economically sustainable manner that would add value to the preparedness strategy for climate change.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI (Accession: MT768046-MT768049).

## AUTHOR CONTRIBUTIONS

MK conceptualized the idea. TJ and JR did the bench works. SS, TJ, and MK drafted the manuscript and worked on literature review. MR critically reviewed the manuscript. All authors approved the final manuscript.

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# Plant Growth Promoting Abilities of Novel *Burkholderia*-Related Genera and Their Interactions With Some Economically Important Tree Species

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A survey of bacterial endophytes associated with the leaves of oil palm and acacias resulted in the isolation of 19 bacterial strains belonging to the genera *Paraburkholderia*, *Caballeronia*, and *Chitinasiproducens*, which are now regarded as distinctively different from the parent genus *Burkholderia*. Most strains possessed one or more plant growth promotion (PGP) traits although nitrogenase activity was present in only a subset of the isolates. The diazotrophic *Paraburkholderia tropica* strain S39-2 with multiple PGP traits and the non-diazotrophic *Chitinasiproducens palmae* strain JS23<sup>T</sup> with a significant level of 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity were selected to investigate the influence of bacterial inoculation on some economically important tree species. Microscopic examination revealed that *P. tropica* S39-2 was rhizospheric as well as endophytic while *C. palmae* JS23<sup>T</sup> was endophytic. *P. tropica* strain S39-2 significantly promoted the growth of oil palm, eucalyptus, and *Jatropha curcas*. Interestingly, the non-diazotrophic, non-auxin producing *C. palmae* JS23<sup>T</sup> strain also significantly promoted the growth of oil palm and eucalyptus although it showed negligible effect on *J. curcas*. Our results suggest that strains belonging to the novel *Burkholderia*-related genera widely promote plant growth via both N-independent and N-dependent mechanisms. Our results also suggest that the induction of defense response may prevent the colonization of an endophyte in plants.

**Keywords:** *Paraburkholderia tropica*, *Chitinasiproducens palmae*, N-fixing leaf endophytes, oil palm, acacia

## INTRODUCTION

Plant-associated bacteria play important roles in plant development and differentiation (Hallmann et al., 1997; Reiter and Sessitsch, 2006). Rhizospheric bacterial communities are known to modulate the bio-availability of mineral nutrients, stimulate plant growth through phytohormone production, and enhance plant resistance to pathogens (Bringel and Couée, 2015). On the other hand, phyllospheric bacteria that inhabit the aerial parts of plants are lesser studied despite the fact that the global phyllosphere measures more than 10<sup>8</sup> km<sup>2</sup> accommodating a diverse bacterial community of about 10<sup>26</sup> bacterial cells (Lindow and Brandl, 2003). It is intriguing how the oxygen-sensitive nitrogen fixation system adapts itself to the photosynthetic O<sub>2</sub>-rich phyllosphere (Dixon and Kahn, 2004).

How the tissue-specific localization of various endophytes is regulated by the plant system is also poorly understood.

*Burkholderia* is a fairly large genus with more than 100 validly described species. It has received little attention from the application point of view due to the fact that the *B. cepacia* complex (Bcc), comprising 20 species, cause opportunistic infections in cystic fibrosis patients while members of the *B. pseudomallei* group cause melioidosis (Cheng and Currie, 2005). *Burkholderia mallei* is the causative agent of the Glanders disease in horses (Nierman et al., 2004). Recent data suggest that *Paraburkholderia* and *Caballeronia* are phylogenetically distinct from the *Burkholderia cepacia* complex and has therefore spawned an interest for their agrobiotechnology applications (Caballero-Mellado et al., 2007; Sawana et al., 2014; Dobritsa and Samadpour, 2016). Diazotrophic *Paraburkholderia* species have been isolated from the rhizosphere, nodules of legume plants, as well as root tissues of non-legume plants (Aizawa et al., 2010; Suárez-Moreno et al., 2012; De Meyer et al., 2013, 2014; Martínez-Aguilar et al., 2013; Sheu et al., 2013, 2015; Steenkamp et al., 2015), while a phosphate solubilizing *Paraburkholderia tropica* strain has been isolated from the rhizosphere of pomegranate (Kaur et al., 2016).

*Paraburkholderia phytofirmans* strain PsJN is a well-studied plant endophyte and has contributed greatly to the understanding of plant–endophyte interactions (Frommel et al., 1991; Conn et al., 1997; Pillay and Nowak, 1997; Barka et al., 2000; Kim et al., 2012b; Kost et al., 2013). *Chitinasiproducens palmarum* isolated from the leaf tissues of oil palm is a newly validated genus genetically distinct from *Burkholderia*, *Caballeronia*, or *Paraburkholderia* (Madhaiyan et al., 2020).

Oil palm is one of the most economically important oil yielding crops in Southeast Asia (Koh and Wilcove, 2007). Acacia and eucalyptus are the dominant forestry species in tropical Southeast Asia, supplying raw material for paper and pulp, plywood, and wood composites. *Jatropha curcas*, on the other hand, is a shrub that grows in poor soils, produces oil-rich seeds, and is seen as an emerging biofuel crop (Madhaiyan et al., 2013b). The growth of such tree crops demands a high availability of soil nutrients, especially nitrogen since forest productivity is directly related to nutrient levels of the soil (Smethurst et al., 2004; Gobert and Plassard, 2008). Inoculation with  $N_2$ -fixing endophytic bacteria is an attractive option to reduce the demand for nitrogen fertilizer and to enhance tree biomass production. This study was therefore undertaken to decipher the mechanisms of plant growth-promoting activities of the *Burkholderia*-related genera, which have not been studied in much detail in the past.

## MATERIALS AND METHODS

### Isolation of Nitrogen-Fixing Endophytic Bacteria

Healthy oil palm leaves and phyllodes of Acacias were collected from various locations of Singapore. The collected samples were placed in plastic bags and processed within 2 h. Sample tissues (~5 g) were surface sterilized by sequential immersion in 90% (v/v) ethanol for 5 min and 10% (v/v) hydrogen peroxide solution

for 10 min followed by repeated washing in sterile water. A 100- $\mu$ l aliquot of the wash water from the third rinse was plated on a rich medium to confirm the efficiency of surface sterilization. Surface-sterilized tissues were macerated, enriched in an N-free medium, diluted serially with phosphate saline buffer, and plated on JNFB (Döbereiner et al., 1995), YEMA (Vincent, 1970), or BAC medium (Estrada-De Los Santos et al., 2001). An aliquot (100  $\mu$ l) of the enriched suspension was simultaneously inoculated in a tube containing semi-solid N-free media. Sub-surface pellicles or colonies that appeared on agar plates after 72 h at 30°C were sub-cultured to obtain pure cultures. Isolates were routinely cultured on their respective agar plates at 30°C under aerobic conditions and stored at –80°C in 7% (v/v) DMSO.

### DNA Amplification, Sequencing, and Phylogenetic Analysis

The phylogenetic positions of the isolated strains were identified by analyzing the complete 16S rRNA gene sequence as previously described (Wilson, 1997). The 16S rRNA gene was amplified from the genomic DNA of the isolated strains using the universal primers 27F and 1492R as described previously (DeLong, 1992; Madhaiyan et al., 2015). Sequencing was done with the BigDye Terminator Cycle Sequencing Kit (Perkin Elmer, Waltham, MA, USA) and samples were run on an Applied Biosystems 3730 XL DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). Sequences were analyzed against the EzTaxon-e Database (<http://eztaxon-e.ezbiocloud.net/>) (Kim et al., 2012a) and aligned using ClustalW tool in MEGA version 6 (Tamura et al., 2013). PCR amplification of the partial *nifH* fragment was performed as described (Pinto-Tomás et al., 2009). A 750-bp fragment of the ACC deaminase gene (*acdS*) was amplified using the primers F1937 and F1938 as described (Blaha et al., 2006). The *nifH* and *acdS* gene sequences were analyzed by BLAST searches against sequences in the GenBank at NCBI.

### Screening for Plant Growth-Promoting Traits

The diazotrophic trait of the isolates was confirmed by the presence of Acetylene Reduction Activity (ARA). The assay was performed using 125 ml serum bottles (Wheaton Industries Inc., Millville, NJ, USA) filled with the nitrogen-free medium (40 ml) of the following composition (per liter): 5 g Glucose, 5 g Mannitol, 0.1 g  $CaCl_2 \cdot 2H_2O$ , 0.1 g  $MgSO_4 \cdot 7H_2O$ , 5 mg  $Na_2MoO_4 \cdot 2H_2O$ , 0.9 g  $K_2HPO_4$ , 0.1 g  $KH_2PO_4$ , 0.01 g  $FeSO_4 \cdot 7H_2O$ , 5 g  $CaCO_3$ , and 1 ml trace element mixture. The trace element mixture comprised (per liter) of 0.1 g  $ZnSO_4 \cdot 7H_2O$ , 0.03 g  $MnCl_2 \cdot 4H_2O$ , 0.3 g  $H_3BO_3$ , 0.2 g  $CoCl_2 \cdot 6H_2O$ , 0.01 g  $CuCl_2 \cdot 2H_2O$ , and 0.02 g  $NiCl_2 \cdot 6H_2O$  in water. The assay was performed by injecting purified acetylene gas into the bottles that were sealed with gas-tight serum stoppers to achieve a yield of 15% acetylene (v/v), followed by incubation for 96 h at 30°C. For nitrogenase switch-off/switch-on assays, the isolates were cultured in N-free medium and supplemented with 1 mM  $NH_4Cl$  or 10%  $O_2$ . Protein concentration of the bacteria used for ARA assay was determined with the Bicinchoninic acid

kit (B9643; Sigma-Aldrich, St. Louis, MO, USA) using bovine serum albumin (BSA) as the standard.

To determine the nitrogenase activity under *in planta* conditions, the ARA was performed using fresh plant tissues that were collected 30 and 60 days after inoculation of the individual bacteria strains. Root samples of the plants were separated from seedlings after carefully removing the adhering soil; placed in 250 ml glass bottles, and sealed with a rubber septum. After removing an equivalent volume of air, acetylene was injected into the bottles to achieve a final concentration of 15% (v/v) and incubated at 30°C for 24 h. Gas samples (0.8 ml for *in planta* samples) were removed at regular intervals with a PTFE-syringe (Hewlett-Packard, Palo Alto, CA, USA) and analyzed in a GCMS-QP2010 Ultra Gas Chromatograph (Shimadzu Corporation, Kyoto, Japan) with a flame ionization detector and GS-Alumina (30 m × 0.53 mm I.D.) column. The operational conditions were helium gas flow 30 ml/min; detector temperature: 200°C; pressure: 4.0 psi. The ethylene produced by the bacteria was quantified using a standard ethylene (C<sub>2</sub>H<sub>4</sub>, Product Number: 00489, Sigma-Aldrich) curve that was prepared in duplicate with concentrations ranging from 1 to 1000 nmol. All the obtained values were expressed after deducting the ethylene value of a blank treatment. The *in planta* ARA values were expressed as nmol C<sub>2</sub>H<sub>4</sub> released day<sup>-1</sup> seedlings<sup>-1</sup> after deducting the plant's background C<sub>2</sub>H<sub>4</sub> emission.

The ability of the strains to catabolize ACC was determined by assessing their growth in DF minimal salts medium (Dworkin and Foster, 1958) supplemented with 3 mM ACC. ACC deaminase activity was measured spectrophotometrically at 540 nm as described previously by Shah et al. (1998) and Honma and Shimomura (1978).

For the extraction of indole compounds, a single colony was used to inoculate 6 mL 2xYT medium supplemented with 100 µg ml<sup>-1</sup> L-tryptophan and incubated at 30°C with agitation (200 rpm) for 4 days. A 2-ml aliquot of the cell-free supernatant was mixed with 100 µl of 10 mM orthophosphoric acid and 4 ml of Salkowski's reagent (1 ml of 0.5 M FeCl<sub>3</sub> in 50 ml of 35% HClO<sub>4</sub>) and incubated at room temperature for 25 min. The concentration of indoles in the culture supernatant was determined spectrophotometrically at 530 nm (UV-1601, Shimadzu) by interpolating against the standard curve of indole-3-acetic acid prepared with concentrations ranging from 0 to 100 µg/ml (Sigma Chemical Co., St. Louis, MO, USA).

To evaluate the phosphate solubilization activity, strains were spot-inoculated on NBRIP agar plates (Nautiyal, 1999) containing 0.5% tricalcium phosphate [Ca<sub>3</sub>(PO<sub>4</sub>)<sub>2</sub>] as the inorganic phosphate source. The plates were incubated at 30°C for 7 days and solubilization of mineral phosphate was qualitatively estimated by measuring the diameter of the clear halo formed as a result of phosphate solubilization around the bacterial colonies.

The ability to produce siderophores by bacterial strains was assayed using the universal Chrome Azurol S (CAS; Sigma-Aldrich) dye assay (Schwyn and Neilands, 1987; Gao et al., 2006).

## Screening for Cell Wall Degrading Endoglucanase Activity

The endoglucanase activity was determined as described previously (Reinhold-Hurek et al., 1993), with some modifications. A plate containing Kim-Wimpenny solid medium with 0.2% carboxymethyl cellulose (CMC) (Kim and Wimpenny, 1981), with or without 0.5% D-glucose, was spotted with 1 µl of an exponentially growing culture that was adjusted to an absorbance at 600 nm to 1.0; air-dried and incubated at 30°C for 3 days. Colonies were flushed off the plate with water and the plate was stained with a 0.1% Congo red solution for 30 min, followed by several washes with 1 M NaCl. The appearance of a clear yellow halo around the colony in a red background indicated a positive staining for endoglucanase activity.

## Growth Inhibitory Activity

*Rhodospiridium toruloides* ATCC 10657 (Hu and Ji, 2016) and *Kosakonia sacchari* strain R4-368 (diazotrophic), the latter previously known as *Enterobacter* species (Madhaiyan et al., 2013a,b), were used as the fungal and bacterial test strains, respectively, for the determination of growth-inhibiting activity of the strains as described by Compant et al. (2005).

## Construction of GFP-Tagged S39-2 and JS23<sup>T</sup> Strains

*Escherichia coli* strain S17-1λ $\pi$  was used to mobilize the Tn5 transposon, constructed in pUT-tac-aph-sfGFP (Supplementary Figure 1), to the genome of S39-2 and JS23<sup>T</sup> strains by biparental mating. Mating was carried out overnight by mixing equal volume of an overnight culture of donor and acceptor cells on a 0.2-µm filter membrane placed on a 2xYT plate. Transformants were selected on succinate media (Nunn and Lidstrom, 1986) with 25 µg ml<sup>-1</sup> kanamycin. The fluorescence was quantified using an Infinite® M200 microplate reader (TECAN, Männedorf, Switzerland). A single transformant that showed high fluorescence with no growth abnormality was selected for plant colonization studies.

The transformant's stability was determined by re-streaking a single colony from selective medium onto a 2xYT agar plate without any selection pressure. After 12 subcultures at a 5-day interval in 2xYT agar plates, colonies were scored for kanamycin resistance and GFP fluorescence. Fresh colonies that were scrapped off the agar plates were washed in sterile distilled water and starved at 4°C for 3 weeks. The suspensions were then plated onto succinate media with or without kanamycin. The colony, cell morphology, and growth of the GFP derivatives were compared with the wild type by culturing in 2xYT and succinate media, respectively.

## Plant Inoculation and Endophytic Colonization of Seedlings

Lemon eucalyptus seeds were obtained from Richters Herbs (Goodwood, Canada). Seeds were surface sterilized by soaking in 70% alcohol (v/v) for 1 min and 15% H<sub>2</sub>O<sub>2</sub> for 5 min followed by thorough rinsing with sterilized distilled water (5 × 15 min each) on a shaking platform. The surface-sterilized



seeds were germinated in sterilized soil that had been autoclaved (121°C, 15 psi for 3 h). Strains JS23<sup>T</sup> and S39-2 were cultured in 2xYT medium for 36 h at 30°C, adjusted to a density of approximately 10<sup>8</sup> cfu ml<sup>-1</sup>, and applied near the root zone at a dose of 2 ml per seedling at 7 days after sowing (DAS). On day 14, the seedlings were transferred from germination trays to plastic pots (15 × 25 cm) containing 750 g non-sterilized red soil (Far East Flora, Singapore). Plants were maintained in a greenhouse with no artificial lighting and air-conditioning. Plant height, number of leaves, number of branches, chlorophyll content, and leaf area were determined at 30 and 60 DAI. Leaf chlorophyll levels were measured using the at LEAF chlorophyll meter (FT Green LLC, Wilmington, DE, USA). The relative chlorophyll concentration was recorded as the ratio of transmittance between the red (650 nm) and infrared (940 nm) emissions through the leaf. Leaf nitrogen (N) content was determined by the combustion method in an elemental analyzer (Vario EL Elemental Analyzer, Elementar, Germany) equipped with a thermal conductivity detector.

Oil palm seedlings derived from tissue culture of a hybrid variety were raised in plastic pots filled with 250 g potting mix (BVB substrates; Stealth Garden, Seaclyff Park, Australia) and maintained in a greenhouse for 60 days. Selected healthy seedlings of uniform size were transferred to 45 × 60 cm pots containing ~5 kg non-sterilized red soil. The root zone of each seedling was inoculated with 5 ml of exponentially grown bacterial cells (10<sup>8</sup> cfu/ml). Plants were maintained in a greenhouse with no artificial lighting and air-conditioning. Plant growth data were recorded at 30, 60, and 90 DAI.

Seeds of *Jatropha curcas* were sown in germination trays filled with potting mixture and inoculated with 2 ml exponentially grown cells (10<sup>8</sup> cfu/ml). Root inoculation was done after germination, and the growth parameters were recorded at 60 DAI. For pot cultivation experiment under N-limiting condition, healthy seedlings derived from surface-sterilized seeds were transferred to pots containing a mixture of perlite, vermiculite, and sand in 1:1:1 ratio (v/v). Bacterial cultures (10<sup>8</sup> cfu ml<sup>-1</sup>; 50 ml pot<sup>-1</sup>) were applied to the root zone around the stem. Plants were maintained in a glasshouse. As a control, plants were watered as needed with a plant nutrient solution containing with 2 mM ammonium chloride (N source). For plants grown under N-limiting condition, a plant nutrient solution without the N source was used.

## Plant Colonization and Monitoring Tissue Localizations by CLSM

To track plant colonization in lemon eucalyptus, seeds were germinated for 7 days under gnotobiotic conditions in multi-well plates containing MS medium (Murashige and Skoog, 1962) and transferred to Phytatray<sup>TM</sup> II plant growth vessels (P5929 Sigma) each containing 200 g of sterilized sand (121°C, 15 psi, 3 h) filled with 40 ml of autoclaved nitrogen-free plant nutrient solution (Iniguez et al., 2004). The plants were cultured in a growth chamber with a temperature of 28°C and 16/8 h day-night cycles. The GFP labeled JS23<sup>T</sup> and S39-2 strains were cultured in succinate medium for 48 h, adjusted to 10<sup>8</sup> cfu

ml<sup>-1</sup>, and inoculated at 5 ml per tray 3 days after seedling transplantation. Endophytic colonization was determined using surface-sterilized shoots (leaf + stem) and roots (30 DAI), which were homogenized with sterile pestle and mortar. Serially diluted samples were plated on R2A agar plates, incubated at 30°C for 3–5 days before colony forming units (CFU) were determined. The efficiency of surface sterilization was monitored by spreading an aliquot of the last wash on a 2xYT agar plate and incubated for 3 days at 30°C.

The localizations of GFP-labeled cells in eucalyptus tissues were examined with confocal laser scanning microscopy (CLSM) at 15, 30, and 45 days after inoculation (DAI). CLSM was carried out using a Zeiss LSM 510 upright confocal microscope with a motorized stage (Carl Zeiss Inc., Oberkochen, Germany) equipped with a multi-argon laser (458/477/488/514 nm), and a He/Ne laser (543 nm and 633 nm). Images were acquired using the Plan-Apochromat 63×/1.4 oil differential objective and analyzed using the Laser Scanning System LSM5 PASCAL software. Bacterial localization in eucalyptus root and shoot tissues was identified from 3-D confocal stacks.

## Nucleotide Sequence Accessions

The 16S rRNA gene sequences of the strains have been submitted to NCBI under the accession numbers KU049648-KU049653, KU140660-KU140661, and KT337490-KT337503. The *acdS* gene and *nifH* gene sequences can be found under the accession numbers KX622566-KX622569 and KX622570-KX622579.

## Statistical Analysis

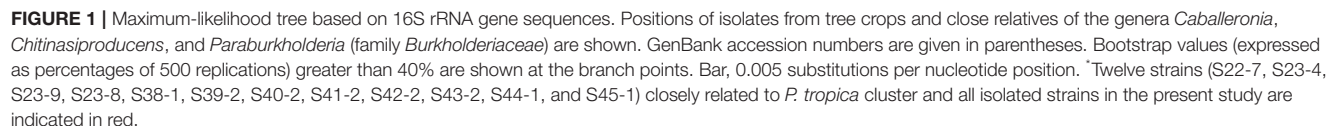
Statistical analyses were carried out using the Statistical Analysis System (SAS) Version 9.4 (SAS Institute Inc., Cary, NC, USA). Analysis of variance (ANOVA) for the endophytic population was carried out using the General Linear Model (GLM) in SAS. The bacterial population data were log-transformed before being subjected to further analysis. The means of the treatment results were subjected to ANOVA and presented using Fisher's protected least significant difference (LSD). Endophytic bacterial inoculation data were subjected to analysis of variance and testing of means by Duncan's multiple range test (DMRT) at  $p \leq 0.05$  using SAS package.

## RESULTS

### Isolation of Endophytic Bacteria From Leaves of Oil Palm and Phyllodes of Acacia

A survey of endophytic bacteria associated with the phyllosphere of oil palm and Acacia led to the isolation of 19 strains belonging to three *Burkholderia* related genera, *Paraburkholderia*, *Caballeronia*, and *Chitinasioproducens* (Figure 1). Recently, the genus *Burkholderia* has been split into three clades: Clade I retaining the genus name *Burkholderia* and consisting mainly of animal and plant pathogens; Clade II was named as genus *Caballeronia* which are mainly composed of strains of environmental origin (Dobritsa and Samadpour, 2016), while Clade III was named as genus *Paraburkholderia*, which are environmental bacteria including plant growth-promoting bacteria and nodule symbionts (Coenye and Vandamme, 2003;





**TABLE 1** | Screening for growth-promoting traits of endophytic isolates from oil palm and acacia leaves.

Isolates	Source (locality) <sup>a</sup>	Close relative (% similarity) <sup>b</sup>	ARA <sup>c</sup>	ACC deaminase <sup>d</sup>	TCP solubilization <sup>e</sup>	Siderophore <sup>f</sup>	Auxins ( $\mu\text{g ml}^{-1}$ ) <sup>g</sup>	EGA <sup>h</sup>	Anti-fungal activity <sup>i</sup>
S51-2	Oil palm leaflet (S2)	<i>P. unamae</i> (100)	60.0 $\pm$ 7.3	11.1 $\pm$ 1.1	17.1 $\pm$ 2.5	2.3 $\pm$ 0.4	5.2 $\pm$ 2.1	8.1 $\pm$ 1.4	+
S22-7	Oil palm leaflet (S1)	<i>P. tropica</i> (100)	–	–	17.7 $\pm$ 1.5	–	3.0 $\pm$ 0.9	5.0 $\pm$ 0.4	–
S23-4	Oil palm leaflet (S1)	<i>P. tropica</i> (100)	–	–	17.0 $\pm$ 2.0	–	2.8 $\pm$ 0.4	4.7 $\pm$ 0.6	–
S23-9	Oil palm leaflet (S1)	<i>P. tropica</i> (100)	–	6.1 $\pm$ 0.6	14.0 $\pm$ 2.0	–	12.8 $\pm$ 3.6	5.0 $\pm$ 0.0	–
S23-8	Oil palm leaflet (S1)	<i>P. tropica</i> (100)	–	10.3 $\pm$ 1.3	16.0 $\pm$ 1.0	–	4.2 $\pm$ 0.2	4.3 $\pm$ 1.2	–
S38-1	Oil palm leaflet (S2)	<i>P. tropica</i> (99.6)	167.6 $\pm$ 19.3	–	18.3 $\pm$ 1.5	–	7.2 $\pm$ 1.0	6.7 $\pm$ 0.6	–
<u>S39-2</u>	Oil palm leaflet (S2)	<i>P. tropica</i> (99.6)	348.6 $\pm$ 55.3	12.4 $\pm$ 1.4	18.7 $\pm$ 3.2	3.3 $\pm$ 0.4	11.0 $\pm$ 2.4	8.7 $\pm$ 2.1	–
S40-2	Oil palm leaflet (S2)	<i>P. tropica</i> (99.6)	18.8 $\pm$ 4.9	–	14.0 $\pm$ 1.0	–	6.4 $\pm$ 1.3	5.7 $\pm$ 0.6	–
S41-2	Oil palm leaflet (S2)	<i>P. tropica</i> (99.7)	25.5 $\pm$ 5.2	9.4 $\pm$ 0.8	18.3 $\pm$ 2.5	–	12.3 $\pm$ 3.6	7.0 $\pm$ 1.0	–
S42-2	Oil palm leaflet (S2)	<i>P. tropica</i> (99.7)	198.7 $\pm$ 24.2	7.1 $\pm$ 1.1	22.3 $\pm$ 0.6	–	12.0 $\pm$ 2.5	8.7 $\pm$ 0.6	–
S43-2	Oil palm leaflet (S2)	<i>P. tropica</i> (99.6)	160.2 $\pm$ 8.5	–	19.3 $\pm$ 1.5	–	7.6 $\pm$ 1.7	7.7 $\pm$ 0.6	–
S44-1	Oil palm leaflet (S2)	<i>P. tropica</i> (99.5)	173.9 $\pm$ 29.5	5.9 $\pm$ 0.5	18.7 $\pm$ 2.5	–	2.0 $\pm$ 0.1	7.3 $\pm$ 0.6	+
S45-1	Oil palm leaflet (S2)	<i>P. tropica</i> (99.6)	49.2 $\pm$ 0.7	9.3 $\pm$ 0.5	19.3 $\pm$ 1.5	–	3.0 $\pm$ 0.7	6.7 $\pm$ 0.6	–
<u>JS23<sup>T</sup></u>	Oil palm leaflet (S3)	<i>C. palmae</i>	–	3.2 $\pm$ 0.2	–	–	1.5 $\pm$ 0.1	–	++
Aca-21	Acacia leaf stalks (S3)	<i>P. sediminicola</i> (99.9)	–	–	14.0 $\pm$ 1.0	–	3.2 $\pm$ 0.4	7.0 $\pm$ 1.0	–
Aca-213	Acacia leaf stalks (S3)	<i>P. aromaticivorans</i> (98.6)	–	8.1 $\pm$ 1.2	12.0 $\pm$ 1.0	–	2.1 $\pm$ 0.5	14.7 $\pm$ 2.5	–
Aca-28	Acacia leaf stalks (S3)	<i>C. mineralivorans</i> (99.5)	–	8.2 $\pm$ 1.2	–	–	5.0 $\pm$ 0.1	–	–
Aca-214	Acacia leaf stalks (S3)	<i>C. mineralivorans</i> (99.7)	–	6.9 $\pm$ 0.8	–	–	6.0 $\pm$ 0.7	–	–
Aca-215	Acacia leaf stalks (S3)	<i>C. mineralivorans</i> (99.6)	–	9.8 $\pm$ 1.8	–	–	9.7 $\pm$ 1.3	–	–

<sup>a</sup>Sample collection in different locations in Singapore in September 2012, Temasek Life Sciences Laboratory (S1), West Coast Park (S2), and Science Park II (S3).

<sup>b</sup>The isolate was identified using the EzTaxon server on the basis of 16S rRNA sequence data.

<sup>c</sup>Acetylene reduction activity (ARA, nitrogenase activity) was performed by same cultural conditions ( $n = 3$ ) and expressed in nmol  $\text{C}_2\text{H}_4/\text{mg}$  of protein/h.

<sup>d</sup>ACC deaminase activity is expressed as nmol  $\alpha$ -Ketobutyrate/min/mg protein. Data represent mean  $\pm$  SD ( $n = 3$ ).

<sup>e</sup>TCP-solubilization plate assay; phosphate solubilization efficiency was determined by a clear halo (diameter in mm) around the bacterial colonies.

<sup>f</sup>Siderophore production comparison among the endophytic leaf isolates strains on a CAS siderophore testing agar. The chelator-iron (III) complex tints the agar with a rich blue background. The orange halo surrounding the colony indicates the excretion of siderophore and its dimension approximates the amount of siderophore excreted.

<sup>g</sup>Indole related compound production by endophytic bacteria strains was estimated in 2xYT broth cultures supplemented with 100  $\mu\text{g ml}^{-1}$  L-tryptophan.

<sup>h</sup>Plate assays for endoglucanase activity (EGA), 0.1% Congo-red stained KW agar plate with glucose. EGA was determined by the appearance on the red background of clear yellowish halos around the points where the bacterium was inoculated.

<sup>i</sup>Relative inhibitory activity against *R. toruloides*.

<sup>T</sup>Type species is published (Madhaiyan et al., 2020). Selected strains (S39-2 and JS23<sup>T</sup>) underlined for further study.

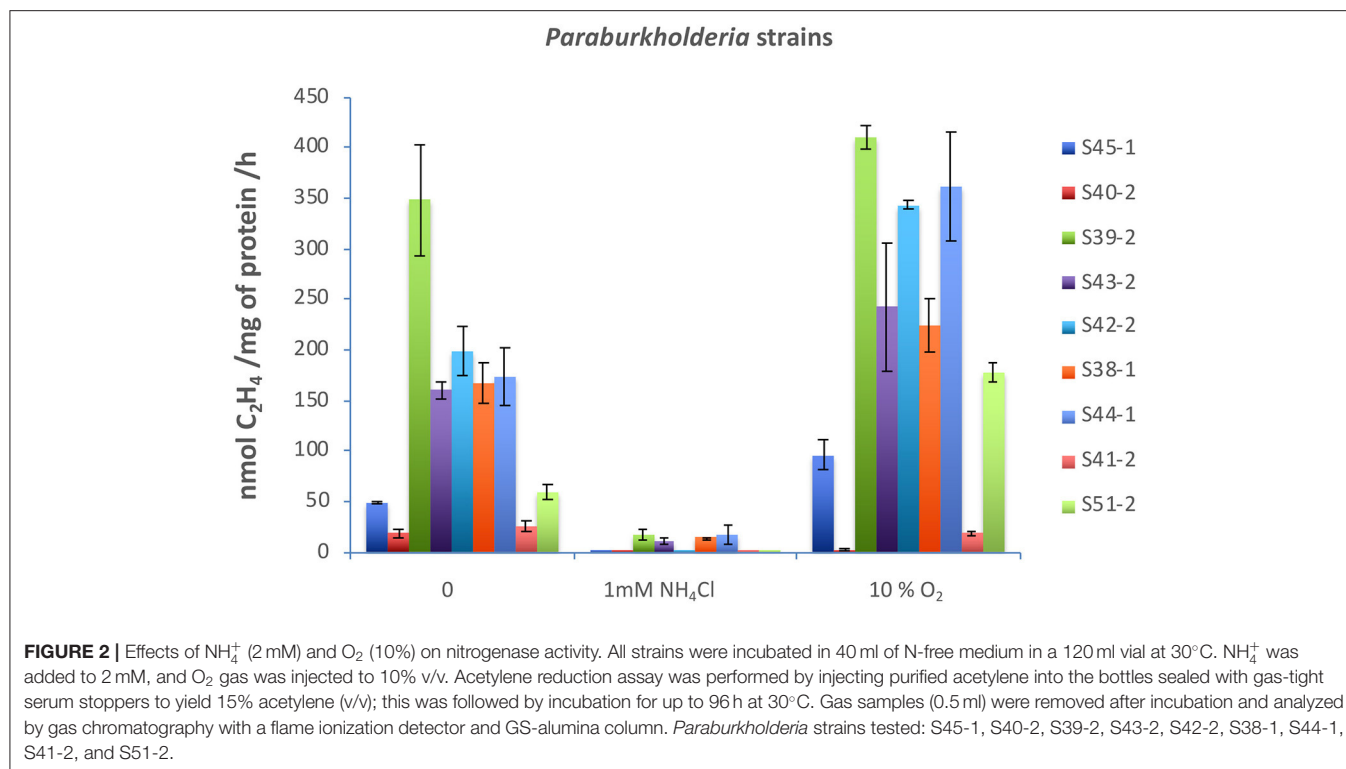
Suárez-Moreno et al., 2012; Sawana et al., 2014; Dobritsa and Samadpour, 2016; Dobritsa et al., 2016). 16S rRNA analysis of the *Burkholderia*-related genera from oil palm and acacias resulted in the identification of 16 distinct strains of *Paraburkholderia*, three strains of *Caballeronia* and one strain belonging to the novel genus, *Chitinasiproducens*. The isolates were closely related to *Caballeronia udeis*, *B. anthina*, *B. vietnamiensis*, *B. ubonensis*, *P. tropica*, *P. sediminicola*, *P. unamae* and *P. phytofirmans* (Table 1, Figure 1). Most of the strains from *Paraburkholderia* could be assigned to *P. tropica*. The endophytic nature of some of these *Paraburkholderia* species has already been demonstrated (Caballero-Mellado et al., 2004; Reis et al., 2004; Suárez-Moreno et al., 2012).

## Nitrogenase Activity

The 12 strains that belong to *P. tropica* showed relatively high nitrogenase activity, ranging from 18.8 to 348.6 nmol  $\text{C}_2\text{H}_4$  (mg

protein)<sup>−1</sup> h<sup>−1</sup>, while the activity was completely absent in four strains even after 96 h of incubation (Table 1). Of the ARA-positive strains, 11 of the 12 strains (91.7%) were derived from oil palm and eight were identified as *P. tropica*. The remaining strains were identified as to *B. anthina*, *B. vietnamiensis* and *P. unamae*. None of four acacias isolates showed ARA activity. The *nifH* genes from the *Burkholderia* strains that had ARA formed a tight cluster with those from other plant-associated *Burkholderia* species (Supplementary Figure 2). Comparisons of the *nifH* sequences generated in this study with those available in GenBank revealed that they shared 96–99% sequence identity with *P. tropica* Ppe8<sup>T</sup> (Reis et al., 2004). The *nifH* gene was not present in the genome of strain JS23<sup>T</sup> as determined by PCR and this has been confirmed by genome sequencing (Madhaiyan et al., 2020).

Nitrogenases are usually highly sensitive to oxygen and ammonium ( $\text{NH}_4^+$ ). Both molybdenum-dependent and



molybdenum-independent nitrogenases are stringently regulated at the transcriptional and post-translational levels (Dixon and Kahn, 2004). Acetylene reduction assay in a medium supplemented with 1 mM NH<sub>4</sub><sup>+</sup> or 10% oxygen showed that, with the exception of five strains (S39-2, S43-2, S38-1, S44-1, and S53-1), none had significant nitrogenase activity when NH<sub>4</sub><sup>+</sup> reached over 1 mM (Figure 2). Surprisingly, nitrogenase activity significantly increased with the supplementation of 10% oxygen in all the strains with the exception of strains S40-2 and S41-2 (Figure 2).

## Indole-Related Compounds and ACC Deaminase Production

Apart from N-fixation, the production of phytohormones, such as indole compounds and ethylene catabolizing ACC deaminase, contribute to the PGP function. Previous studies have demonstrated auxin production in *P. kururiensis*, *P. phytofirmans*, and *P. unamae* (Caballero-Mellado et al., 2007; Sun et al., 2009). We found that all the isolates produced indole compounds from the precursor L-tryptophan (Table 1). The activity was higher in the extracts of *P. unamae* S51-2, *P. tropica* S39-2, and *P. tropica* S23-8, producing 11.1, 12.4, and 10.3 nmol  $\alpha$ -ketobutyrate mg<sup>-1</sup> h<sup>-1</sup> proteins, respectively. In addition, most strains showed ACC deaminase activity as evidenced by their ability to grow on media containing 3 mM ACC with the exception of six strains (Table 1). Sequence analysis of PCR products using the specific primer pair F1936/F1938

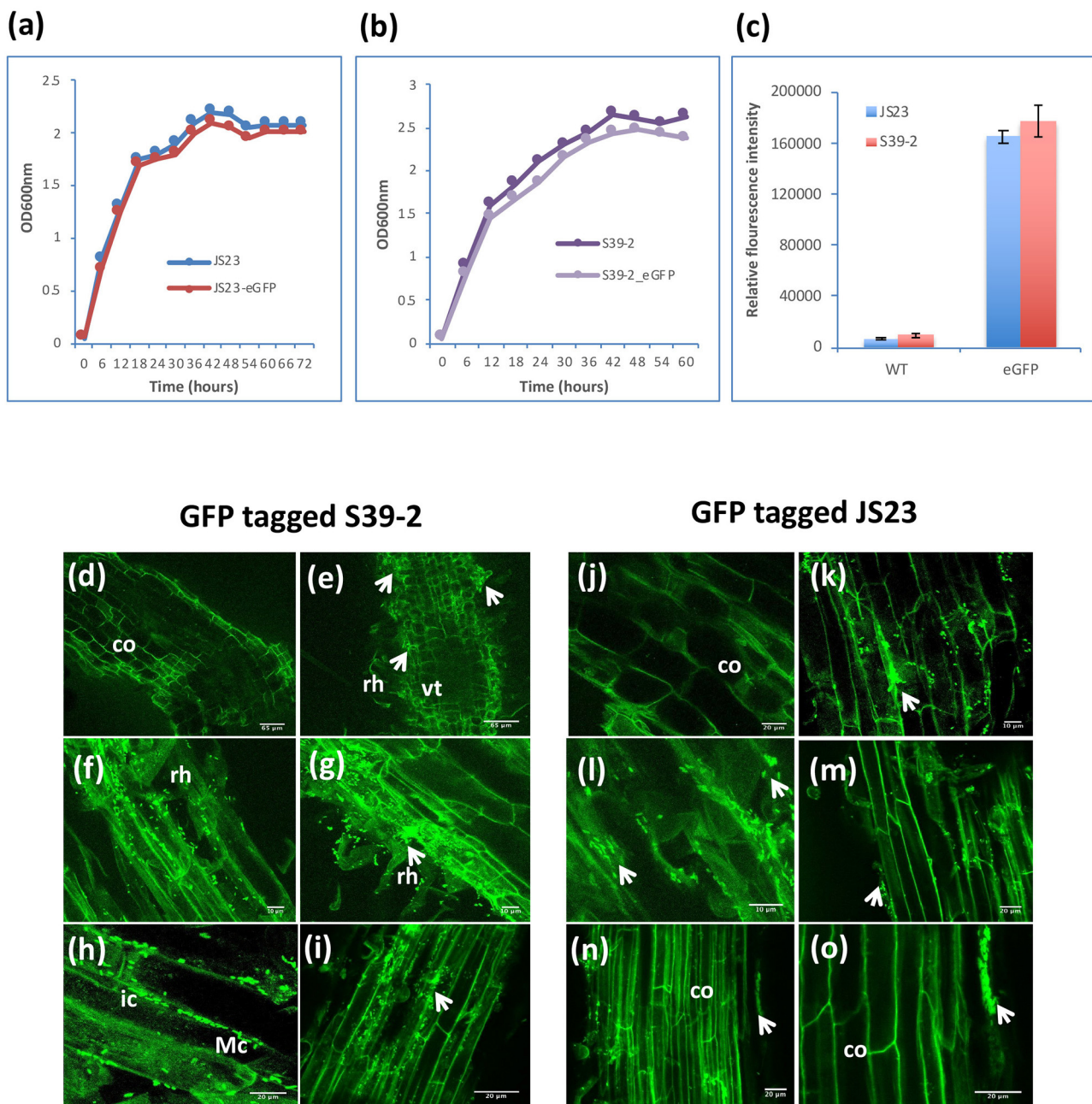
(Blaha et al., 2006) confirmed the presence of *acdS* genes in *P. unamae* S51-2, *P. aromaticivorans* Aca-213, and *P. tropica* S39-2.

## Phosphorus Solubilization and Siderophore Production

Phosphorus solubilization is an important PGP trait and it is very common among *Paraburkholderia* species (Ghosh et al., 2016; Kaur et al., 2016). In the presence of inorganic phosphate in NBRIP medium (Nautiyal, 1999), all N-fixing strains produced a clear zone on the plates, indicating solubilization of inorganic phosphate (Table 1 and Supplementary Figure 3A). These strains were taxonomically placed in *B. anthina*, *B. vietnamiensis*, *P. unamae*, and *P. tropica*. Two of the N-fixing strains also produced siderophores, which are responsible for chelating Fe<sup>3+</sup> ion (Table 1 and Supplementary Figure 3B).

## Endoglucanase and Anti-microbial Activity

Subsequent to their colonization of the root surface, endophytic bacteria usually enter the inner tissues. Such endophytic bacteria secrete cell wall-degrading endo-1,4-glucanase which is believed to be important for plant colonization (Compant et al., 2005; Reinhold-Hurek et al., 2006; Fan et al., 2016). In this study, four strains showed clear endoglucanase activity when cultured in KW medium supplemented with CMC and glucose (Table 1). Three strains, *C. palmarum* JS23<sup>T</sup>, *P. tropica* S44-1, and *P. unamae* S51-2, weakly inhibited the growth of the red yeast, *Rhodospiridium toruloides*. None of the isolates was able to inhibit the growth of endophytic bacterium *K. sacchari* R4-368 (Table 1).



**FIGURE 3 |** Colonization of GFP-tagged S39-2 and JS23<sup>T</sup>. **(a, b)** Cell density of wild-type (WT) and GFP-tagged JS23<sup>T</sup> and S39-2 in liquid culture (2xYT medium). **(c)** Relative fluorescence intensity of WT and GFP-tagged cells in culture medium was quantified in Tecan plate reader (Tecan i-Control, infinite 200; 476 nm excitation wavelength and 506 nm emission wavelength) using 96 flat bottom white polystyrol plate (Corning). Error bars are standard deviations ( $n = 3$ ). **(d, j)** uninoculated roots. **(e-i)** Root tissues inoculated with S39-2-gfp on 15 DAI **(d-f)** and 45 DAI **(h, i)**. **(k-o)** JS23<sup>T</sup>-gfp inoculated root tissues on 15 DAI **(k, l)** and 45 DAI **(m-o)**. DAI, days after inoculation; rh, root hairs; co, cortical cells; arrowhead indicate micro-colonies (Mc); vt, vascular tissue; ic, intercellular colonization. Scale bars = 10 μm **(f, g, k, and l)**, 20 μm **(h, i, j, m, n, and o)**, and 65 μm **(d, e)**.

## Colonization of Lemon Eucalyptus Seedlings

Considering their PGP characteristics, strains S39-2 and JS23<sup>T</sup> originally isolated from oil palm were selected to gain further insights on their interactions with their host. Strain S39-2

had high nitrogenase activity and endoglucanase activity, while strain JS23<sup>T</sup> lacked both traits. Both strains produced ACC deaminase and IAA (**Table 1**). To facilitate the tracking of plant colonization by the bacterial strains, they were labeled with GFP by integrating a *T5::egfp* gene cassette into the chromosome



**TABLE 2 |** Colonization of eucalyptus tissues by *gfp*-tagged strains.

Treatments	Epiphytic (rhizoplane) population ( $\times 10^9$ cfu g <sup>-1</sup> )	Endophytic population ( $\times 10^7$ cfu g <sup>-1</sup> )	
		Root	Leaf + stem
<i>P. tropica</i> S39-2- <i>gfp</i>	14.3 $\pm$ 2.5 a	3.7 $\pm$ 2.2	0.67 $\pm$ 0.2
<i>C. palmae</i> JS23- <i>gfp</i>	3.3 $\pm$ 0.6 b	0	0
Uninoculated control	0	0	0
LSD ( $p \leq 0.05$ )	2.08	–	–

Data represent mean  $\pm$  SD ( $n = 3$ ). The data are statistically analyzed using DMRT. Within each vertical column, values followed by the same letter are not statistically different according to Fisher's protected LSD at  $p \leq 0.05$  levels.

Bacteria populations were determined at 30 DAI by selection on Kanamycin-containing solid medium (25  $\mu$ g ml<sup>-1</sup>). Results are derived from 3 replicates.

via Tn5 transposition. The selected GFP-tagged strains were confirmed to have indistinguishable cell morphology and growth rate from the respective parent (**Figures 3a,b**) and showed high fluorescence (**Figure 3c**).

Inoculation of GFP-tagged strains on eucalyptus seedlings under sterile conditions showed that both *C. palmae* JS23-*gfp* and *P. tropica* S39-2-*gfp* were able to efficiently colonize the rhizosphere of the seedlings. Micro-colonies of JS23-*gfp* and S39-2-*gfp* could be found on the surface of roots or root hairs (**Figures 3d–g, j–m**). The rhizoplane colonization pattern of strains JS23-*gfp* and S39-2-*gfp* was similar to previous reports where rhizosphere bacteria colonize the nutrient rich zones of root hairs, emerging lateral roots or root tips that support bacterial proliferation (Walker et al., 2003; Bais et al., 2006). However, persistent intercellular colonization was observed only with S39-2-*gfp* (**Figures 3h,i**), but not with JS23-*gfp* (**Figures 3n,o**). It was observed that JS23-*gfp* strain had a weaker GFP signal on the root surface and the signal in internal tissue was only obvious at 15 DAI. Surprisingly, GFP signal in the internal tissues disappeared at 45 DAI in JS23-*gfp* inoculated plants. To further confirm the GFP imaging data, the titers of JS23-*gfp* and S39-2-*gfp* in different parts of the plants were determined. Strain JS23<sup>T</sup> had significantly lower cell density on the rhizoplane, and the endophytic population was not observed at 30 DAI (**Table 2**). This is in stark contrast to strain S39-2, which had  $3.7 \pm 2.2$  and  $0.67 \pm 0.2 \times 10^7$  cfu g<sup>-1</sup> tissues in roots and shoots (stem + leaf), respectively, at 30 DAI.

## Plant Growth Promoting Effect on Tree Species

Strains S39-2 and JS23<sup>T</sup> were inoculated on eucalyptus seedlings. After 60 days, the inoculated plants displayed a ~30.4% increase in plant height over the control plants. The number of leaves and branches, leaf area, and chlorophyll contents were also significantly higher in the inoculated plants (**Figures 4A–E**). Compared to the mock-inoculated plants, strain S39-2-inoculated plants showed a significantly higher biomass yield, with a 60.4% increase in root biomass and a 44.2% increase in shoot biomass (**Figure 4F**). Strain S39-2 displayed a clear *in planta* nitrogenase activity, which was associated with a 53.5% increase in leaf nitrogen content over control plants. In contrast, clear *in planta* AR-activity was not observed in control plants. Consistent with this finding, the leaf nitrogen

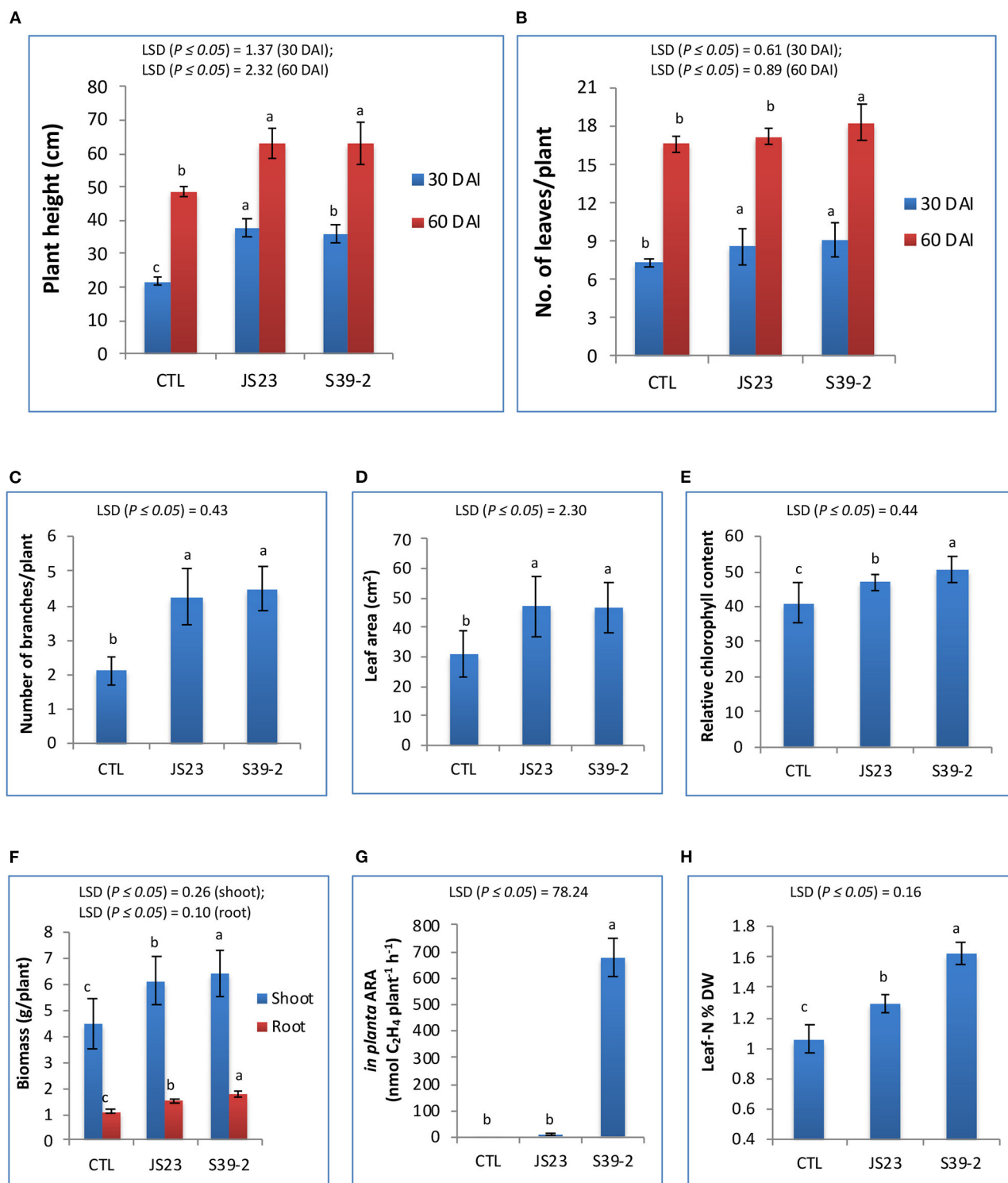
content was not altered (**Figures 4G,H**). The trace level of AR-activity detected in JS23<sup>T</sup> inoculated plants was not significantly different from the control and is likely to have resulted from aerial cross-contamination during the cultivation. Surprisingly, this non-diazotroph with relatively low auxin production displayed significant PGP activity in eucalyptus.

Similarly, both strains were able to promote the growth of oil palm seedlings (**Table 3**). The growth promotion results were statistically significant at 30, 60, and 90 DAI. Strain S39-2 treated seedlings had a higher bacterial population in roots ( $21.8 \pm 2.7 \times 10^5$  cfu g<sup>-1</sup> tissues) than in leaflets ( $6.3 \pm 0.54 \times 10^5$  cfu g<sup>-1</sup> tissues) while no bacteria were recovered at 30 DAI from strain JS23<sup>T</sup> treated seedlings (**Table 3**). Furthermore, we tested the PGP activity of strains JS23<sup>T</sup> and S39-2 on *Jatropha curcas*, a tree plant that holds promise for biofuel production. Strain JS23<sup>T</sup> showed very little PGP activity while S39-2 showed strong PGP activity (**Table 4**). Strain S39-2 was able to colonize the root, stem, and leaf tissues of *Jatropha*, yielding population densities of  $63.8 \pm 4.8 \times 10^6$ ,  $9.8 \pm 1.8 \times 10^6$ , and  $0.57 \pm 0.02 \times 10^6$  cfu g<sup>-1</sup> tissues, respectively, at 60 DAI, while JS23<sup>T</sup> cells were not recovered from the surface-sterilized root, stem, or leaf tissues (**Table 4**).

## DISCUSSION

### Endophytic Bacteria From Acacias and Oil Palm Phyllosphere and Their PGP Characteristics

Plant productivity is closely related to soil nutrient and climatic conditions. Since fertilizer application is not normally practiced in forestry, improvements in genotypes and the rhizospheric microbiome are critical for boosting tree productivity. The phyllosphere harbors diverse bacteria and the dominant bacterial taxa usually include diazotrophs and methylophiles (methanol- and other one-carbon compound-consuming). This study focused on the diversity of *Burkholderia*-related genera within leaf tissues of acacias and oil palm. It is evident that members of the genus *Paraburkholderia* were dominant, followed by *Caballeronia*. Notably, *Chitinasioproducens palmae* is a new addition to the existing list of *Burkholderia*-related and tree plants associated bacteria (Madhaiyan et al., 2020). Members of N<sub>2</sub>-fixing *Paraburkholderia* species have often been isolated as



**FIGURE 4 |** PGP effects of non-diazotrophic and diazotrophic isolates on eucalyptus seedlings. The effect on plant height (A), number of leaves (B), number of branches (C), leaf area (D), relative chlorophyll content (E), total biomass of leaves, stems and roots (F), *in planta* ARA activity (G) and leaf N content (H) was measured at 30 and 60 DAI with strain JS23<sup>T</sup> in lemon eucalyptus grown in small pot containing 750 g of red soil. Mean values  $\pm$  SD are presented ( $n = 18$ ). The data were statistically analyzed using DMRT. Different letters indicate significant differences of the treatments according to least significant difference (LSD) at  $p \leq 0.05$  levels. DAI, days after inoculation; CTL, uninoculated control.

**TABLE 3** | Effect of bacterial inoculation on the growth of oil palm seedlings.

Treatments	Plant height (cm)			Number of leaflets plant <sup>-1</sup>			Endophytic Population ( $\times 10^5$ cfu g <sup>-1</sup> )	
	DAI			DAI			Root	Leaflet
	30	60	90	30	60	90		
<i>P. tropica</i> S39-2	39.3 $\pm$ 1.7 a	52.2 $\pm$ 3.4 a	60.0 $\pm$ 2.5 a	7.9 $\pm$ 0.6 a	9.1 $\pm$ 0.8 a	10.3 $\pm$ 0.9 a	21.8 $\pm$ 2.7	6.3 $\pm$ 0.54
<i>C. palmae</i> JS23 <sup>T</sup>	36.7 $\pm$ 3.1 b	51.3 $\pm$ 2.2 a	58.6 $\pm$ 2.6 a	7.6 $\pm$ 0.5 ba	8.0 $\pm$ 0.5 b	10.2 $\pm$ 0.8 a	0	0
Uninoculated control	29.6 $\pm$ 1.6 c	39.4 $\pm$ 3.2 b	43.2 $\pm$ 3.2 b	7.0 $\pm$ 0.5 b	7.3 $\pm$ 0.9 c	9.6 $\pm$ 0.9 b	0	0
LSD ( $p \leq 0.05$ )	2.03	2.69	2.88	0.64	0.65	0.62	–	–

Data represent mean  $\pm$  SD ( $n = 9$ ). The data are statistically analyzed using DMRT. Within each vertical column, values followed by the same letter are not statistically different according to Fisher's protected LSD at  $p \leq 0.05$  levels. Endophytic bacterial populations were determined at 30 DAI by selection on R2A agar medium. DAI, days after inoculation.

**TABLE 4** | Endophytic population of *Burkholderia* related strains in various tissues of *Jatropha* at 60 DAI under sterile N-limiting conditions.

Treatments	Fresh weight (g plant <sup>-1</sup> )	Dry weight (mg plant <sup>-1</sup> )	Endophytic population ( $\times 10^6$ cfu g <sup>-1</sup> )		
			Root	Stem	Leaf
<i>P. tropica</i> S39-2	1.7 $\pm$ 0.3 a	306.7 $\pm$ 10.7 a	63.8 $\pm$ 4.8	9.8 $\pm$ 1.8	0.57 $\pm$ 0.02
<i>C. palmae</i> JS23 <sup>T</sup>	1.5 $\pm$ 0.1 b	226.7 $\pm$ 18.3 b	0	0	0
Uninoculated control	1.3 $\pm$ 0.1 c	226.7 $\pm$ 19.0 b	0	0	0
LSD ( $p \leq 0.05$ )	0.131	10.39	–	–	–

Data represent mean  $\pm$  SD ( $n = 12$ ). The data are statistically analyzed using DMRT. Within each vertical column, values followed by the same letter are not statistically different according to Fisher's LSD at  $p \leq 0.05$  levels. Endophytic bacterial populations were determined at 60 DAI by selection on R2A agar medium.

rhizospheric and/or in root endophytic bacteria from various crops (Viallard et al., 1998; Compant et al., 2005; Theocharis et al., 2012). Our studies added a new insight into the diversity of *Burkholderia*-related bacteria in plant leaves, and our data suggested that they play an important role in tree crop health and growth.

Consistent with many reports on endophytes, the colonies formed were yellowish, round, smooth, and convex, 1–2 mm in diameter (Estrada-De Los Santos et al., 2001). The observed congruence between phylogenetic trees of 16S rRNA and *nifH* genes suggest that the common ancestor of *Paraburkholderia* was a diazotroph, and this function has been inherited in most species with a few exceptions (Suárez-Moreno et al., 2012). The ability to fix nitrogen is a major feature of plant-growth promoting *Paraburkholderia* species (Martínez-Aguilar et al., 2008). Although *Paraburkholderia* species were isolated from enriched N-free media, some strains were negative for the AR assay since the ability to grow on N-free medium or the presence of the *nifH* gene does not necessarily warrant nitrogenase activity (Madhaiyan et al., 2015). It is likely that these bacteria are not true diazotrophs despite their ability to grow in a culture medium without added nitrogen (Castanheira et al., 2016). The strains might have lost the ability during the purification procedure since it has been speculated that dinitrogen fixers grow best in the presence of other heterotrophic bacteria which support nitrogen fixers by physical or biochemical activities (Wiegel and Schlegel, 1976). *Paraburkholderia* isolates from oil palm belonging to *P. tropica* had higher ARA (Table 1), but none of the isolates from acacias reduced acetylene. It is surprising that the N-fixing activity was not significantly inhibited with 10% oxygen supplementation in many of the leaf isolates, suggesting the

possibility of oxygen resistance mechanism in the leaf endophytes (Figure 3).

Phytohormones regulate many aspects of plant growth, development, and responses to stress. Previous studies have reported IAA production in different *Paraburkholderia* species, such as *P. kururiensis*, *P. phytofirmans*, and *P. unamae* (Caballero-Mellado et al., 2007; Sun et al., 2009). Our data with *P. tropica* are consistent with these reports as the majority of strains strongly produce auxins. ACC deaminase activity was found in all three genera of strains reported here although some strains appeared to have lost the gene (Table 1), suggesting their common role in inhibiting ethylene-mediated leaf senescence. This is consistent with previous reports on *Burkholderia* and *Paraburkholderia* that were of non-leaf origin (Suárez-Moreno et al., 2012). The member of the genus *Paraburkholderia* usually has a strong capacity to solubilize inorganic phosphates. On the other hand, this is rarely found in *Caballeronia* and *Chitiniasiproducens* (Table 1). Siderophore production was also rarely encountered in the leaf isolates, suggesting that it play a minor role in the phyllosphere.

## Plant Colonization and Growth Promotion by Strains S39-2 and JS23

The strains selected for plant inoculation studies, S39-2 and JS23<sup>T</sup>, significantly promoted the growth of seedlings of oil palm and eucalyptus despite the fact that JS23<sup>T</sup> had only weak auxin production, moderate ACC deaminase activity, and total lack of nitrogenase activity (Table 1). Further, JS23<sup>T</sup> failed to persistently colonize the internal tissues of lemon

eucalyptus, palm, and *Jatropha curcas*. This suggests that JS23<sup>T</sup> promoted plant growth mainly with the ACC deaminase activity in the rhizosphere. It remains possible that these bacteria promoted plant growth via other mechanisms in the oil palm rhizosphere, where its inoculation led to a significant increase in leaf number and plant height throughout the 90-day observation period (Table 3). Unlike S39-2, JS23<sup>T</sup> displayed only weak PGP activity in *Jatropha curcas*. This suggests that there was a close interaction between the plants and the bacteria with regard to its colonization and growth promotion.

Although the mechanisms for these plant-bacteria interactions await further investigations, our GFP-tagged strains provided some interesting hints. JS23-gfp and S39-2-gfp differed significantly in their pattern of colonization in lemon eucalyptus (Figure 3). The most obvious difference was the long-term colonization pattern: while S39-2-gfp was able to sustain long-term colonization as an endophyte, JS23-gfp appeared to trigger some defense response that led to its gradual removal from the host tissues. Coincidentally, this strain was also able to inhibit the growth of red yeast. In addition, JS23-gfp appeared to trigger a similar defense response in oil palm and *Jatropha curcas* as no bacteria was observed in the leaves of both plants.

## CONCLUSION

*Paraburkholderia tropica* S39-2 isolated as a leaf endophyte from oil palm possesses multiple plant growth promotion traits and is able to colonize and promote the growth of oil palm and *Jatropha*. It may be developed as an efficient bioinoculant for tree species to reduce fertilizer usage; improve crop productivity, and reduce greenhouse gas emission. The strains S39-2 and JS23<sup>T</sup> can also serve as valuable tools for further studies of bacteria–host interactions of the *Burkholderia* related taxa, particularly with regard to understanding endophyte–plant interactions and their utilization for enhancing the sustainability of trees and conventional crop production.

## DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: <https://www.ncbi.nlm.nih.gov/>, given in the article.

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## AUTHOR CONTRIBUTIONS

MM and TA performed all the experiments, isolation, characterization, and bioassays for bacteria and plants performed data analysis and prepared the manuscript. GS edited the manuscript. LC participated in plant inoculation experiments and prepared plant materials. LJ designed the research plans and supervised the whole study and revised the manuscript. All authors read and approved the final manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.618305/full#supplementary-material>

**Supplementary Figure 1** | pUT-tac-aph-sGFP plasmid (8776 bp). aph: kanamycin resistance gene; bla: ampicillin resistance gene; sfGFP: super-folder GFP.

**Supplementary Figure 2** | Phylogenetic tree based on *nifH* gene sequences showing the position of isolates from tree crops. GenBank accession numbers are given in parentheses. Bootstrap values (expressed as percentages of 1000 replications) greater than 50% are shown at the branch points. Bar, 0.05 substitutions per nucleotide position. The gold box represents *nifH* positive strains, eight strains closely related to *P. tropica*, one strain each from *P. unamae* and *B. vietnamiensis*. The blue box represents symbiotic *Paraburkholderia* strains.

**Supplementary Figure 3** | (A) Phosphate solubilizing endophytic leaf isolates on NBRIIP agar medium at 7 days. (B) CAS agar plate: siderophore production comparison among the endophytic leaf isolates on a CAS siderophore testing agar. The chelator–iron (III) complex tints the agar with a rich blue background. The orange halo surrounding the colony indicates the excretion of siderophore, and its dimension approximates the amount of siderophore excreted. Strains: *P. tropica* strains S43-2, S42-2, S45-1, S44-1, S23-8, S22-7, S23-9, S23-4, S39-2, S38-1, S40-2, and S41-2, *C. palmarum* strain JS23<sup>T</sup>, *P. unamae* strain S51-2, *P. sediminicola* strain Aca-21, *P. aromaticivorans* strain Aca-213, *B. anthina* strains S25-8 and S21-2, *B. vietnamiensis* strain S53-1, and *C. mineralivorans* strains Aca-28, Aca-214 and Aca-215.



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# Microbial Spectra, Physiological Response and Bioremediation Potential of *Phragmites australis* for Agricultural Production

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Common reed (*Phragmites australis*) can invade and dominate in its natural habitat which is mainly wetlands. It can tolerate harsh environments as well as remediate polluted and environmental degraded sites such as mine dumps and other polluted wastelands. For this reason, this can be a very critical reed to reclaim wastelands for agricultural use to ensure sustainability. The present review manuscript examined the microbial spectra of *P. australis* as recorded in various recent studies, its physiological response when growing under stress as well as complementation between rhizosphere microbes and physiological responses which result in plant growth promotion in the process of phytoremediation. Microbes associated with *P. australis* include *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, *Fusobacteria*, *Actinobacteria*, and *Planctomycetes* families of bacteria among others. Some of these microbes and arbuscular mycorrhizal fungi have facilitated plant growth and phytoremediation by *P. australis*. This is worthwhile considering that there are vast areas of polluted and wasted land which require reclamation for agricultural use. Common reed with its associated rhizosphere microbes can be utilized in these land reclamation efforts. This present study suggests further work to identify microbes which when administered to *P. australis* can stimulate its growth in polluted environments and help in land reclamation efforts for agricultural use.

**Keywords:** bioremediation, microbial spectra, physiological response, *Phragmites australis*, agricultural production

## INTRODUCTION

Common reed (*Phragmites australis*) is an invasive helophytic grass which has great impact on the ecosystem. It is tall, slender with a bare stem and plume-like inflorescence. *Phragmites australis* is found in brackish and freshwater wetlands, temperate and tropical regions of the world (Den et al., 1989; Brix, 1999; Meyerson et al., 2000). The ability of this reed to proliferate and survive in diverse environmental conditions and invade the environment could be traced to its high productivity (Kettenring et al., 2012; Douhovnikoff and Hazelton, 2014; Eller et al., 2014; Saltonstall et al., 2014). The common reed can competitively displace indigenous vegetation; hence, it is referred to as an invasive plant. In places where it has become a nuisance, control measures are devised to limit its spread and its encroachment in the natural arena. Methods to curb its spread include cutting and burning, flooding, the use of natural enemies, and application of herbicides. Some of the methods are effective but some show very little success (Reimer, 1976; Thompson and Shay, 1985; Monterio et al., 1999; Ailstock et al., 2001; Gusewell, 2003; Relyea, 2005; Avers et al., 2007).

Despite the need to control its spread *P. australis* has been found useful as a bioremediator of polluted environments due to its ability to survive under stress (Windham et al., 2003; Weis and Weis, 2004; Duman et al., 2007; Bragato et al., 2009; Cerne et al., 2011). The ability of *P. australis* to remediate the environment results from its various genetic and physiological characteristics and these include having the ability to create a rhizosphere environment which encourages the habitation and proliferation of certain rhizosphere microbes. The common reed exudes enzymes and other cell contents which make its rhizosphere conducive for habitation by a myriad of mutually beneficial microbes. Nejla et al. (2014) observed a significant positive correlation between soil dehydrogenase activity (DHA) and pentachlorophenol (PCP) removal in polluted soils with the aid of microorganisms present in the reed's rhizosphere. This implied that microorganisms in the rhizosphere of *P. australis* and the enzyme activities promoted the biodegradation of PCP and the reclamation of the land. Work done by Cheema et al. (2009) and Yang et al. (2011) supported the findings of Nejla et al. (2014), whereby they observed both microbial and enzymatic activities as influenced by the root exudates promoted the remediation of the environment and freeing it of contaminants.

A plethora of the studies confirmed the findings that the rhizosphere of *P. australis* harbors diverse microorganisms that act as growth-promoting microbes which aid the growth of the reed and promote the remediation of the environment (Kadlec and Wallace, 2008; Jiang et al., 2013; Li et al., 2013; Zou et al., 2013; Bouali et al., 2014). This present review collated information on the microbial spectra associated with the rhizosphere of *P. australis* and the physiological response of the reed to environmental stress as well as the reed-microbes interaction that promotes the growth of the reed and enhance its bioremediation potential in the reclamation of land for agricultural use.

## DISTRIBUTION, IMPACT, AND USES OF *PHRAGMITES AUSTRALIS*

The common reed originates from Europe. However, traces of a North American origin exist (Saltonstall, 2002). Research involving molecular markers showed that the species that were not a native pedigree of the reed were introduced in North America, and these species are behind the sporadic increase of the reed in North America (Chambers et al., 1999; Saltonstall, 2002). Catling and Mitrow (2012) pointed out that the abundance of the reed in North America is an attribute of the sporadic, but similar-looking European subspecies of the reed. Pollution, eutrophication, and shoreline development are notable factors contributing to the distribution and abundance of the reed in North America for the past 150 years (Marks et al., 1994; Chambers et al., 1999). Chambers et al. (1999) further reported that the distribution and abundance of the reed increased across the continent.

Very little is known about this reed on the African continent. However, some work has been done in southern Africa where *P. australis* is considered native based on pollen fossil records

which point to presence in the southern African region since the Late Quaternary period (Scott, 1982). Due to intense mining in South Africa and the need to rehabilitate mine dumps and acid mine wetlands, this reed has been very important as a primary remediator of many of these polluted sites. This led to the prevalence of the common reed in aquatic and semi-aquatic areas especially in riverbeds and wet places (Gibbs et al., 1990; Van Oudtshoorn, 1999; Leistner, 2000) as well as in various heavy metal polluted areas adjacent to the mines. The common reed displaces indigenous vegetation through its competitive ability hence reducing the biodiversity of native plants (Catling and Mitrow, 2012). It forms thickets of vegetation because of high biomass formed by the reed leading to the blockage of light rays needed for the growth of the native fauna. Consequently, native plants less competitive than *P. australis* receive less sunlight, photosynthesize poorly, and are eventually crowded out and displaced.

Physiologically, the reed produces gallic acid which is broken down in the presence of ultraviolet light rays to form mesoxalic acid, a toxic chemical that hinders the growth of susceptible plants and seedlings native in the area (Thimmaraju et al., 2009). Controlling the growth of this common reed is a global concern and burning of the reed and its use as a forage for goats (Jolly, 2017) are believed to be the most effective methods for its control. The reed has some important uses despite its negative impact to the environment and biodiversity. The reed is used in weapon production like spears used for game hunting. It also provides shelter for birds and other kinds of animals. Some of its parts like the rootstocks are ground into flour or made into a thin liquid food of oatmeal and can be roasted in a moist state and eaten (Peterson, 2010). The reed is also known for its bioremediation role in most polluted environments because of its ability to thrive well in harsh conditions and the microbe-plant interaction that is predominant with reeds and promote their growth as well as enhance their remediation potential.

## MICROBIAL CONSORTIA ASSOCIATED WITH *PHRAGMITES AUSTRALIS* IN ENVIRONMENTAL REMEDIATION

Plants are inhabited by microbes either as endophytes or as ectophytes or as pathogens. As colonizers of plants, microbes form either mutually beneficial relationships with plants playing crucial roles in recycling of nutrients and breaking down of pollutants (Srivastava et al., 2017; Lyu et al., 2020). Plant parts which are most likely inhabited by ectophytes are those which have high nutrient levels such as secretion organs. The rhizosphere is one of the environment-plant interfaces most colonized by ectophytic microbes and provides environment laden with oxygen and nutrients for microbes' proliferation (Stottmeister et al., 2003). Microorganisms dominating the rhizosphere of macrophytes have been recorded to play important biological functions which include nutrient acquisition (Pii et al., 2015) that enhances growth and fitness, disease suppression (Mendes et al., 2011), and stress tolerance of the macrophytes (de Zelicourt et al.,



2013). These important plant–microbe interactions have led to expanded research into these associations and how they impact the remediation potentials of the macrophytes.

Remediation of polluted environments by plants is partly dependent on the interaction between plants and their associated microbes. Plant growth-promoting rhizobacteria in synergy with the associated plant have been proven to play a major role to clean-up of pollutants from polluted soils. However, several endophytes, mycorrhizae and algae contribute immensely to environmental remediation. Root zone microbes have been beneficial in constructed wetlands to remove soil contaminants. The interplay of root zone microbes in association with plants has been revealed in a plethora of studies which include the study by Zhang et al. (2021) which showed that adjusting plant–bacteria interactions in the rhizosphere community of plants is an important aspect of phytoremediation. Similarly, Wang et al. (2020) found that the presence of denitrifying bacteria, in the root zone, guarantees high  $\text{NO}_3\text{-N}$  removal efficiency from saturated soils. These established principles form the basis of constructing efficient wetlands for the removal of contaminants from soil.

Constructed wetlands have been adopted for the past decades in the remediation of contaminated environments because the method is cost-effective and devoid of any environmental damage (Kadlec and Wallace, 2008; Li et al., 2013; Zou et al., 2013; Bouali et al., 2014). Chandra et al. (2012) pointed out that *P. australis* has been employed in wetlands remediation of contaminated environment in both tropical and temperate part of the world. Ravit et al. (2003) stated that the plants' high biomass, root depth, ability to thrive well and breakdown pollutants, and ability to adapt easily are the bases upon which the reed and other species of plants used in bioremediation are selected. However, the effectiveness of the constructed wetlands in the treatment of polluted environment is dependent on the microbial consortia present in the rhizosphere of plant species selected for the bioremediation (Tian et al., 2014). Some of these microbes enhance plants' development (Jiang et al., 2013) and possibly promote the bioremediation process.

Shaw et al. (2006) views the rhizosphere as an exceptional zone around the root that is known for complex biological activities involving many microorganisms. Raaijmakers et al. (2009) pointed out that among the various microbes present in the rhizosphere, bacterial populations play a vital role in most activities in the rhizosphere because of their high level of host specificity. The various communities of bacteria present in the rhizosphere assist plants in the acquisition of inorganic nutrients, promote nitrogen uptake as well as protecting plants against attack by pathogenic fungi (Cocking, 2003; Berg et al., 2005; Uroz et al., 2007). Because of the importance of bacteria in natural ecosystems, it becomes necessary to unravel the bacterial diversity and possibly the bacteria–plant interactions in the habitats and how they aid in polluted land reclamation for crop production.

Microbial consortia of many wetland plants' roots have been investigated via culture-dependent and molecular methods (Jiang et al., 2013; Li et al., 2013; Abed et al., 2018). The era of high-throughput genomic technologies accelerated the discovery of root zones microbes as well as their biological activities. The

uncovering of the microbial consortia and microbial metabolic activity was through the new fields of metagenomics and metatranscriptomics. Notably studies include Kumar et al. (2018) which uncovered the rhizobacteria population composition of barley and alfalfa in oil-contaminated soils. Another noteworthy study is that of Brereton et al. (2020) which cataloged the rhizosphere microbiome of *Festuca arundinacea*, *Salix miyabeana* and *Medicago sativa* in contaminated soil. In addition, Kalu et al. (2021) investigated the fungal and metabolome diversity of rhizosphere and endosphere of *P. australis* in an acid mine-polluted environment. Furthermore, Mang and Ntushelo (2021) investigated the influence of acid mine water on the diversity and metabolite shift of microbial populations of the common reed. Obieze et al. (2020) investigated the functional attributes and response of bacterial communities to nature-based fertilization during hydrocarbon remediation. Bledsoe et al. (2020) observed increased bacterial diversity in bulk soils and plant rhizospheres in a long-term nutrient enriched oligotroph-dominated wetland. Hu et al. (2021) investigated the composition and co-occurrence patterns of *P. australis* rhizosphere bacterial community and observed the characterization of the rhizosphere by *Arthrobacter*, *Pseudomonas*, *Trichococcus*, and *Ramlibacter* that also played a crucial role in the regulation of plant fitness and nutrient cycling. Lyu et al. (2020) observed that bacterial phyla enriched in the rhizosphere of *P. australis* were found to be putative keystone taxa and might be involved in the regulation of bacterial interactions and plant growth. The investigation of the sediment microbiomes associated with the rhizosphere of emergent macrophytes in a shallow, subtropical lake by Huang et al. (2020) suggested that rhizosphere microbiome communities are influenced by the presence of macrophyte roots, with oxygenated rhizosphere and surface sediment communities being more diverse, and organized into more interconnected co-occurrence networks.

Metatranscriptomic studies which have accelerated our understanding of rhizosphere microbes in relation to phytoremediation include those by Yergeau et al. (2018) and Gonzalez et al. (2018) which both revealed the metatranscriptomics of the root zone in plants growing in contaminated soils with huge implications for phytoremediation. The exudates from the roots of plants are known to promote the growth and actions of rhizosphere associated microbes (Jiang et al., 2013; Zou et al., 2013). Most of the constructed wetlands are based on the principle of microbes–host plant interaction enhanced through the exudates from the plant. Abed et al. (2014) pointed out that there are very little wetlands built for bioremediation of oil polluted water. Few studies done fronted well-constructed wetlands as an effective bioremediation method for hydrocarbons contaminated water (Zou et al., 2013; Tian et al., 2014). One of the largest surface flows constructed wetland system in Oman, Arabian Gulf region, for oil-produced water remediation is predominated by *P. australis* (Abed et al., 2014). However, the knowledge of the microbial consortia of the reed's rhizosphere in oil-polluted wetlands is still minimal. Abed et al. (2018) reported bacterial communities in the rhizosphere of the reed from an oil-polluted wetland using molecular (Illumina MiSeq sequencing) and culture-based methods, and showed that

the dominant phyla belonged to *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*.

Integration of constructed wetland into the landscape could provide an efficient remediation of organic pollutants (Lorah and Voytek, 2004). *Phragmites* sp. and *Typha angustifolia* known as wetland plants have been shown in various studies to possess the potential of remediating chlorinated pollutants (Ma and Burken, 2002; Miglioranza et al., 2004; Zhang et al., 2005; Gomez-Hermosillo et al., 2006; Monferran et al., 2007; Ma and Havelka, 2009; Faure et al., 2012; San et al., 2013). Furthermore, some studies done have shown that most mineralization of recalcitrant organic contaminants occur at the rhizosphere (Kuiper et al., 2004; Krutz et al., 2005; Kidd et al., 2008; Gerhardt et al., 2009). A study of San et al. (2014) used pyrosequencing approaches to show that the rhizosphere of the reed in organochlorine contaminated soil were dominated by the phyla *Proteobacteria*. Furthermore, they identified *Sphingomonas* sp., *Pseudomonas* sp., *Devosia* sp. and *Sphingobium* sp. to be persistent in the organochlorine's environment indicating them as potential bioremediation microorganisms. Ding et al. (2021b) identified the following genera *Rhodobacter*, *Catellibacterium*, *Hydrogenophaga*, *Geothrix* and *Aeromonas* as colonizers of the rhizosphere of *P. australis* and these facilitate the removal  $\text{NH}_4^+$ -N and chemical oxygen demand from the constructed wetland.

The significance of microbes-plant interaction has prompted many studies to be focussed on the interactions between microbes and *P. australis*. In wetlands colonized by the reed, the endophytic bacteria clustered into phyla *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Fusobacteria* and small portion of unidentified bacteria have the potential to promote phytoremediation (Li et al., 2010). However, Borsodi et al. (2007) observed less diverse periphyton bacterial communities that were clustered into phyla *Proteobacteria*, *Firmicutes* and *Actinobacteria* in the reed using culture-dependent methods. Vladar et al. (2008) identified *Desulfovibrio*, *Desulfotomaculum*, and *Desulfobulbus* as the reed rhizosphere's sulfate-reducing bacteria. Work done by Zhang et al. (2013) on bacterial diversity of the rhizosphere of three ecotypes of the reed using the pyrosequencing approach showed the following phyla *Proteobacteria*, *Actinobacteria*, *Bacteroidetes*, *Chloroflexi*, *Gemmatimonadetes* and *Planctomycetes* to be the dominant bacterial cluster although differences in bacterial diversity existed in the different ecotypes.

A brief description of the roles of the bacterial communities colonizing the rhizosphere of *P. australis* provided an indication of their role in enhancing the growth of this reed, promoting their bioremediation ability necessary to reclaim polluted agricultural lands for expanded agricultural production. The genera *Methylophilales*, *Nitrosomonadales*, and *Desulfuromonadales* belonging to the phylum *Proteobacteria* have been reported to play a crucial role in nitrogen, sulfur, and global carbon recycling that enhances the growth of the reed and promote their phytoremediation ability (Ansola et al., 2014). *Bacteroidetes* has been reported to be actively involved in nitrogen fixation, a

major component of the nitrogen cycle needed to enrich the soil and promote crop production in various species of halophytes (Alishahi et al., 2020). *Cyanobacteria* promote degradation of organic pollutants and enhance the process of the carbon cycle (Savage et al., 2010; Wang et al., 2016). *Betaproteobacteria* has varieties of ammonia oxidizing bacteria that enhance the removal of excess nitrogen that could constitute a major challenge to the growth of crops (Wang et al., 2013). Other denitrifying bacteria associated with the rhizosphere of *P. australis* include *Catellibacterium* (Kong et al., 2019), *Hydrogenophaga* (Xing et al., 2018), *Aeromonas* (Sun et al., 2019), and *Geothrix* (Zhang et al., 2010). *Sediminibacterium* was reported to play vital role in the biodegradation of vinyl chloride (Wilson et al., 2016). *Acidovorax* enhances the removal of heavy metals (Zhang et al., 2019). *Geobacter* promotes the removal of amino acids and organic acids in systems under suitable conditions (Lu et al., 2015). *Bacillus* biodegrades various organic compounds necessary for dissolved organic carbon reduction (Guan et al., 2015). *Nitrosospora* ammonia oxidizing bacteria promote nitrogen cycling (Dong and Reddy, 2012). *Flavobacterium* promotes denitrification treatment nitrogenous contaminants (Pishgar et al., 2019). *Thauera* stimulate organic matter removal through enzyme secretion pathway that enhances chemical oxygen demand removal efficiency (Sanchez et al., 2018). The above alluded roles of these bacterial communities colonizing this rhizosphere of this reed contribute immensely to the growth of the reed while promoting the bioremediation potential as well as reclamation of contaminated agricultural land and enhancing agricultural production.

As previously mentioned in this review, the advancement in the sequencing technologies and computational analysis have unveiled knowledge on spectra of microorganisms which colonize the rhizosphere. Alegria et al. (2016) stated that the wetland plants microbiota could promote phytodepuration. Pietrangelo et al. (2018) showed composition and functional capability of bacteria microbiota of the rhizosphere of *P. australis* and *T. latifolia* using Illumina MiSeq sequencing techniques that the rhizosphere is dominated by *Actinobacteria*, *Firmicutes*, *Proteobacteria*, and *Planctomycetes*. However, the microbiota assemblage compositions and their potential contribution to phytodepuration needs further research. Table 1 provides a summary of the microbial spectra associated with *P. australis* in the remediation of polluted environments.

Aquatic macrophytes control their physiological activity to enhance their adaptation to changes in the environment. *P. australis* enhances its ability to survive under flooding conditions by increasing the rate of evapotranspiration to enhance its protection and uptake of nutrients (Zhao et al., 2012; Srivastava et al., 2014). Furthermore, these macrophytes, through plant residue decomposition, nutrients uptake, and root exudates, modify the physiochemical parameters of the soil to enable their proliferation and possibly the remediation of the environment (Luigimaria et al., 2014; Hallin et al., 2015; Packer et al., 2017). Hence, the next subsection of this review looked at the physiological response of *P. australis* to environmental stress.

**TABLE 1** | Summary of the microbial spectra associated with *P. australis* in the remediation of polluted environment.

Remediation sites	Source of microorganism	Methods of identification	Phylum/Family of organisms	References
Oil-polluted wetlands	Rhizosphere	Molecular (Illumina MiSeq sequencing) and culture-based	<i>Proteobacteria</i> , <i>Bacteroidetes</i> , and <i>Firmicutes</i>	Abed et al. (2018)
Organochlorines contaminated sites	Rhizosphere	Molecular (Pyrosequencing approach)	<i>Proteobacteria</i>	San et al. (2014)
Constructed wetland	Endophytes	Culture-independent method and	<i>Proteobacteria</i> , <i>Firmicutes</i> , <i>Bacteroidetes</i> , <i>Fusobacteria</i>	Li et al. (2010)
Lake	Periphyton Samples	Culture-based method	<i>Proteobacteria</i> , <i>Firmicutes</i> and <i>Actinobacteria</i>	Borsodi et al. (2007)
Lake	Rhizosphere	Culture-based and molecular method	<i>Proteobacteria</i> <i>Firmicutes</i>	Vladar et al. (2008)
Natural wetland	Rhizosphere	Molecular method (pyrosequencing)	<i>Proteobacteria</i> , <i>Actinobacteria</i> , <i>Bacteroidetes</i> , <i>Gemmatimonadetes</i> , <i>Planctomycetes</i> , <i>Acidobacteria</i> , <i>Aquificae</i> , <i>Caldiseica</i> , <i>Chlamydiae</i> , <i>Chlorobi</i> , <i>Chloroflexi</i> , <i>Cyanobacteria</i> , <i>Deferribacteres</i> , <i>Deinococcus-Thermus</i> , <i>Elusimicrobia</i> , <i>Fibrobacteres</i> , <i>Lentisphaerae</i> , <i>Planctomycetes</i> , <i>Spirochaetes</i> , <i>Tenericutes</i> , <i>Verrucomicrobia</i>	Zhang et al. (2013)
Natural wetland	Rhizosphere	Illumina MiSeq sequencing techniques	<i>Actinobacteria</i> , <i>Firmicutes</i> , <i>Proteobacteria</i> , and <i>Planctomycetes</i>	Pietrangelo et al. (2018)
Tailing dam of Mintails Mogale Gold Mine and Sibanye Gold Mine	Rhizosphere and endosphere	MiSeq high-throughput technology	<i>Ascomycota</i> and <i>Basidiomycota</i>	Kalu et al. (2021)
Constructed wetland	Rhizosphere	Quantitative polymerase chain reaction (qPCR)	<i>Rhodobacter</i> , <i>Catellibacterium</i> , <i>Hydrogenophaga</i> , <i>Geothrix</i> and <i>Aeromonas</i>	Ding et al. (2021b)

## PHYSIOLOGICAL RESPONSE OF *PHRAGMITES AUSTRALIS* IN STRESSED ENVIRONMENT

Response of plants to environmental stress is dynamic and it involves physiological, metabolic, and molecular responses which all constitute plant fitness. Some plants only survive stress but have their growth and reproduction retarded. However, some survive and still manage to grow and proliferate in the presence of stress. The focus of this section is the physiological response

of *P. australis* to stress in polluted environments. The authors demonstrate the unique physiological response which enables *P. australis* dominance in polluted environments.

Physiological response of plants and microorganisms in a stressed environment varies with the types and levels of stress. The physiological response involves the production of metabolites that promotes their survival or remediation potential. In view of the ability of *P. australis* to thrive well in stressed environments, a myriad of studies investigated the physiological dynamics of this reed when growing under

stressed environments as a factor that contributes to their survival. Majken et al. (2005) showed that *P. australis* responds physiologically to water deficit stress through the production of the metabolite proline. Proline is believed to enhance the plant survival in the water deficit environment. In a regime of drought and flooding Wen et al. (2017) found that *P. australis* net photosynthetic rate, stomatal conductance, intercellular CO<sub>2</sub>, and transpiration rates decreased with prolonged drought stress and the delay in subsequent flooding after the drought. However, this reed is able to increase its physiological response even before it receives flooding under which it copes better. This shows a balance between water conservation and growth. This is probably a coping measure to conserve water to ensure water retention within the plant during water scarcity but on the other hand, maintain a foliage that allows the plant to undertake its various biological and ecological functions. In the saline-alkaline marsh in which the reed was growing, it accumulated more Na<sup>+</sup> in the shoots after long-term drought stress showing a self-regulatory mechanism of ion balance in different organs with increasing drought stress. Most recently, Ding and Sun (2021) found that various depths of flooding of *P. australis* triggered varying physiologic responses with leaf blades maintaining high enzyme activity and proline content while leaf sheaths maintained the greatest amount of soluble protein again demonstrating an orchestrated physiological response to flooding characterized by tissue specialization.

Similarly, in an earlier study Ding et al. (2021a), in a more targeted *P. australis* study about the role of tissue in partitioning various metals found that leaf sheaths had the highest potential to store metals of all the organs observed. The highest translocation factor for Fe was observed from the stems to the leaf sheaths and a higher bio-concentration factor for Mn was found in the leaf blades and leaf sheaths with Cd and Zn higher bio-concentration factors observed in the stems. This demonstrated tissue specialization in *P. australis* in stress resistance. Investigating the enrichment characteristics and biological response of *P. australis* to sulfamethoxazole and ofloxacin residues, Lv et al. (2020) found that sulfamethoxazole and ofloxacin accumulated in the plant in the rank root > leaf > stem and accumulation and transport of ofloxacin was higher than that of sulfamethoxazole. Besides these few studies, other studies have uncovered the physiologic responses of *P. australis* to stress and found results which have implications for the use of this reed as a phytoremediator. This includes the study of Wu et al. (2020) who investigated the responses of *P. australis* to Cu stress using a combined approach which employed morphology, physiology, and proteomics. Dayou et al. (2021) investigated trait-based adaptability of *P. australis* to the effects of soil water and salinity in the Yellow River Delta. The authors observed reduction in the average height and stem diameter with increase in leaf water content and thickness as well as salinity stress tolerant strategy in *P. australis* that enables the reed to dominate the river. Wahman et al. (2021) evaluated the changes in the metabolome profiles of *P. australis* when exposed to stress caused by drugs using a serial coupling of reversed-phase liquid chromatography and hydrophilic interaction liquid chromatography combined with

accurate high-resolution time-of-flight mass spectrometer (TOF-MS) and observed variation in the metabolites shift in respect to different drugs. Strikingly, an increase in the production of quercetin was observed by the authors in the plant after diclofenac incubation.

Pflugmacher et al. (2001) observed the production of glutathione conjugate and cysteine conjugate in all cormus part of *P. australis* in the complete metabolism of cyanobacterial toxin microcystin and enzymes such as glutathione S-transferases (sGST) that enhances the complete breakdown of the toxins. Sauvêtre et al. (2018) observed the production of metabolites involving GSH conjugation and 2,3-dihydroxylation, as well as acridine related compounds in *Armoracia rusticana* (hair root culture) treated with endophytic bacteria from *P. australis* in response to carbamazepine (CBZ) exposure. Carbamazepine is known as a recalcitrant pharmaceutical pollutant in the aquatic environment. In their work, higher removal rate of CBZ and metabolite production were observed when the endophytes were introduced. This implied that the endophytes could enhance the development of the plant and promote the breakdown of CBZ.

In addition, Luisa et al. (2004) reported increased production of phytochelators, and antioxidant enzymes such as glutathione reductase, glutathione-S-transferase, catalase, ascorbate peroxidase, dehydroascorbate reductase, guaiacol peroxidase in *P. australis* when exposed to increased concentration of Cd. Sulaiman and Alfadul (2013) also observed increased production of metabolites such as malondialdehyde, aspartate, glutamate, serine, histidine, glycine, threonine, cysteine, valine, methionine, phenylalanine, isoleucine, leucine, lysine, proline, and the activities of antioxidant enzymes such as superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and peroxidase in response to the increase concentration Cd, Zn, Cu, and Pb. *P. australis*' increase production of metabolites and antioxidant enzymes are stress response of the plant to the increase concentrations of heavy metals which enhance its survival and sequestration of heavy metals by the plant. **Table 2** provides a summary of metabolites produced as well as synthesized enzymes by *P. australis* in a stressed environment. The interaction between *P. australis* and associated rhizospheric microbes and endophytes is believed to initiate physiological response leading to the production of diverse metabolites either by the reed or associated microbes that have the tendency to promote growth and bioremediation potential of the reed.

## INTERACTION OF THE STRESS INDUCED PHYSIOLOGICAL RESPONSES AND RHIZOSPHERE MICROBES OF *PHRAGMITES AUSTRALIS* AS FACILITATOR OF GROWTH AND BIOREMEDIATION POTENTIAL OF THE REED

The plant environment from the roots to the apex is a continuum of physiological and metabolic activity under various influences either internal such as genetic or external such



**TABLE 2 |** Summary of the metabolites produced, and enzymes synthesized by *P. australis* in response to stress.

Causes of the stress	Metabolites produced	Enzymes secreted	Suggested purpose of metabolites and enzyme secretion	References
Water deficit	Proline		Maintaining the water level of the plant for its survival	Majken et al. (2005)
Cyanobacterial toxin microcystin (MC-LR)	Glutathione conjugate and cysteine conjugate	Glutathione S-transferases (sGST)	Breakdown of the toxin	Pflugmacher et al. (2001)
Carbamazepine (CBZ)	GSH conjugation and 2,3-dihydroxylation, as well as acridine related compounds		Breakdown of recalcitrant pharmaceutical pollutant (CBZ)	Sauvêtre et al. (2018)
Cadmium (Cd)	Phytochelatin (PC)	Glutathione reductase, Glutathione-S-transferase, Catalase, Ascorbate peroxidase, Dehydroascorbate reductase, Guaiacol peroxidase	Removal of Cd	Luisa et al. (2004)
Cd, Zn, Cu, and Pb	Malondialdehyde (MDA), Aspartate, Glutamate, Serine, Histidine, Glycine, Threonine, Cysteine, Valine, Methionine, Phenylalanine, Isoleucine, Leucine, Lysine, Proline	Superoxide dismutase, catalase, ascorbate peroxidase, glutathione peroxidase and peroxidase	Removal of the heavy metals	Sulaiman and Alfadul (2013)
Presence of drugs	Quercetin		Removal of the drugs from the environment	Wahman et al. (2021)

as the environment characterized by the climate, ecological interactions, and the condition of the soil. Given this proven fact, it is logical that rhizosphere microbes influence the response of the plant to environmental stress. This has been proven in many studies, but we limit our focus on the effect of rhizosphere microbes on the physiological responses of *P. australis* to stress, in particular stress related to soil pollution. We also look at the use of rhizosphere microbes to improve the bioremediation efforts of *P. australis*. These include the use of rhizosphere microbes to first degrade complex chains of pollutant compounds into their smaller subunits for easier adsorption by the reed.

For the reclamation of polluted land needed for the growth of crops and sustainability of agricultural production, there is a serious need to unravel various microbial communities in association with stress resistant plants which include macrophytes like *P. australis* and the physiological mechanisms adopted by the plants that could aid in the remediation of the contaminated land. Toyama et al. (2009) evaluated the biodegradation of bisphenol A (BPA) and bisphenol F (BPF) in rhizosphere sediment of *P. australis*. Bisphenols are endocrine disrupting chemicals with the potentials to cause adverse effect on human health and other animals when released on

land and absorbed by crops (Chen et al., 2002; Crain et al., 2007). Consequent upon the adverse effect of bisphenols, there existed the need to remediate the environment contaminated with these chemicals. The authors observed a high rate of BPA and BPF removal and the presence of A BPA-degrading bacterium, *Novosphingobium* sp. strain TYA-1, and a BPF-degrading bacterium, *Sphingobium yanoikuyae* strain TYF-1. The results suggested that the interactions of *P. australis* and these bacteria can speed up the rate of removal of bisphenols from the sediment (Toyama et al., 2009).

Nejla et al. (2014) examined the phytoremediation potential of *P. australis* grown in pentachlorophenol and cadmium co-contaminated soils. They observed significant positive correlation between soil dehydrogenase activity (DHA) and pentachlorophenol (PCP) removal in planted soil implying that *P. australis* enhanced the biodegradation of PCP through the activities of enzymes and the microorganisms in the rhizosphere of the plant. The degradation of cyanobacteria toxin microcystin by *P. australis* (Pflugmacher et al., 2001) provided a good indication of the application of the reed in the bioremediation of polluted agricultural land and the reclamation of the land for agricultural practices. Physiology and rhizosphere microbiology

**TABLE 3 |** Remediation ability of *P. australis* in diverse polluted environment.

Nature of the environment	Bioremediation potential	References
Fe contaminated	100% bioaccumulation of Fe	Batty (2003)
Cu, Cd, Ni, Pb, and Zn contaminated aqueous solution	Adsorption of Cu, Cd, Ni, Pb, and Zn	Southichak et al. (2006)
As and Sb contaminated	Removal of As and Sb	Ghassemzadeh et al. (2008)
Cu contaminated	High Cu tolerance	Ali et al. (2002)
Zn and Mn contaminated	High root accumulation of Zn and Mn	Peltier et al. (2003)
Cu, Cd, Cr, Ni, Fe, Pb and Zn contaminated	Removal of Cu, Cd, Cr, Ni, Fe, Pb and Zn	Menka and Tripathi (2015)
Cd, Cr, Hg, Mn, Ni, Pb, and Zn contaminated	Cd, Cr, Hg, Mn, Ni, Pb, and Zn accumulation in the order root> rhizome> leaf> stem	Bonanno and Lo Giudice (2010)
As and trace metal contaminated	Phytostabilization of As and trace metals	Paola et al. (2018)
Cd, Cu, Pb, and Zn contaminated sediments	Bioaccumulation of Cd, Cu, Pb, and Zn	Fawazy et al. (2012)
Trace elements (Ag, Al, As, B, Ba, Be, Co, Fe, Mo, Pd, Pt, Rh, Sb, Se, Sr, Ti, and V) contaminated	Removal of Ag, Al, As, B, Ba, Be, Co, Fe, Mo, Pd, Pt, Rh, Sb, Se, Sr, Ti, and V in the order root>rhizome>leaf>stem	Bonanno (2011)
Co, Cr, Cu, Fe, Cd, Ni, Mn, and Zn contaminated	High bioaccumulation of Co, Cr, Cu, Fe, Ni, Mn, and Zn in the roots and Cd, and Pb in the leaves	Rzymiski et al. (2014)
Heavy metal (Co, Ni, Mo, Cd, Pb, Cr, Cu, Fe, Mn, Zn and Hg) and trace metal (As, Se, Ba) contaminated estuarine sediments	Decrease the metals in the order Fe > Mn > Zn > Pb > Ba > Cr > As > Cu > Ni > Co > Mo > Cd > Se > Hg	Cicero-Fernández et al. (2017)
Bisphenol A (BPA) and bisphenol F (BPF) contaminated	High rate of BPA and BPF removal in the presence of A BPA-degrading bacterium, <i>Novosphingobium</i> sp. strain TYA-1, and a BPF-degrading bacterium, <i>Sphingobium yanoikuyae</i> strain TYF-1) in the rhizosphere sediment of <i>P. australis</i>	Toyama et al. (2009)
pentachlorophenol and cadmium co-contaminated soils	Enhancement of the biodegradation of PCP through the activities of soil dehydrogenase (DHA) and the microorganisms in the rhizosphere of the plant	Nejia et al. (2014)
Butachlor contaminated	Degradation of butachlor	Yang et al. (2011)
Urban runoff treatment	Reduction of biological oxygen demand (BOD), and remove large amount of nitrogen	Byoung-Hwa and Miklas (2007)
Cyanobacterial toxin microcystin (MC-LR) contaminated	Complete remediation of cyanobacterial toxin microcystin (MC-LR)	Pflugmacher et al. (2001)
Ni and Pb contaminated Lake Burullus, Egypt	Phytoextraction of Ni and Pb	Eid et al. (2021)

were also completed in the work of Fahid et al. (2020) who found that *P. australis* vegetated with three bacterial strains belonging to *Acinetobacter* and *Bacillus* had an improved capacity of hydrocarbon extraction from diesel contaminated water. Likewise, *P. australis* grew more rapidly in the presence of the bacteria which were also hydrocarbon degrading strains. This was more of a complementation exercise as the ability of the reed to adsorb hydrocarbons was probably because the long chain hydrocarbons had first been degraded by the bacteria to make them easier for adsorption by the reed. Plant growth promotion by rhizosphere microbes of the *P. australis* has been proven in several studies, and logically growth promotion can be seen to aid phytoremediation in places where this reed grows as a remediator of a polluted site. Riva et al. (2019) found that cultivable microbes isolated from the rhizosphere and the interior of *P. australis* in a constructed wetland in Morocco improved growth of *J. acutus* and its ability to remove azo-dyes. This indicates that the rhizosphere of *P. australis* is an enrichment niche for microbes which can be effectively used in phytoremediation even using a different plant species. Similarly,

Saleem et al. (2019) investigated the feasibility of *P. australis* in combination with *Acinetobacter*, *Bacillus*, and *Pseudomonas* in phenol degradation. The authors observed the colonization of the reed's rhizosphere by the microbes which aid in their growth and phenol degradation.

You et al. (2021) investigated the effect of arbuscular mycorrhizal fungi on the growth and toxic element uptake by *P. australis* under Zn/Cd stress. The authors observed that the reduction of Zn and Cd toxicity in the reed as impacted by the actions of arbuscular mycorrhizal fungi via the increase of the reed biomass and regulatory patterns under different Zn/Cd concentrations. Wu et al. (2020) found that inoculation of *P. australis* with arbuscular mycorrhizal fungi can relieve this plant of photosynthesis inhibition caused by Cu stress as a result, promote the growth of the reed. This study proved the existence of a symbiotic relationship between the fungi and the plant, and this can be manipulated in several bioremediation efforts. **Table 3** provided a summary of the remediation potentials of *P. australis*. The ability of *P. australis* to bioaccumulate heavy metals could be an attribute of the plant's growing periods and physiology (Windham et al., 2001). Furthermore, the

bioremediation potential of *P. australis* could be attributed to the interaction between the reed and the associated microbes which is culminated with a lot of physiological response involving the production of metabolites and various enzymatic activities that can enhance the growth of the reed. The association between *P. australis* and its rhizosphere microbes has implications for phytoremediation of wastelands that can be reclaimed for agricultural use.

*Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, *Fusobacteria*, *Actinobacteria*, and *Planctomycetes* are the major families of bacteria dominating the rhizosphere of *P. australis*. Some species belonging to this families as well as some arbuscular mycorrhizal fungi have been found to play major roles in the biological activities that promotes the growth of the reed and the reed's remediation potential. However, further research is required to unveil other species of bacteria and

other microbes that can promote the growth of *P. australis* in polluted environment and enhance the phytoremediation process necessary for the reclamation of agricultural land for sustainable agricultural production.

## AUTHOR CONTRIBUTIONS

CK, MR, and KN drafted the manuscript. KN also provided oversight of the writing process. All authors have read and approved the manuscript.

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# Diversity of Rhizo-Bacteriome of *Crocus sativus* Grown at Various Geographical Locations and Cataloging of Putative PGPRs

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Earlier plant growth promoting rhizo-bacteria (PGPRs) were isolated from the plants, by cultivation based techniques and the interaction was mostly thought to be bilateral. The routine bilateral study, with no information on the associated microbiome, could be one of the reasons for the limited success of PGPRs in the field conditions. Keeping in view the role of PGPRs in rhizo-bacteriome on the growth and production of plant, the present study was aimed at studying the diversity of the rhizo-bacteriome of saffron grown across three geographical locations namely Kashmir, Kishtwar and Bengaluru. Variation in the rhizo-bacteriome of saffron growing across 10 different sites from 3 geographical locations was studied using 16S rDNA amplicon metagenomic sequencing. 16 bacterial phyla, 261 genera and 73 bacterial species were cataloged from all the rhizosphere samples. *Proteobacteria* was a dominant phylum in all the rhizosphere samples. Rhizo-bacteriome of saffron grown in Kishtwar was found to be significantly different from the rhizo-bacteriome of saffron grown in Kashmir and Bengaluru. Interestingly, the rhizo-bacteriome of saffron grown in Bengaluru was very similar to the saffron grown in Kashmir, thereby indicating that the rhizo-bacteriome in saffron is “plant driven” as the corm sown in Bengaluru were from Kashmir. Despite variation in rhizo-bacteriome, core rhizo-bacteriome in saffron was identified that was represented by 53 genera and eight bacterial species belonging to 11 phyla irrespective of their geographical distribution. In addition, 21 PGPRs were reported for the first time from the saffron rhizosphere. The high yielding saffron field Wuyan was found to have the highest number of PGPRs; this indicates that the presence of PGPR is important for yield enhancement than diversity. The two PGPR *Rhizobium leguminosarum* and *Luteibacter rhizovicinus* were reported from all the locations except Kishtwar that had escaped isolation in our previous attempts using cultivation based techniques. It is being proposed instead of going for random isolation and screening for PGPRs from plant rhizosphere, an alternate strategy using metagenomic cataloging of the rhizo-bacteriome community and cultivation of the dominant PGPR should be undertaken. This strategy will help in the selection of dominant PGPRs, specific to the plant in question.

**Keywords:** PGPR—plant growth-promoting rhizobacteria, *Crocus sativus*, rhizo-bacteriome, core microbiome, 16S rDNA



## INTRODUCTION

Microbiomics is a fast-growing field in which collective genomes of microorganisms, of a given community (a microbiota) are investigated. Literature is replete, with the studies on the microbiomes of environmental samples such as soil and water (Gui-Feng et al., 2020), human gut and skin (Parelo, 2020), plant rhizosphere, cormosphere and phyllosphere (Stone et al., 2018; Bhagat et al., 2021; Rüger et al., 2021). The plant microbiome is reported to be critical to host adaptation, productivity and health (Zhao et al., 2019; Trivedi et al., 2020). The plant microbiome acts as a reservoir of microbes, that directly influences the structure and composition of the plant, promotes plant growth, increases stress tolerance, mediates local patterns of nutrient cycling and can also be used as molecular markers (Bakker et al., 2013; Coats and Rumpho, 2014; Trivedi et al., 2020; Bhagat et al., 2021). Earlier microbiome associated with different plants would be studied mostly using cultivation based techniques. However, it is a fact now, that not more than 1% of bacteria can be cultivated by routine cultivation and 99% remained uncultivated (Steen et al., 2019). In order to study microbiomes associated with any niche, cultivation independent technique metagenomics, complements the cultivation based techniques. In metagenomics, genomes of the bacterial communities are extracted collectively and sequenced directly. Metagenomics has revolutionized the study of complex microbial communities, as it overcomes the limitation of cultivation based methods, as far as cataloging of bacteria present and functions performed by them, in any niche is concerned (Boughner and Singh, 2016; Alteio et al., 2020; Taş et al., 2021). Further, the attention has shifted from plant-microbe interaction to plant-microbiome interaction and the role of microbiome in plant growth promotion (Kour et al., 2019; Yadav et al., 2020). The rhizosphere microbiome's contribution to plants has been acknowledged by giving it the title of "plant's second genome" (Ofek-Lalzar et al., 2014; Yin et al., 2020). Plant growth promotion by Plant growth promoting bacteria (PGPBs) has been reported in various plants such as in rice (Chauhan et al., 2019), wheat (Çakmakçı et al., 2017), maize (Zerrouk et al., 2019), tomato (Cordero et al., 2018), soya bean (De Gregorio et al., 2017) and saffron (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016; Kour et al., 2018; Magotra et al., 2021).

*Crocus sativus*, commonly known as saffron, is the world's costliest spice with medicinal and cosmetic value (Zhao et al., 2019; Bhooma et al., 2020). 1 kg of saffron costs about 11,000 USD/Kg (Ambardar et al., 2014). The other reasons for the selection of the *Crocus sativus*, as a test organism, in addition to its economical status is, (i) reference microbiome of root and corm during various growth stages has been cataloged previously, (ii) PGPBs have been isolated and evaluated for their efficacy, (iii) and most importantly, it is reported to be a monogenetic herb world over with the uniform genotype, hence variation in rhizo-bacteriome and its effect on yield was the question worth asking. Variation in the quality and yield of saffron growing across different locations has been reported due to various factors such as epigenetic, climate change, soil characteristics and microbes associated with plants. It is a sterile triploid plant ( $2n = 3x$

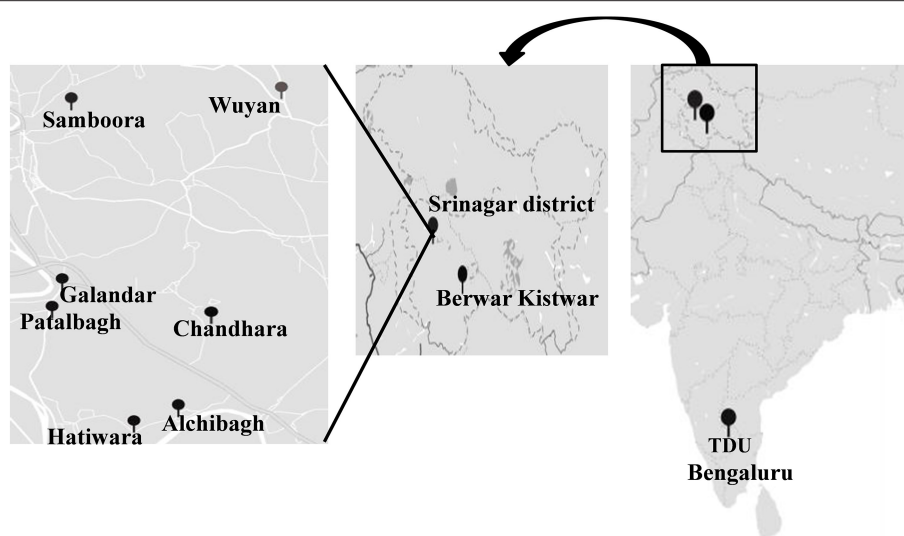
$= 24$ ) and reproduces vegetatively by underground bulb-like, starch-storing organs, known as corms (Wani et al., 2018; Nemati et al., 2019). The herb has an interesting biannual life cycle that is characterized by three distinct growth stages, dormant stage from July to August, flowering stage from October to November and vegetative stage from January to May. The flowering stage lasts ~30 days a year. Each flower has three red trumpets like stigmas (2 to 3 cm long) which when dried, are commercialized as a saffron spice (Yasmin and Nehvi, 2013). The bacteriome and mycobioime of saffron rhizosphere were, for the first time, reported by our group (Ambardar et al., 2014, 2016). In addition, PGPBs were isolated and evaluated in the course of the study (Ambardar and Vakhlu, 2013; Ambardar, 2014; Kour et al., 2018; Magotra et al., 2021). Recently, the cormosphere bacteriome of saffron was compared across the geographical locations from India and Morocco and preliminary data suggests that the microbiome is location specific and simultaneously, there is a core microbiome that is common to all locations (Bhagat et al., 2021). Such reports of core and location specific microbiome have also been reported in other plants such as, rice (Eyre et al., 2019); coffee (Fulthorpe et al., 2020); switch grass (Grady et al., 2019); red sage (Chen et al., 2018), wheat (Kuzniar et al., 2020) etc.

Since saffron is reported to be a monogenetic plant and has the same genotype worldwide, the present study was undertaken to explore the variation in the rhizo-bacteriome associated with the plant grown across different geographical locations in India to evaluate the effect of geography on rhizo-bacteriome of the saffron. Rhizo-bacteriome associated with a saffron plant was compared and a core rhizo-bacteriome was identified using 16S rDNA gene targeted metagenomic analysis during the flowering stage in November 2016. In addition, an inventory of putative PGPRs present in the rhizo-bacteriome was made and compared across these locations and a correlation with the yield was attempted.

## MATERIALS AND METHODS

### Sample Collection

Saffron rhizosphere samples were collected from 10 locations including eight saffron fields from Kashmir district (Wuyan, Patalbagh\_upper range, Patalbagh\_lower range, Alchibagh, Galandar, Chandhara, Hatiwara, Samboora); one from Berwar saffron field from Kishtwar, Jammu district and one laboratory experiment conducted in Bengaluru (**Figure 1**). The details of all the saffron fields, geographical coordinates (latitude and longitude), fertilizers usage, total production, and total area of cultivation have been tabulated in **Table 1**. Composite sampling was performed from all the fields wherein the *Crocus sativus* rhizosphere samples were collected from the three corners of five fields of each location and all the 15 samples per location were pooled together to form composite samples (Ambardar and Vakhlu, 2013; Ambardar, 2014). Rhizosphere samples were collected by uprooting the plants and vigorously shaking by hand. The soil that remained adheres to the roots after vigorous shaking was taken as rhizosphere soil. The roots were washed in normal



**FIGURE 1 |** Geographic location of sample collection sites on the map of India. TDU (The University of Trans-Disciplinary Health Sciences and Technology), Bengaluru, (Berwar) Kistwar and 8 locations with in Srinagar district (Wuyan, Patalbagh\_upper range, Patalbagh\_lower range, Alchibagh, Galandar, Chandhara, Hatiwara and Samboora).

saline (0.85% NaCl) that was taken further for metagenomic DNA extraction.

A separate experiment was conducted in which saffron corms were collected from Kashmir, Pampore during the dormant stage of the saffron life cycle (July, 2016). These corms were then planted in 10 pots (5 corms/pot) filled with the garden soil at Trans-Disciplinary University, Bengaluru (Karnataka). These pots were kept in open under natural conditions (temperature 20°C) for a period of 3 months (August–November 2016). The rhizosphere samples from 15 plants were collected randomly and pooled together to form a composite sample during the flowering stage similar to field samples. This experiment was performed to address the question whether the root microbiome is soil driven or plant driven. Root microbiome was harvested using same design and method as in case of field samples.

### Rhizosphere Metagenomic DNA Extraction

Metagenomic DNA was extracted from rhizosphere samples using a MoBio Power soil DNA extraction kit (MoBio Laboratories Inc. Carlsbad, CA, USA) following the manufacturer's instructions. The DNA quality and quantity were determined by using a Nano Drop device (Thermo Scientific, Wilmington, DE) and electrophoresis on 0.8% agarose gel, with 1 kb plus ladder as molecular weight marker.

### 16S rDNA Sequencing of the Extracted Rhizosphere Metagenome

A 16S rDNA sequencing library was constructed targeting the V3 and V4 hyper-variable regions of the 16S rDNA gene, according to the 16S rDNA metagenomic sequencing library preparation protocol (Illumina, San Diego, CA, USA). The PCR was performed with 12 ng template DNA and region-specific primers with Illumina index and sequencing adapters

(forward\_primer: 5'-TCGTCGGCAGCGTCAGATGTGTAT AAGAGACAGTCGTCGGCAGCGT CAGATGTGTATAAGA GACAGCCTACGGGNGGCWGCAG-3'; reverse primer: 5' GTC TCGTG GGCTCGGAGATGTGTATAAGAGACAGGTCTCG TGGGCTCGGAGATGTGTATAAGAGAC AGGACTACHV GGGTATCTAATCC-3') using KAPA HiFi Hot Start Ready Mix (KAPA Biosystems, Wilmington, MA, USA). The amplicons were purified using the Agencourt AMPure XP system (Beckman Coulter Genomics, Brea, CA, USA). Subsequently, purified PCR products were visualized after gel electrophoresis and quantified with a Qubit dsDNA HS Assay Kit (Thermo Scientific) on a Qubit 3.0 fluorometer. The PCR products of all the samples were pooled to 4 nM concentration, prior to sequencing, and were analyzed on an Agilent 2,200 Tape Station (Agilent Technologies, Santa Clara, CA, USA) for quantity and quality analysis. The pooled sample (4 nM) was denatured with 0.2 N NaOH, diluted further to 4 pM concentration, and combined with 20% (v/v) denatured 4 pM PhiX as control, following Illumina's guidelines. Samples were sequenced on the MiSeq sequencing platform (Illumina) using a 2 × 250 cycle V3 kit, following standard Illumina sequencing protocols.

### Data Analysis

Raw data of 16S rDNA sequencing was analyzed for a quality check using the FastQc tool kit (Brown et al., 2017). The adapter and low quality reads ( $Q < 30$  Phred score) were trimmed using the Cutadapt tool of Trimalore (Jiang et al., 2014). The reads with phred score above 30 were selected for QIIME1 pipeline analysis (version 1.9.1). The QIIME 1 pipeline (Quantitative Insights into Microbial Ecology version 1.9.1) was used to process and filter multiplexed sequence reads. The sequencing reads were grouped into an operational taxonomic unit (OTUs) that were further clustered against GreenGenes\_13\_8 sequences to

**TABLE 1 |** Area under cultivation and production data of saffron from various saffron fields under study. Saffron production was maximum in the Wuyan fields and lowest in Kishtwar fields.

Sample id	Description	Coordinates		Area under cultivation in (canals)	Area under cultivation in (Hectare)	Fertilizer used	Production for year 2016 (Kg/hectare/year)	Total Production
		Latitude N	Longitude E					
Sample 1	Kishtwar	33.311591	75.776622	101	5.1		1.26	6.4
Sample 2	Alchibagh	33.964289	74.946839	600	30.36		2	60.72
Sample 3	Galandar	33.988966	74.924863	2,000	101.18		2	202.36
Sample 4	Hatiwara	33.961191	74.938362	2,100	106.23		2	212.46
Sample 5	Patalbagh_upper range	33.983498	74.923041	2,000	101.18	DAP (Diammonium phosphate—5 kg), MOP (Muriate of potash—3 kg), Potash, Urea (1.5 Kg) A and Vermicompost	2	202.36
Sample 6	Chandhara	33.98243	74.953026	12000	607.03		2	1214.06
Sample 7	Patalbagh_lower range	33.983498	74.923041	200	10.2		3	30.6
Sample	Samboora	34.024078	74.926665	2500	126.46		2.5	316.15
Sample 9	Wuyan	34.026266	74.966175	10000	505.86		5	2529.3
Sample 10	Bengaluru	13.123444	77.547963	Experiment in pots	Experiment in pots	—	—	—

The details of the fertilizer used was provided by SKAUST, Kashmir, India.

**TABLE 2 |** 16S rDNA amplicon sequencing reads, total phyla, genera and species cataloged in saffron rhizosphere from various saffron fields under study.

Sample id	Description	16S rDNA amplicon sequencing PE reads	Phyla	Genera	Species
Sample 1	Kishtwar	68,384	16	138	37
Sample 2	Alchibagh	90,658	15	147	39
Sample 3	Galandar	67,052	14	130	31
Sample 4	Hatiwara	88,215	16	140	34
Sample 5	Patalbagh_upper range	51,918	12	122	29
Sample 6	Chandhara	59,826	16	143	28
Sample 7	Patalbagh_lower range	97,484	13	111	31
Sample 8	Samboora	75,753	15	122	34
Sample 9	Wuyan	63,972	15	154	43
Sample 10	Bengaluru	49,388	16	143	30
Total (unique phyla, Genera and species)			16	261	73

ascertain the taxonomical affiliation of bacteria (McDonald et al., 2012). Reads failing to hit the reference were subsequently clustered *de novo* at the 97% similarity level using the UCLUST greedy algorithm. Chimeric sequences were identified by the UCHIME algorithm of USEARCH61 and removed. OTU sequences were aligned using PYNAST. OTU taxonomy was determined using the Green Genes taxonomy database (Caporaso et al., 2010).

Statistical Analysis of Metagenomic Sequence Data

The bacterial community associated with each sample was compared for alpha and beta diversity analysis using Qiime 1 (Sinclair et al., 2015). For alpha diversity analysis, rarefaction curves and diversity indices like Chao1, Shannon, Simpson, phylogenetic diversity were calculated for estimating the richness and evenness in all the samples (Chao, 1987; Gotelli and Colwell, 2011). For rarefaction curves, the operational taxonomy units OTU table was rarified to an even depth of 1,00,000 sequences per sample, to avoid biases generated by the difference in sequencing depth. Rarefaction curves (97% identity) were plotted between the number of observed OTUs (cluster count) and the number of reads of the samples. In order to evaluate  $\beta$  diversity, PCOA plots, venn diagram and heat maps were constructed. In addition, a circos plot was generated for representing the core rhizo-bacteriome across all locations using the tool Circos Version 0.63-9 (Krzywinski et al., 2009).

RESULTS

Ten rhizosphere samples were collected from 8 different saffron fields from Kashmir and 1 from Kishtwar (Jammu

and Kashmir); and 1 from corms grown in pots at Trans-Disciplinary University, Bengaluru (Karnataka). The saffron fields were selected from different geographical locations having variations in total production of saffron as represented in **Table 1**. Wuyan in Kashmir had maximum saffron production and Kishtwar in Jammu had minimum production in 2016, the year of sample collection (**Table 1**). The corms were collected from Kashmir and sown in pots in Bengaluru to test the hypothesis that rhizo-bacteriome in saffron is plant driven, hence lacks production data, as flowering needs special climatic conditions. Rhizosphere samples were analyzed for bacterial diversity using 16S rDNA sequencing and the total number of 16S rDNA sequence reads per sample ranged between 51,918 and 97,484 (**Table 2**). The bacterial communities associated with all the rhizosphere samples were classified up to the phylum, genus and species level (**Table 2**).

## Rhizo-Bacterial Diversity

Bacterial diversity associated with all the 10 rhizospheric samples was cataloged into 16 phyla, 261 genera and 73 species (**Table 2**). Out of 16 phyla, *Proteobacteria* was the dominant phyla in all the samples with higher relative abundance in Kashmir (avg 59.1%) followed by Bengaluru (49%) and Kishtwar (31.28%). However, *Firmicutes* and *Planctomycetes* were relatively abundant in Kishtwar (20, 10%) as compared to Kashmir (avg 4.9, 3.12%) and Bengaluru (5, 4%) respectively (**Figure 2**).

Out of total of 261 bacterial genera cataloged, the abundance of 4 bacterial genera namely *Bacillus*, *Lysobacter*, *Rhodoplanes* and *Janthiobacterium* were observed. *Bacillus* was dominant at four locations namely Kishtwar (54%), Hatiwara (24%), Patalbagh\_lower range (22%) and Bengaluru (15%), (**Figure 3**). However, *Bacillus* was also found co-dominant with other genera at two locations namely Wuyan [*Bacillus* (16%) and *Rhodoplane* (17%)] and Galandar [(*Bacillus* (12%) and *Lysobacter* (13%)]]. The abundance of *Bacillus* was comparatively less in rest of the four locations i.e., Alchibagh (4%), Chandhara (7%), Samboora (8%), and Patalbagh\_upper (11%). *Janthiobacterium* was dominant in Alchibagh (19%). *Lysobacter* was the most abundant bacterial genera in two locations namely Patalbagh\_upper range (15%) and Samboora (18%), whereas *Lysobacter* (15%) shared dominance with *Rhodoplanes* (14%) in Chandhara (**Figure 3**).

53 genera out of 261 bacterial genera were common in all the samples, are considered as the core rhizo-bacteriome that has been discussed in detail under core rhizo-bacteriome section. on comparing all the 10 samples, unique genera specific to each location were also identified. The maximum number of unique bacterial genera were identified in Wuyan (19) followed by Alchibagh (10), Galandar and Kishtwar (6 each), Chandhara (4), Patalbagh\_lower range and Samboora (2 each), Patalbagh\_upper range, Hatiwara and Bengaluru (1 each), (**Figure 4**).

73 bacterial species were present in all the samples, out of which, 3 bacterial species i.e., *Bacillus flexus*, *Lysobacter brunescens* and *Janthiobacterium lividum* were dominant; but their abundance varied (**Figure 5**). *Bacillus flexus* was abundant in seven locations namely Kishtwar (49%), Patalbagh\_lower range (38%), Hatiwara (35%), Bengaluru (31%), Galandar (28%),

Wuyan (22%) and Patalbagh\_upper range (20%). However, *Janthiobacterium lividum* (19%) was abundant in Alchibagh (**Figure 5**). *Lysobacter brunescens* was abundant in rest of the two locations namely Samboora (14%) and Chandhara (40%). Out of 73 bacterial species, 8 bacterial species were considered as the core rhizo-bacteriome (common in all the samples) that has been discussed in core rhizo-bacteriome section. In addition to core species, each sample has unique bacterial species that were specific to that particular location and absent in all other samples. Kishtwar had four unique bacterial species namely *Bacillus marisflavi*, *Macroccoccus caseolyticus*, *Myroides odoratimimus* and *Staphylococcus scui* whereas two species were unique in Alchibagh namely *Methylostenora mobilis* and *Sphingomonas suberifaciens* and Galandar namely *Paenibacillus barengoltzii* and *Paracoccus marcusii*. Only one unique species was cataloged from Patalbagh\_upper range (*Vellonellas dispar*), Wuyan (*Paenibacillus ginsengarvi*) and Samboora (*Roseomonas mucosa*). However, Bengaluru, Hatiwara, Chandhara and Patalbagh\_lower range did not have any unique bacterial species (**Figure 6**).

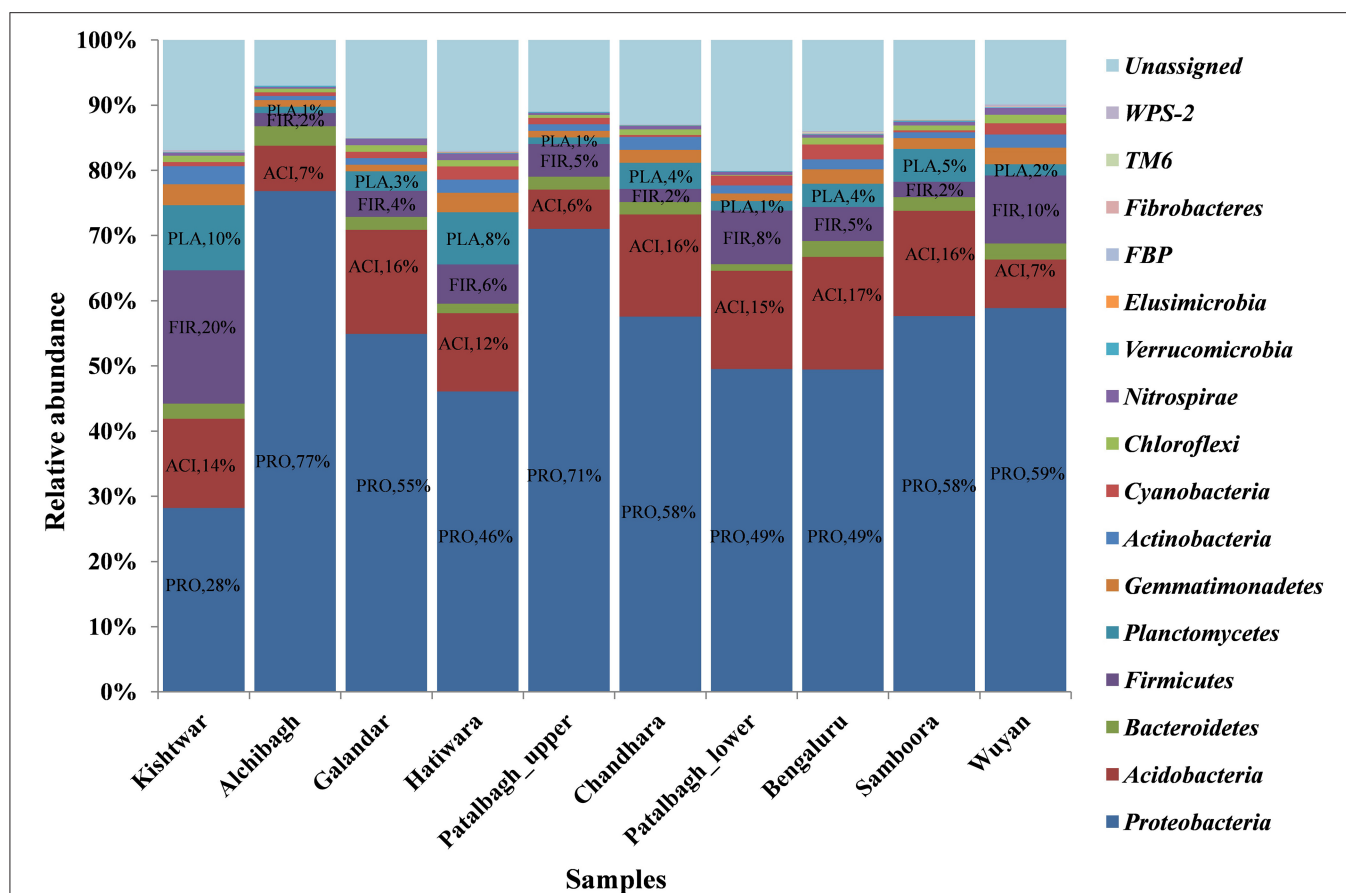
## Statistical Analysis of Rhizo-Bacterial Diversity

The bacterial diversity of each sample was also analyzed using the alpha diversity indices i.e., Chao1, Simpson, phylogenetic diversity and Shanon (**Table 3**), rarefaction curves (**Figure 7**), PCOA plots (**Figure 8**) generated by Qiime software. Rarefaction curves (97% identity) of rhizosphere samples did not reach plateau indicating bacterial diversity was well-represented but could increase on repetitive sampling (**Figure 7**). The bacterial community in saffron rhizosphere from Samboora field was diverse as compared to other sample, as depicted by higher number of different species (OTUs) in the rarefaction curve. This result was further complemented by diversity indices such as Chao1, phylogenetic diversity, Shannon and Simpson indexes were also higher in the case of Samboora (**Table 3**). Beta diversity analysis using PCOA plots was done to evaluate the significant variation in bacterial diversity among all the samples. Beta bacterial diversity of Kishtwar was significantly different from Kashmir and Bengaluru, as it does not cluster with the rest of the samples in PCOA plots (**Figure 8**).

## Core Rhizo-Bacteriome of Saffron

Despite the variation in the rhizobacterial community associated with saffron plant across different locations, the core rhizo-bacteriome that remains constant in the saffron growing across different geographical locations was identified. Core rhizo-bacteriome in saffron consisted of 11 phyla, out of total of 16 phyla i.e., *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Nitrospirae*, *Planctomycetes*, *Verrucomicrobia* and *Actionobacteria*. At genera level, it constituted 53 out of 261 genera and eight bacterial species out of 73 bacterial species. The bacterial species that constituted the core comprised of *Bacillus flexus*, *Bacillus muralis*, *Edaphobacter modestum*, *Lysobacter brunescens*, *Pseudoxanthomonas mexicana*, *Psychrobacter celer*, *Roseateles*





**FIGURE 2 |** Comparison of rhizo-bacteriome of saffron from different geographical location at phylum level representing dominance of *Proteobacteria* in all 10 samples. The relative abundance of *Proteobacteria* is less in Kishtwar (Jammu) as compared to all the samples from Kashmir and Bengaluru whereas Firmicutes and Planctomycetes are more in Kishtwar as compared to others. PRO, *Proteobacteria*; FIR, *Firmicutes*; ACI, *Acidobacteria*; PLA, *Planctomycetes*.

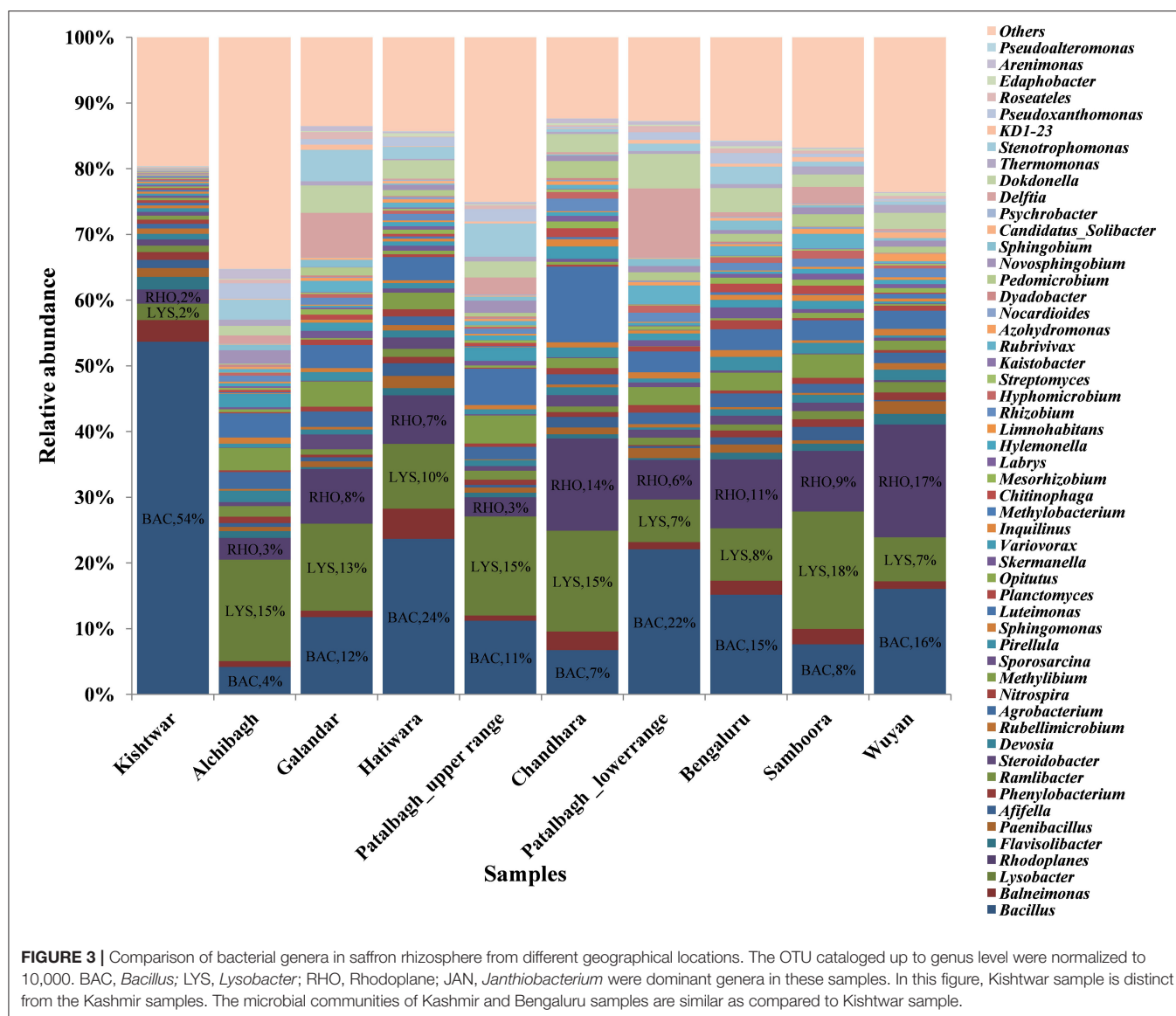
depolymerans and *Variovorax paradoxus*. The core rhizo-bacteriome in saffron is also well-represented in heat maps at genus and species level and with circos plot (Figures 9–11).

## DISCUSSION

Metagenomic approaches using NGS technologies can be used to explore the taxonomic and functional diversity of bacteria associated with the plants, thereby accelerating the research specific to the effect of plant bacteriome on plant health (Parvathi et al., 2019; Nathan et al., 2020). The present study was aimed at (i) exploring variation in the rhizo-bacteriome of the saffron grown across 10 sites from 3 geographical locations namely Kashmir, Kishtwar and Bengaluru, (ii) identify the core rhizo-bacteriome that persist in saffron irrespective of its geographical locations (if any), and (iii) presence of PGPRs in rhizo-bacteriome and its correlation with yield. The selected fields varied in the saffron production with Wuyan in Kashmir with the highest production i.e., 5 kg/hectare) whereas the fields in Kishtwar, Jammu with lowest production i.e., 2 kg/hectare in the year 2016 (Table 1).

## Comparative Rhizo-Bacteriome Diversity

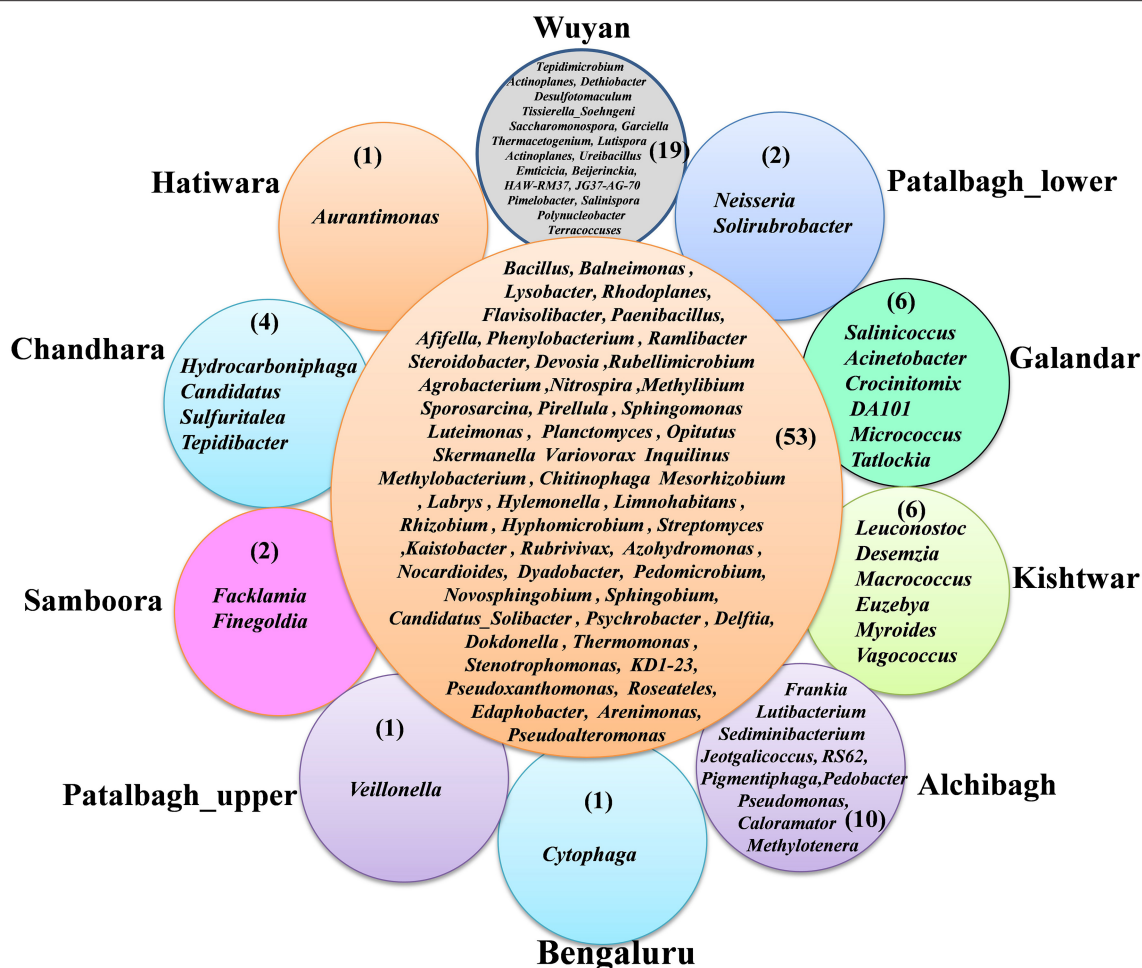
Out of 16 bacterial phyla, *Proteobacteria* was the most abundant phylum in all the samples (Figure 2). This is in accordance with a previously published report on saffron rhizosphere by our group (Ambardar and Vakhlu, 2013; Ambardar et al., 2014, 2016). *Proteobacteria* have been also reported to be the dominant phyla in the rhizosphere of other plants such as, *Gossypium hirsutum*, *Artemisia argyi*, *Ageratum conyzoides*, *Erigeron annuus*, *Bidens biternata*, *Euphorbia hirta* and *Viola japonica* (Qiao et al., 2017; Lei et al., 2019). *Proteobacteria* are known to regulate nutrients (carbon, nitrogen, sulfur) cycling in the environment that enhance plant growth (Rampelotto et al., 2013; Mukhtar et al., 2020). In our recent publication, *Proteobacteria* was also found to be the most abundant phylum in the cormosphere of saffron from Kashmir and Kishtwar, as well as in Morocco (Bhagat et al., 2021). On comparing all the samples, the rhizo-bacterial diversity of saffron growing in Kishtwar was found distinct from the rest of the samples as the relative abundance of *Proteobacteria* (PRO-31.28%) was comparatively less whereas the relative abundance of *Firmicutes* (FIR-20%) and *Planctomycetes* (PLA-10%) were more as compared to Kashmir (PRO-59.1%, FIR-4.9%, PLA-3.12%) and Bengaluru (PRO-49%, FIR-5%, PLA-4%), (Figure 2). This



was further complemented by beta diversity analysis wherein rhizo-bacterial diversity of saffron grown in Kishtwar did not cluster with the rest of the samples based on the PCOA plots and heat maps at genus and species level (Figures 8–10) respectively. Similar to rhizo-bacteriome, cormo-bacteriome of Kishtwar was found significantly different than that of Kashmir and surprisingly, relatively similar to Morocco in our recent report (Bhagat et al., 2021). The significant variation in the rhizo-bacterial diversity of saffron growing in Kishtwar from rest of the samples can be preliminarily attributed to corms (plant) as Kishtwar is closer to Kashmir geographically than Morocco. Though saffron is reported to be a sterile monogenetic triploid plant, with no genetic variation and reproduces vegetatively by corms (Nemati et al., 2019), however, the variation in the yield and quality of saffron has been reported worldwide (Cardone et al., 2021). Climate and soil are thought to be two major abiotic

factors (Cardone et al., 2019, 2020) and epigenetic influences have been reported to be one of the biotic factors to affect the quality and yield of saffron (Chen et al., 2021). In the preliminary observation, it seems that Cormo/rhizo-bacteriome has a role to play and is “plant driven” because rhizo-bacteriome of saffron grown in different climates and soil at Bengaluru was similar to that of Kashmir. The reason was the corms that were collected from Kashmir and grown in Bengaluru indicating that rhizo-bacteriome in saffron could be plant driven.

Comparison of rarefaction curve (97% identity) of all the samples indicated that the bacterial community in saffron rhizosphere from Samboora field in Kashmir was more diverse as compared to other samples, as the number of different species (OTUs) was higher here. This result was further complemented by diversity indices like Chao1, phylogenetic diversity, Shannon and Simpson indexes which were also higher in the case of



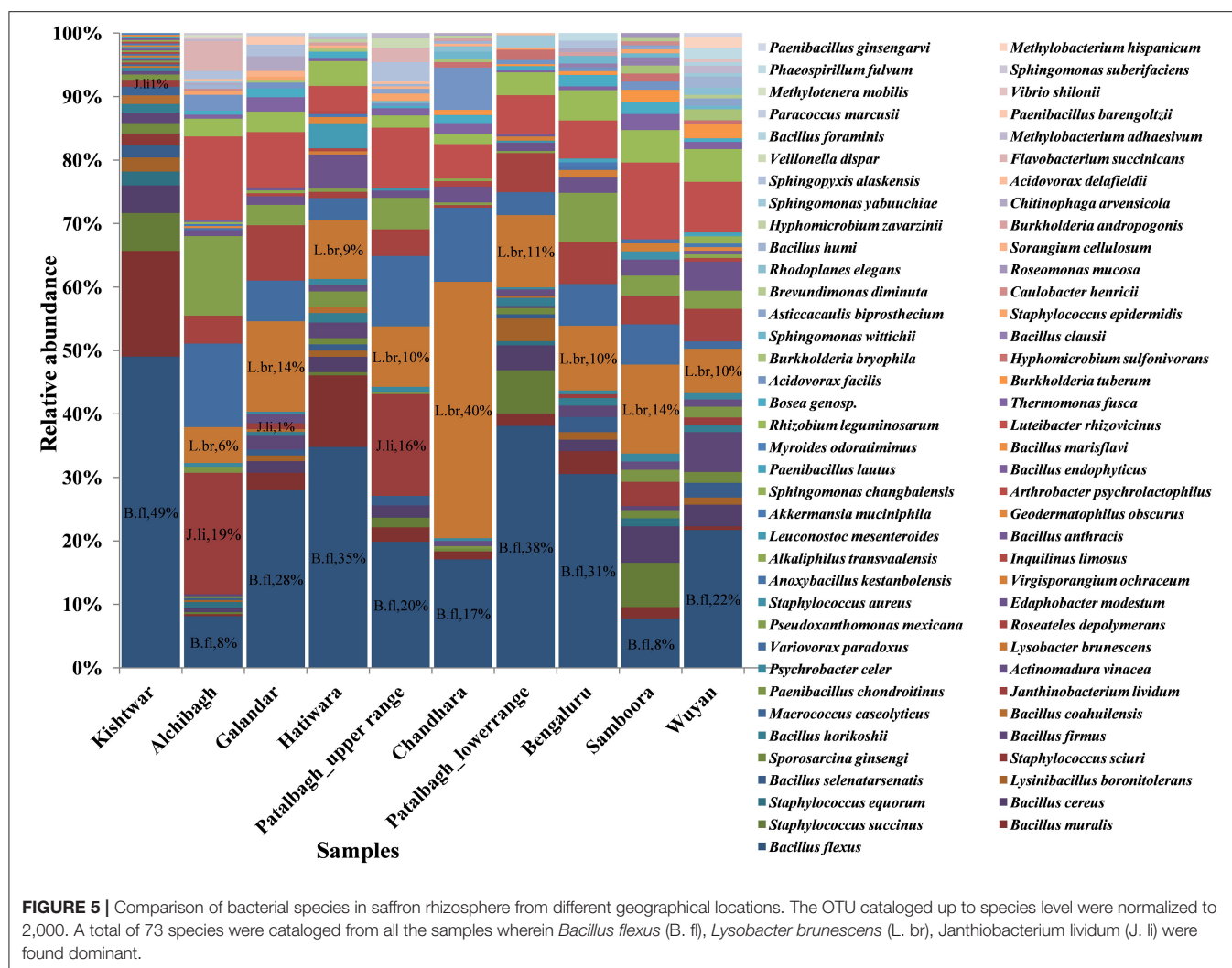
**FIGURE 4 |** Radical Venn diagram representing the unique genera identified in 10 different locations. Wuyan-Kashmir has the maximum number (19) of unique genera followed by 10 in Alchibagh, 6 in Gullandar and Kishtwar, 4 in Chandhara, 2 in Samboora and Patalbagh\_lower range and 1 genera in Hatiwara, Bengaluru, Patalbagh\_upper range.

Samboora (Table 3). However, higher bacterial diversity in the rhizosphere of saffron growing in Samboora field does not affect the saffron production positively as saffron yield in Samboora (2.5 kg/hectare) was less than that of Wuyan fields (5 kg/hectare) that had less diversity than Samboora.

The dominance of three bacterial species, *Bacillus flexus*, *Lysobacter brunescens* and *Janthiobacterium lividium* were observed in the rhizo-bacteriome of saffron grown in all the 10 locations (Figures 3, 5). *Bacillus flexus* was dominant in the rhizosphere of saffron grown in seven locations namely Kishtwar, Bengaluru, Wuyan, Galandar, Hatiwara, Patalbagh\_upper range and Patalbagh\_lower range. *Lysobacter brunescens* was dominant in Chandhara and Samboora; and *Janthobacterium lividium* in Alchibagh (Figure 5). Dominant bacterial genera identified in the saffron rhizosphere (*Bacillus*, *Lysobacter*, *Janthobacterium* and *Rhodoplanes*) have been reported from plant rhizospheres such as saffron (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016), green pepper (Liu et al., 2019), grapevine (Sacca et al., 2019) and oilseed rape (Gkarmiri et al.,

2017), etc. However, only *Bacillus flexus* have been reported in *Limoneum sinense* and rice (Roy et al., 2020; Xiong et al., 2020) whereas *Lysobacter brunescens* and *Janthobacterium lividium* have not been reported from any plant so far.

In our previous studies, *Pseudomonas* was found dominant in the saffron rhizospheres by cloning based 16S rDNA metagenomic approach wherein full length 16S rDNA was amplified, TA cloned and sequenced using Sanger sequencing (Ambardar et al., 2014). Surprisingly, in the present study, *Pseudomonas* was only present in the saffron field from Alchibagh and absent in all other samples. The absence of *Pseudomonas* could be attributed to DNA extraction protocol and PCR primers and cloning approaches that were different from the present study. In a previous study, the metagenomic DNA was extracted manually using four protocols and pooled together and full length 16S rDNA primers followed by TA cloning were used to catalogue the diversity (Ambardar et al., 2014), whereas, in the present study, a commercial kit was used for DNA extraction followed by amplification of only V3-V4 region of 16S rDNA.



In addition to this, the previous sampling was done in the year 2009 and present sampling was done after about 5 years in 2016, therefore climate change and floods in between in Kashmir can also be probable reasons, though this needs further investigation.

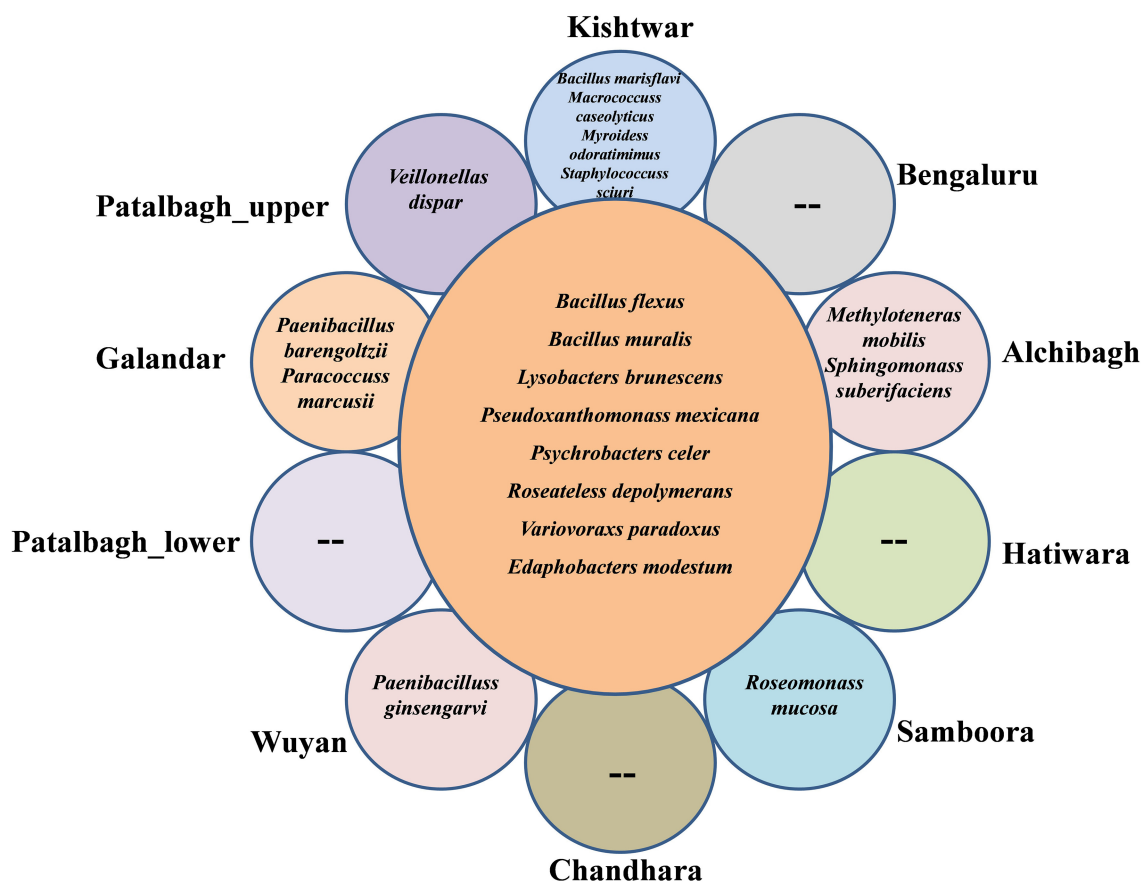
Various bacterial species, being reported in the present study, have been also reported by our group earlier by cultivation based method and were characterized *in-vitro* and *in-vivo* as well (Ambardar and Vakhlu, 2013; Ambardar et al., 2014; Kour et al., 2018; Bhagat et al., 2021). However, in the present study, six bacterial phyla, 212 bacterial genera and 70 bacterial species have been reported from the rhizosphere of saffron for the first time. Though, the rhizo-bacteria cataloged in the present study have been reported from other plants, but not reported from the cormosphere and rhizosphere saffron by our group earlier.

## Core Rhizo-Bacteriome of Saffron

Comparative analysis of rhizo-bacteriome of saffron across different sites revealed the presence of various common bacterial phyla, genera and species representing the core rhizo-bacteriome of saffron. Core rhizo-bacteriome in saffron was represented

by 53 bacterial genera and eight bacterial species namely *Bacillus flexus*, *Bacillus muralis*, *Edaphobacter modestum*, *Lysobacter brunescens*, *Pseudoxanthomonas mexicana*, *Psychrobacter celer*, *Roseateles depolymerans*, *Variovorax paradoxus*. These bacteria mostly belonged to 11 bacterial phyla namely *Proteobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, *Cyanobacteria*, *Gemmatimonadetes*, *Bacteroidetes*, *Nitrospirae*, *Planctomycetes* and *Actionobacteria*. Core rhizo-bacteriome in saffron has been illustrated in the heat maps at genus & species level and with circos plot (Figures 9–11) respectively. Recently, core bacteriome associated with the corms of saffron were compared across different geographical locations i.e., Kashmir, Kishtwar and Morocco. 24 bacteria genera belonging to the phylum *Proteobacteria*, *Actinobacteria*, *Bacteroidetes* and *Verrucomicrobia* made the core of cormo-bacteriome in saffron (Bhagat et al., 2021). Core rhizo-bacteriome in saffron represents 20.3% (53 out of 261) of the whole rhizo-bacteriome which was found comparatively less than the core cormo-bacteriome i.e., 32.8% of total cormo-bacteriome, however the number of bacterial genera constituting the core was more





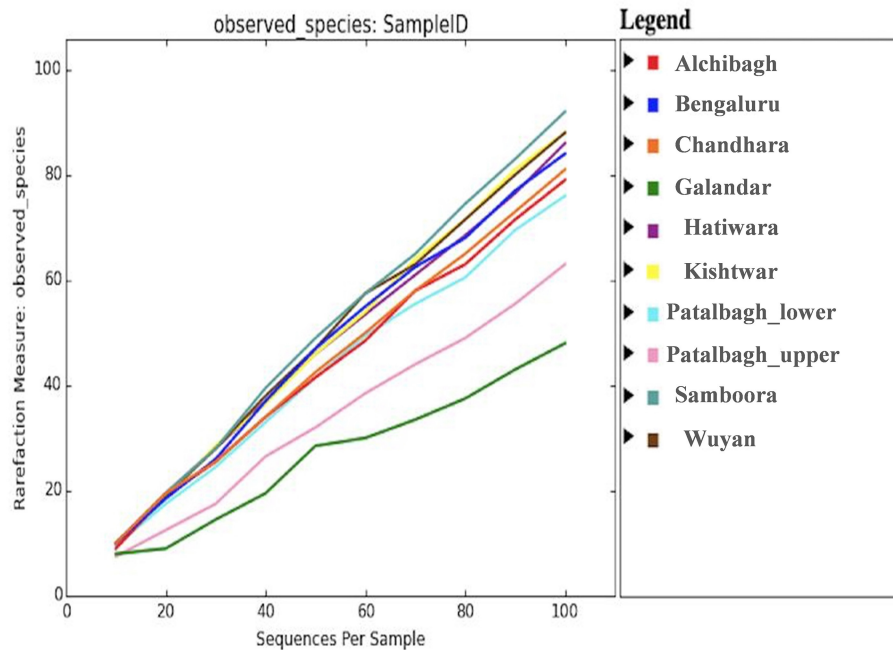
**FIGURE 6 |** Radical Venn diagram representing the number of unique and common species identified in 10 different locations. 8 common species were identified in 10 samples. Kishtwar had maximum number (4) of unique bacterial species followed by 2 bacterial species each in Alchibagh and Galandar whereas only 1 bacterial species in Patalbagh\_upper range, Wuyan and Samboora. Bengaluru, Hatiwara, Chandhara and patalbagh\_lower range did not have any unique bacterial species.

in rhizosphere (53 out of 261) as compared to cormosphere (24 out of 73), (Bhagat et al., 2021). Core microbiome of various plants have been studied such as wheat (Kuzniar et al., 2020), tomato (Cordero et al., 2020), *Gymnadenia conopsea* (Lin et al., 2020), coffee (Fulthorpe et al., 2020), switch grass (Grady et al., 2019), *Phaseolus vulgaris* (Pérez-Jaramillo et al., 2019), *Oryza sativa* (Eyre et al., 2019), Vineyards soil (Coller et al., 2019), *Dalbergia spruceana* (Skaltsas et al., 2019), and *Salvia miltiorrhiza* (Chen et al., 2018), etc. Core microbiome of 21 *Salvia miltiorrhiza* seeds represented 54% of the whole microbiome cataloged from seven different geographic origins (Chen et al., 2018). In the case of *Phaseolus vulgaris* rhizosphere, the core microbiome represented 25.9% of the total microbiome in native and agricultural soils (Pérez-Jaramillo et al., 2019). The core microbiome of 30 phylogenetically diverse angiosperm plants constitutes 40% of the whole microbiome (Fitzpatrick et al., 2018). Core rhizo-bacteriome in saffron was 20.3% of the total microbiome, which was comparatively less than *Salvia miltiorrhiza*, *Phaseolus vulgaris* and Angiosperm plants. In rice seeds, the core microbiome was enriched in *Rhizobium*, *Pantoea*, *Sphingomonas*, *Methylobacterium*, *Xanthomonas*, *Paenibacillus*, *Alternaria*, and *Occultifur* (Wang

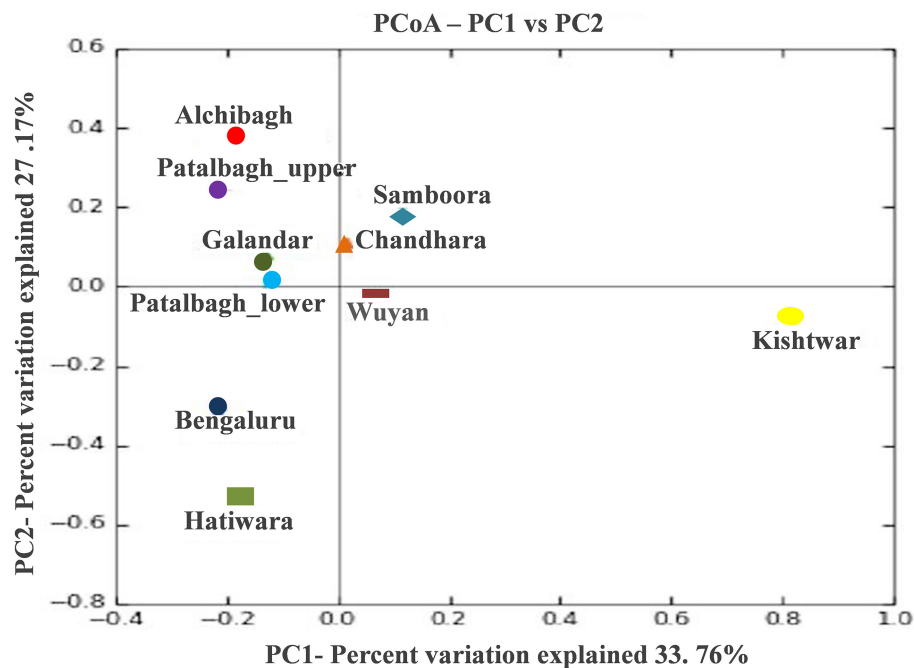
**TABLE 3 |** Diversity indices were the maximum in Samboora representing maximum bacteria diversity and richness in rhizosphere of saffron growing in Samboora as compared to other samples.

Diversity indices → samples	Chao1	Simpson	Shannon	Phylogenetic diversity
Kishtwar	590	0.9698	5.9	15
Alchibagh	534	0.9854	6.3	13
Galandar	621	0.9776	6.1	18
Hatiwara	566	0.7246	3.7	10
Patalbagh_upper range	504	0.9856	6.3	15
Chandhara	628	0.9854	6.3	18
Patalbagh_lower range	467	0.9678	5.8	17
Samboora	2,050	0.9862	6.4	20
Bengaluru	978	0.8566	4.7	15

et al., 2020). Saffron's core rhizo-bacteriome was enriched with *Bacillus*, *Rhizobium*, *Sphingomonas*, *Agrobacterium*, and *Methylobacterium* which was similar to the core microbiome in rice.



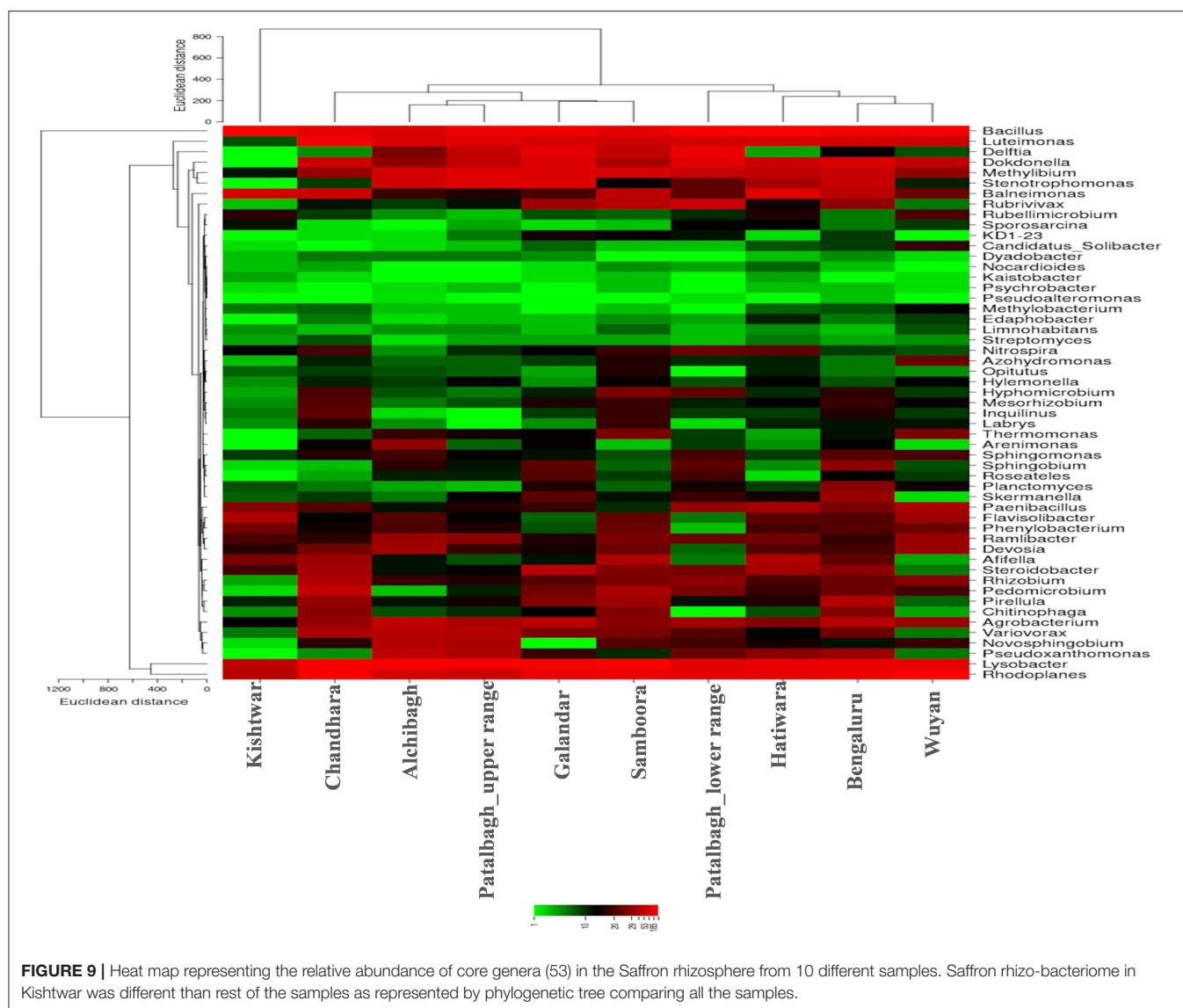
**FIGURE 7** | Rarefaction curve representing the bacterial diversity across 10 samples where in Samboora is more diverse and Galandar is least diverse as represented by the number of species observed as per plot.



**FIGURE 8** | PCoA plots representing the bacterial diversity of Kishtwar is significantly different from rest of sample as it doesn't cluster with rest of the sample.

Out of 53 core rhizosphere bacteria identified in the present study, 19 bacteria were also reported previously from the rhizosphere and cormosphere of saffron grown in Kashmir,

by our group using culture dependent and independent approaches (Ambardar and Vakhlu, 2013; Ambardar, 2014; Ambardar et al., 2014, 2016; Kour et al., 2018; Bhagat et al.,

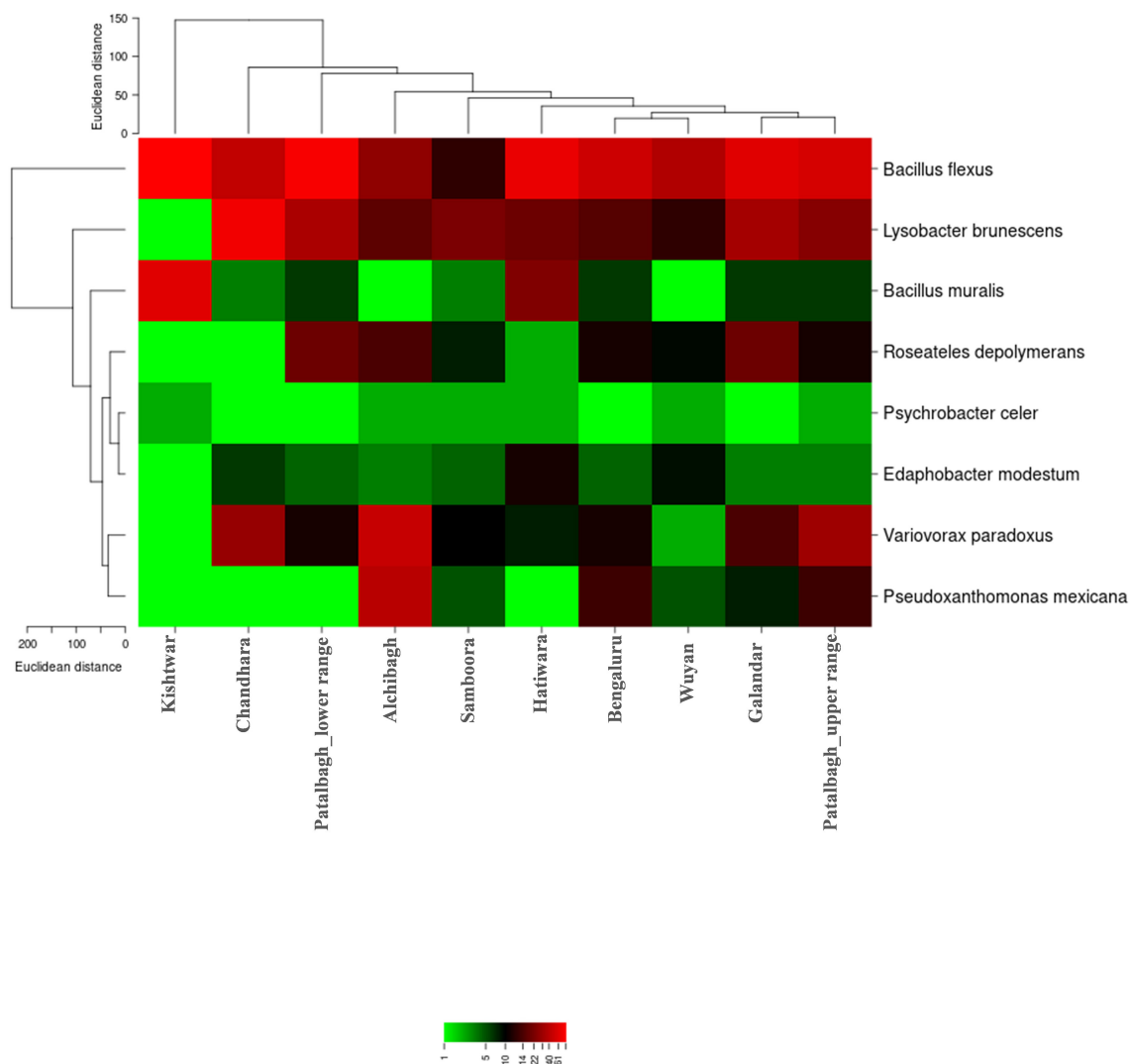


2021). Interestingly, the core rhizo-bacteriome of saffron also harbored some common bacteria as in previously published core cormo-bacteriome. 11 bacterial genera namely *Agrobacterium*, *Chitinophaga*, *Dyadobacter*, *Mesorhizobium*, *Methylobacterium*, *Nocardioideis*, *Opiritutus*, *Rhizobium*, *Sphingomonas*, *Streptomyces* and *Varivorax* were common in both the underground organs of saffron that is corm (Bhagat et al., 2021) and roots. In addition, *Agrobacterium*, *Bacillus*, *Delftia*, *Rhizobium*, and *Variovorax* have also been reported from rhizosphere and cormosphere of saffron, grown in Morocco using cultivation dependent methods (Chamkhi et al., 2018). Though we could not capture the genera reported in Morocco cormo/rhizosphere by earlier used techniques but with the present technique they were captured and found to be present in Kashmir. However, 34 bacterial genera of the core rhizo-bacteriome of saffron grown in India were reported for the first time in the saffron, in the present study.

Eight core bacterial species identified in the present study was also reported from other plants such as *Bacillus flexus* from rice (Roy et al., 2020), *Bacillus muralis* from peanut (Jiang et al., 2016), *Edaphobacter modestum* from *Magnolia grandiflora* (Stone and Jackson, 2016), *Pseudoxanthomonas mexicana* from maize and peanut rhizosphere (Geng et al., 2018; Youseif, 2018), *Roseateles depolymerans* from ginger (Chen et al., 2014), *Variovorax paradoxus* from mustard roots (Belimov et al., 2005). However, *Lysobacter brunescens* and *Psychrobacter celer* has not been reported from any plant so far.

## Plant Growth Promoting Bacteria From Saffron Rhizo-Bacteriome

64.1% of total core rhizo-bacteriome i.e., 34 out of 53 bacteria has been reported as PGPRs from saffron and other plants (Supplementary Table 1). While exploring the core rhizo-bacteriome for PGPR, it was found that 21 bacteria have been



**FIGURE 10 |** Heat map representing the relative abundance of core bacterial species (8) in the Saffron rhizosphere from 10 different samples. Saffron rhizo-bacteriome in Kishtwar was different than rest of the samples as represented by phylogenetic tree comparing all the samples.

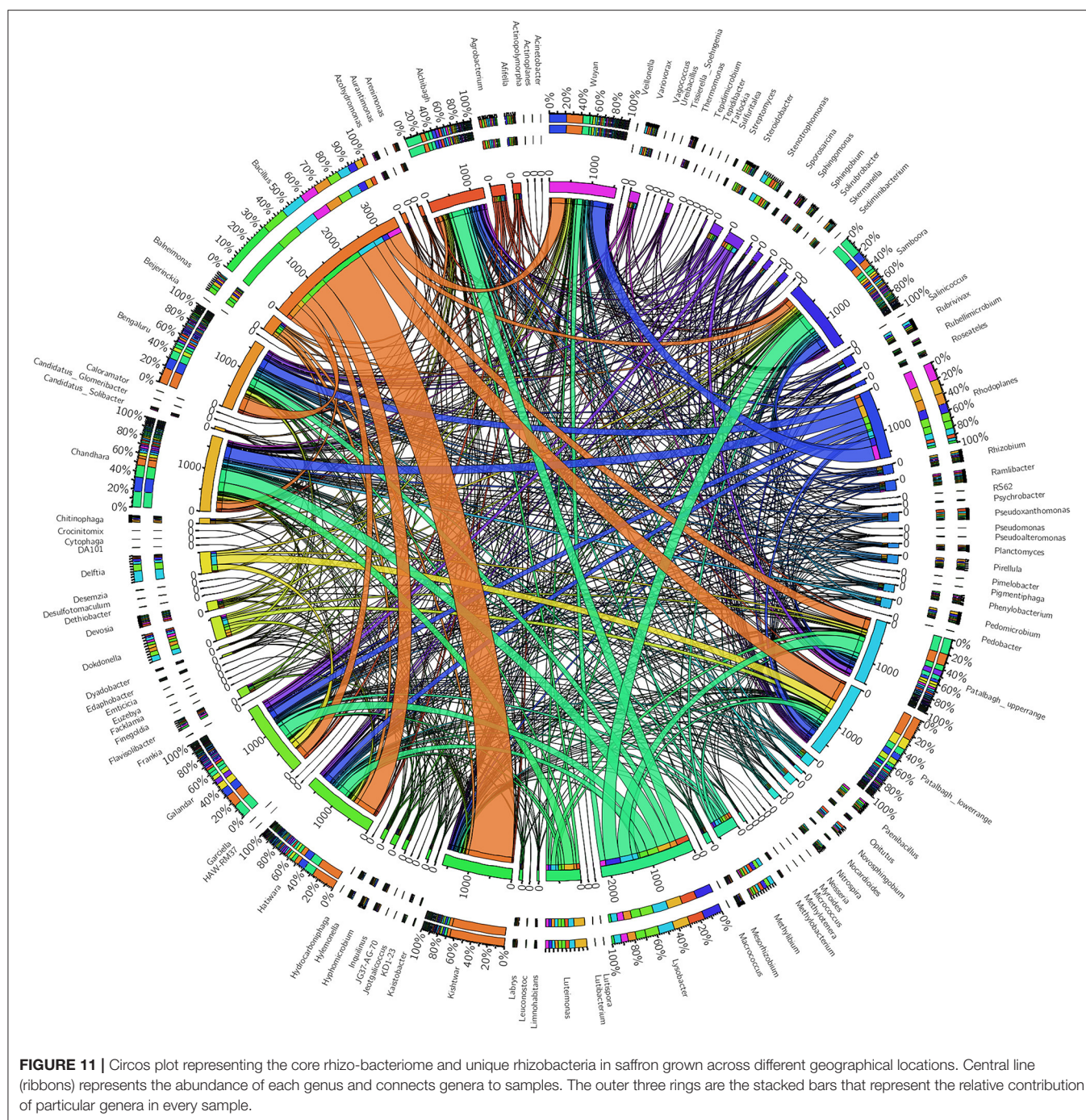
reported as PGPB for the first time from saffron in the present study as previously used methods failed to capture them. The core rhizo-bacteria that are being reported as PGPRs for the first in saffron are *Azospirillum*, *Agromyces*, *Agrobacterium*, *Bradyrhizobium*, *Burkholderia*, *Dokdonella*, *Edaphobacter*, *Flavobacterium*, *Flavisolibacter*, *Frankia*, *Leuconostoc*, *Luteimonas*, *Lysobacter*, *Phenylobacterium*, *Pseudoxanthomonas*, *Mesorhizobium*, *Nocardia*, *Novosphingobium*, *Rhizobium*, *Rubellimicrobium*, *Variovorax* etc.

In addition to core rhizo-bacteriome, saffron rhizo-bacteriomes of each location were enriched with some specific bacteria that were unique to that particular location. An inventory of unique PGPBs of all these bacteria across different locations was made based on the previous reports of PGPBs from saffron and other plants. The location specific unique PGPBs details have been summarized in **Supplementary Table 2**.

Wuyan had the maximum number of unique genera i.e., 19 out of which 5 are reported as PGPB i.e., *Actinoplanes*, *Beijerinckia*, *Desulfotomaculum*, *Dethiobacter* and *Ureibacillus*. In other locations unique number of genera was 4 in Chandhara, 2 in Samboora, 1 in Hatiwara and 1 in patalbagh\_upper range but none of them have been reported as PGPB. Kishtwar had six unique genera and three genera namely *Desemzia*, *Macroccoccus* and *Myroides* have been reported as PGPB (**Supplementary Table 2**). In addition, Alchibagh had 10 unique genera and four have been reported as PGPB. Galandar had six unique genera and three PGPB reported, Patalbagh\_upper had two and Bengaluru has one unique genera, both locations have one PGPB reported (**Supplementary Table 2**).

In the present study, *Bacillus flexus*, *Lysobacter brunescens* and *Janthiobacterium lividium* were the dominant bacterial species identified in saffron rhizo-bacteriome. *Bacillus* genus





dominant in the rhizosphere microbiome is predominantly used as plant growth promoting bacteria, due to their ability to colonize the roots rapidly, competitive colonization potential, production of various phytohormone and conversion of complex nutrients such as phosphorous and nitrogen into simple absorbable forms (Aloo et al., 2019; Enebe and Babalola, 2019; Hashem et al., 2019; Kashyap et al., 2019). *Bacillus flexus* KLBMP 4941 in *Limoneum sinense* (Xiong et al., 2020) and *B. flexus* U8 in rice (Roy et al., 2020) have been reported as PGPR. However, *Lysobacter brunescens* and

*Janthobacterium lividium* have not been reported so far from any plant.

In addition to *Bacillus flexus* other bacterial species of core rhizo-bacteriome reported as PGPR are *Bacillus muralis*, *Pseudoxanthomonas mexicana*, *Roseateles depolymerans* and *Variovorax paradoxus* (Supplementary Table 3). *Bacillus muralis* strain HS4 has been reported as PGPR in peanut (Jiang et al., 2016). *Pseudoxanthomonas Mexicana* has been reported as PGPR from rhizosphere of maize and peanut (Geng et al., 2018; Youseif, 2018). *Roseateles depolymerans* has been isolated as an endophyte

from the seedling stage of ginger and reported to have PGPR properties (Chen et al., 2014) and *Variovorax paradoxus* has been reported as PGPR from Indian mustard roots (Belimov et al., 2005). *Lysobacter brunescens*, *Edaphobacter modestum* and *Psychrobacter celer* have not been reported as PGPB so far (Supplementary Table 3).

Among different locations, Kishtwar had four unique species (*Bacillus marisflavi*, *Macroccoccus caseolyticus*, *Myroides odoratimimus* and *Staphylococcus scui*) and all are reported as PGPB in different plant. In addition, Galandar from Kashmir had only one PGPB (*Paracoccus marcusii*) reported. All other locations have no unique species reported PGPB so far (Supplementary Table 4).

Altogether, 110 bacterial genera (out of total 261) and 21 bacterial species (out of total 73), cataloged in present study, have also been reported to be plant growth promoting bacteria from saffron and other plants (Supplementary Table 5). Plant growth promoting bacterial species cataloged in saffron are *Acidovorax facilis*, *Bacillus cereus*, *Bacillus firmus*, *Bacillus flexus*, *Bacillus horikoshii*, *Bacillus marisflavi*, *Bacillus muralis*, *Brevundimonas diminuta*, *Burkholderia bryophila*, *Burkholderia tuberum*, *Luteibacter rhizovicius*, *Macroccoccus caseolyticus*, *Myroides odoratimimus*, *Paracoccus marcusii*, *Pseudoxanthomonas mexicana*, *Rhizobium leguminosarum*, *Roseateles depolymerans*, *Staphylococcus epidermidis*, *Staphylococcus equorum*, *Staphylococcus sciuri*, *Staphylococcus succinus* and *Variovorax paradoxus*.

On comparison of PGPB from each sample, Wuyan had maximum number of PGPBs (76 out of 154) followed by Galandar (75 out of 132), Alchibagh (73 out of 147), Chandhara (72 out of 143), Hatiwara (73 out of 140), Kishtwar (66 out of 139), Bengaluru (63 out of 143), Samboora (62 out of 124), Patalbagh\_lower (61 out of 111) whereas Patalbagh\_upper (60 out of 122) with the minimum number of PGPBs (Supplementary Table 6). The highest number of PGPB in the Wuyan sample could be correlated to the yield as the production in Wuyan is two times as compared to other fields. In the previous report by our group, PGP *Bacillus amyloliquefaciens* W2 for growth promotion of saffron and corm rot inhibition were isolated from the Wuyan by cultivation based method (Gupta and Vakhlu, 2015). However, in present study a well-reported PGPR *Rhizobium leguminosarum* and *Luteibacter rhizovicius* was relatively abundant in all the nine locations, including Wuyan and completely absent in Kishtwar. *Rhizobium leguminosarum* possess various PGP characteristics such as phosphorous solubilization, siderophore, IAA, HCN production and nitrogen fixation which enhance the growth of host plants in pea plant, lettuce and carrot (Mishra et al., 2009; Tank and Saraf, 2010; Flores-Félix et al., 2013; Gopalakrishnan et al., 2015). *Luteibacter rhizovicius* MIMR1 have been reported as plant growth promoting bacteria that promotes root development in barley (Guglielmetti et al., 2013). Absence of *Rhizobium leguminosarum* and *Luteibacter rhizovicius* may be attributed to the low production status of Kishtwar as compared to Kashmir, but the real confirmation will come only after their evaluation *in-vivo*. The presence of unique location specific PGPB at places other than Wuyan, with a lower yield than

Wuyan indicates that the potential of PGP bacteria varies and maybe all the PGPB are not as effective as that of Wuyan fields. The PGPB cataloge in the present and previous studies in saffron by metagenomic approaches will be captured by culture based technique and evaluated for PGP potential in the future.

## CONCLUSION

The present study investigated the variation in rhizo-bacteriome of saffron grown across different locations i.e., Kashmir, Kishtwar and Bengaluru in India. The rhizo-bacteriome of saffron grown in Kishtwar was found significantly different from the saffron grown in Kashmir and Bengaluru. Interestingly, rhizo-bacteriome in saffron seems to be “plant driven” though in our earlier report we have suggested the bacteriome of corm is location specific. This need to be further confirmed as for cormo-bacteriome corms were directly taken from the fields and analyzed whereas in the present study, corms from Kashmir were grown in the pots with garden soil from Bengaluru and analyzed. Despite growing in non-native soil, the similarity of Bengaluru rhizo-bacteriome to Kashmir rhizo-bacteriome indicates that it is plant driven. This needs to be studied extensively further by analyzing rhizo-bacteriome of saffron roots developed from corms from Kishtwar and Kashmir, cultivated in Bengaluru in non-native soil. Further, the total PGPBs and unique PGPBs were highest in the Wuyan field that has maximum production indicating, a correlation between number of PGPBs and production. However, unique and common PGPB were cataloged across geographical locations in the present study, in the future they will be cultivated by media engineering (for those that are difficult to cultivate) and evaluated *in-vivo* to estimate their actual efficacy.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA687631>.

## AUTHOR CONTRIBUTIONS

SA, JV, and MG have designed the experiments. SA conducted the experiments, analyzed the sequencing data, and experiment regarding growth of saffron plants in Bengaluru. SA and NB wrote the manuscript, analyzed the taxonomy data. MG conceptualized and supervised the study. JV guided in collection of samples from Kashmir and Kishtwar. All authors contributed to the article and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fsufs.2021.644230/full#supplementary-material>

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# Organic Fertilizer Based on *Rhizophagus intraradices*: Valorization in a Farming Environment for Maize in the South, Center and North of Benin

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Maize plays an important role in agricultural production systems in all agro-ecological zones of Benin. Despite its importance, its production faces many constraints including soil fertility. One of the ecological technologies aimed at improving agricultural production is the use of soil microorganisms including arbuscular mycorrhizal fungi (AMF). This study aims to evaluate the effectiveness of *Rhizophagus intraradices*, an indigenous strain, on maize productivity in farmers' areas in the Research and Development (RD) sites of the North (Ouénou), Center (Miniffi), and South (Zouzouvou). Three maize producers were selected at each RD site, for nine maize producers. The experimental design was a randomized complete block of three treatments with three replications. The different treatments were (i) Control–farmer's practice, (ii) *R. intraradices* + 50% of the recommended dose of NPK and urea, and (iii) 100% of the recommended dose of NPK and urea. Soil samples from the different RD sites were taken at a depth of 0–20 cm before sowing for chemical analysis. The different growth parameters (height, crown diameter, and leaf area), grain yield, and endomycorrhizal infection of maize plants were evaluated. The results showed that the soils were moderately acidic ( $5.5 \leq \text{pH}$  waters  $\leq 6.8$ ) and low in organic matter ( $0.95 \leq 33 \text{ OM} \leq 1.17$ ) regardless of the study area. The greater maize grain yield was recorded with application of 100% of the recommended dose of NPK and urea, and *R. intraradices* + 50% of the recommended dose of NPK and urea. In the RD sites at the South, Center, and North recorded with *R. intraradices* + 50% of recommended dose of NPK and urea, the grain yields of 1.9, 3.4, and 1.74 t/ha with an increase of 28, 38.21, and 13.21%, respectively, compared with farmer's practice. Mycorrhization frequencies in plants treated with  $\text{Ri}\frac{1}{2} \text{N}_{15}\text{P}_{15}\text{K}_{15}$  vary between 37.44 and 51.67% in the three zones. The results of the current study have proven the potential use of *R. intraradices* in sustainable intensification of maize production in Benin.

**Keywords:** *Rhizophagus intraradices*, farming environment, ecological systems, yield, maize, Benin

## INTRODUCTION

The decadent productivity of agricultural speculation, in general, and that of maize, in particular, in Benin, a West African country, could be justified by certain constraints including climate change, declining soil fertility illustrated by nitrogen and phosphorus deficiencies (Benbrahim et al., 2004; Balogoun et al., 2014) caused by poor farming practices such as slash-and-burn agriculture. In tropical and subtropical countries, phosphorus is essential because of its natural unavailability in soils (Hailemariam et al., 2013), and nitrogen (N) is a limiting factor for cereal crops (Batamoussi et al., 2014). In this context, the satisfaction of food needs, in general, and maize demands, in particular, will have to be based on productivity improvement. Thus, producers resort to intensive use of inputs, especially mineral fertilizers, which are leached and easily diluted into rivers, lakes, and streams with adverse consequences on the environment and the health of humans and animals. According to Alalaoui (2007), the prolonged use of mineral fertilizers without organic inputs leads to the depletion of soil organic matter, which is more sensitive to wind and rain erosion.

For these reasons, it is imperative to have an understanding of the processes underlying the bioavailability of soil nutrients to plants, as well as the soil–root interactions of microorganisms (Eisenhauer, 2017; Bi et al., 2018). One of the oldest and most widespread mutualistic associations of microorganisms concern symbiosis in which a particular soil fungi called arbuscular mycorrhizal fungi (AMF) colonize the roots of most (74%) of the terrestrial plant families (van der Heijden et al., 2015). These fungi belong to the family of glomeromycetes, which includes at least 313 characterized species. AMF are key elements of soil fertility (Bedini et al., 2018). Several examples suggest the use of AMF for the promotion of plant performance (growth, survival, and tolerance) because they improve nutrition (water and minerals), photosynthesis, protection against biotic and abiotic stresses, regulation of development processes (flowering, fruit formation, rooting, etc.), (Bedini et al., 2018), and participate in soil structuring (Alqarawi et al., 2014). However, the wide use of mycorrhizal inocula in agriculture remains a challenge due to their cost, variability, quality, and effects on the plant such as their incompatibility with high levels of phosphorus (P) in the soil (Usharani et al., 2014; Berruti et al., 2016).

However, knowledge and understanding of the mechanisms that govern the functioning of these AMF communities, particularly in poor tropical agrosystems where sustainable management of generally low soil nutrient resources, must take into account the benefits of indigenous microorganisms. Fortunately, with the advent of molecular biology, considerable advances in AMF identification and nomenclature have been noted (Oehl et al., 2011).

Although the importance of intraspecific plant diversity and AMF for ecosystem functioning has often been highlighted (Wall et al., 2015), the interactive influences on their respective and reciprocal performance are still not well-understood (Sendek et al., 2019).

Although work on AMF diversity and use is not legion in Benin (Tchabi et al., 2008; Balogoun et al., 2014), it is,

nonetheless, oriented toward speculation other than maize in small geographical study areas and has mainly concerned exotic strains of AMF. However, Benin has indigenous strains of AMF in regions characterized by different types of climate and soils from North to South through the center and which could each present significant specificity to be taken into account in the analyses. The objective of this study is to evaluate the effect of the fungus *Rhizophagus intraradices* on maize growth and yield at three Research and Development (RD) sites in Benin.

## MATERIALS AND METHODS

### Materials

The maize variety 2,000 SYNEE-W was used during the experimentation at the different sites where the trials were carried out. It is an extra-early variety, 75 days old, developed by the International Institute of Tropical Agriculture (IITA) and the Institut National des Recherches Agricoles du Bénin (INRAB). It is a variety that presents a good resistance to the rot of the stem, to the maize streak virus (MSV) of the *Mastrevirus* genus, to the American rust, to the helminthosporiose caused by the *Cochliobolus heterostrophus* fungus. In addition, it tolerates drought (MAEP, 2016).

The mycorrhizal inoculum of the *Rhizophagus intraradices* species used was isolated, identified, and characterized on the basis of morphological criteria (diameter, color, ornamentation, thickness of the spore wall) from the rhizosphere of maize soils in different agro-ecological zones of Benin (Aguégué et al., 2021). This mycorrhizal fungus was preserved at the Laboratory of Biology and Molecular Typing in Microbiology of Faculty of Sciences and Techniques of University of Abomey-Calavi (FAST/UAC). The inocula were produced and multiplied by associating spores of *Rhizophagus intraradices* with sorghum seedlings. Sorghum seeds were disinfected in a bleach solution (5%), then rinsed and soaked in sterile distilled water for 24 h. Sorghum plants were grown in glasshouses in pots containing sterilized substrate consisting of a mixture of clay and peat (2:1 v/v), for 4 months to ensure good sporulation of the strains. After 4 months of cultivation, the inoculum, consisting of spores and root fragment mixture, was collected (Rivera et al., 2003).

### Study Area

The tests were installed on Research and Development sites respectively in the South at Zouzouvou (Commune of Djakotomey), in the Center at Miniffi (Commune of Dassa) and in the North at Ouénou (Commune of N'Dali), (Figure 1). In each of the zones, the trials were set up at three (3) different producers. The choice of sites was made taking into account the fact that they are the sites of Research and Development programs and that declining soil fertility is a priority constraint. The sites are flat with a maximum 2% slope and are not flooded.

### Determination of Soil Chemical Parameters

Soil samples were taken at a depth of 0–20 cm (Adjanohoun et al., 2011) at various sites in the South (Zouzouvou), Center (Miniffi), and North (Ouénou). A 500-g composite sample was collected at each site prior to the installation of the experimental

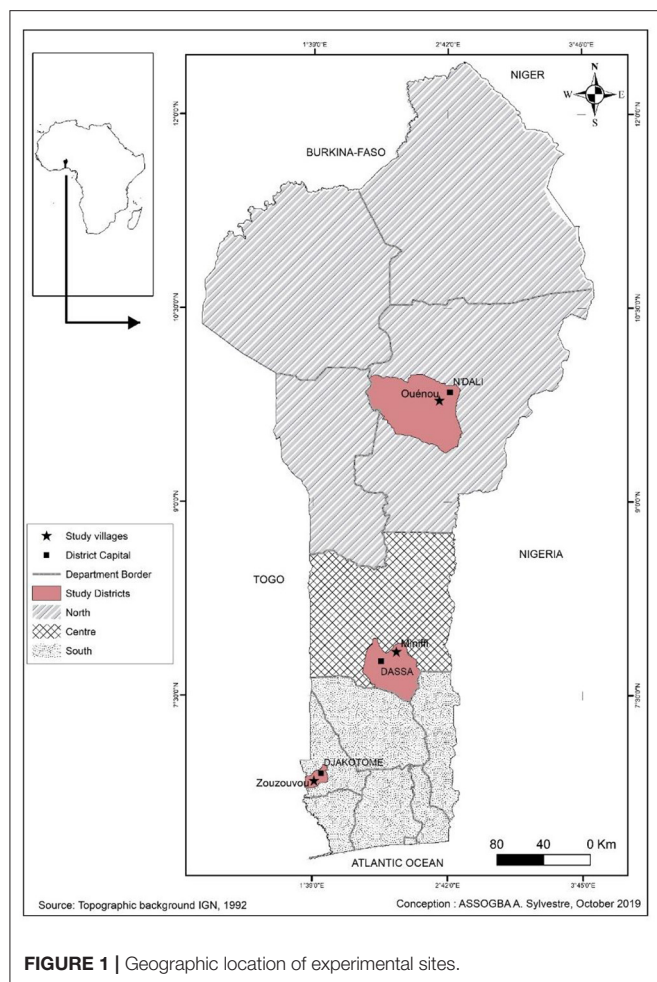


FIGURE 1 | Geographic location of experimental sites.

device. At each site, a mixture of soil samples was collected using an auger at a depth of 0–20 cm. Five (05) sampling points were randomly selected. Four (04) of the five (5) sampling points are each located on the four cardinal points (North–South–West–East). The fifth sampling point is located approximately at the junction of the four (04) preceding points. These samples were sent to the Laboratoire des Sciences du Sol, Eau et Environnement (LSSEE) of the Institut National des Recherches Agricoles du Bénin (INRAB). These analyses consisted of the determination of pH (water), (by glass electrode in a soil/water ratio of 1/2.5), organic matter and carbon (Walkley and Black, 1934), assimilable phosphorus (Bray and Kurtz, 1945), total nitrogen (Kjedahl, 1883), and exchangeable bases by the method of Metson (1957) with ammonium acetate at a pH equal to 7.

## Experimental Device

The plowing was done at a depth of 20 cm using a hoe on each site. Each 12.8 m<sup>2</sup> (4 × 3.2 m) elementary plot had four lines of 4-m long. The trials were installed at three growers on each site. At each grower, the experimental design used was a completely randomized block of three treatments with three replicates. Each treatment had, thus, covered three elementary plots (1 plot × 3

replicates) separated by alleys 1-m wide. The treatments were defined as follows:

- T1: Farmer's practice (control);
- T2: Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (*R. intraradices* + 50% of the recommended dose of NPK and urea);
- T3: N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (100% of the recommended dose of NPK and urea).

Farmer's practice technique in the present study is characterized by the application of the recommended dose of mineral fertilizer N<sub>15</sub>P<sub>15</sub>K<sub>15</sub> on the 15th day after sowing and urea on the 45th day after sowing. However, for treatments T2 and T3, the mineral fertilizer N<sub>15</sub>P<sub>15</sub>K<sub>15</sub> was applied on the day of sowing, and urea was applied on the 45th day after sowing. The recommended rate of mineral fertilizer for the maize crop used in this study is 200 kg ha<sup>-1</sup> of N<sub>15</sub>P<sub>15</sub>K<sub>15</sub> and 100 kg ha<sup>-1</sup> of urea (46% N).

## Sowing and Inoculation of Maize Seeds

Before sowing, the maize seeds were coated with the inoculum of *Rhizophagus intraradices*. Seed coating was done according to the methodology described by Fernandez et al. (2000). A 1 kg of the inoculum was mixed with 600 ml of distilled water to obtain a paste to which 10 kg of seeds were added for mixing. The coated seeds were dried at room temperature for 12 h. Sowing was done in plots about 5-cm deep at a spacing of 0.80 × 0.40 m, i.e., a density of 31,250 plants/ha (Hernandez et al., 1995). On the day of sowing, a bottom manuring at the rate of 200 kg/ha of fertilizer (N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>) was applied in accordance with the experimental protocol. Then, on the 40<sup>th</sup> day after sowing (DAS), maintenance manure consisting of urea (40% N) was applied in accordance with the experimental design. Data for the various growth, yield, and mycorrhization parameters were collected on the two central lines of the 6.4-m<sup>2</sup> working plot at each site.

## Evaluation of Growth Parameters

The height of the maize plants and the diameter at the collar of the plants were collected on 12 plants from the two central lines of each elementary plot, every 15 days from DAS until the 60th DAS on the different RD sites. The height of a maize plant was measured with a graduated ruler, the diameter was measured with a caliper at the plant collar, and the leaf area was estimated at the 60th DAS by the product of the length and width of the leaves multiplied by 0.75 (Ruget et al., 1996).

## Evaluation of Grain Yield

Corn grain yield was assessed at harvest (80th DAS). Corn cobs were harvested, dispatched, and shelled per plant and per elementary plot. Grain moisture percentage was determined using a moisture meter (LDS-1F). The average grain yield values of the maize plants were determined by Equation 1 used by Ferro Valdés et al. (2013):

$$R = \frac{P \times 10.000}{S \times 1.000} \times \frac{14}{\% H} \quad (1)$$

Where,

$R$  = the average maize grain yield in t ha<sup>-1</sup>;



**TABLE 1** | Chemical characteristics of the study soil.

Characteristics	pH <sub>(water)</sub>	OM (%)	P-ass (ppm)	C/N	Sum of exchangeable bases (meq/100 g)	CEC (meq/100 g)
Miniffi	6.20	1.16	11.75	13.33	7.84	8
Ouénou	5.54	0.95	6.91	6.88	1.82	4.64
Zouzouvou	5.6	1.17	2	10.75	2.47	6.5

pH<sub>(water)</sub>; OM, organic matter; P-ass, assimilable phosphorus; C, carbon; N, Nitrogen; CEC, cation exchange capacity.

**TABLE 2** | ANOVA results of height and collar diameter.

Sources of variation	Height		Collar diameter	
	F-value	p-value	F-value	p-value
(Intercept)	334.5508	<0.0001	1,148.9774	<0.0001
Time	<b>140.5530</b>	<b>&lt;0.0001</b>	<b>128.8457</b>	<b>&lt;0.0001</b>
Treatment	0.1180	0.8888	<b>74.4053</b>	<b>&lt;0.0001</b>
Areas	<b>56.2697</b>	<b>&lt;0.0001</b>	<b>4.3320</b>	<b>0.0161</b>
Time:treatment	1.3592	0.2622	0.2867	0.7514
Time:areas	<b>22.5223</b>	<b>&lt;0.0001</b>	<b>4.0523</b>	<b>0.0207</b>
Treatment:areas	1.5527	0.1941	<b>20.1609</b>	<b>&lt;0.0001</b>
Time:treatment:areas	0.5470	0.7017	0.7745	0.5447

The bold values show the significance level of the different variables under study.

P = the maize grain weight in kilograms (kg);

S = the harvest area in m<sup>2</sup>, and

% H = the grain moisture content in %.

## Evaluation of Endo Mycorrhizal Infection of Maize Plant Roots

Corn root samples were collected at the 80th DAS. After staining with trypan blue according to the method described by Phillips and Hayman (1970), arbuscular mycorrhizal fungi associated with maize plant roots were observed with binoculars (XSP-BM-2CEA. 2013). Estimation of mycorrhizal infection of roots was carried out according to the intersection method described by Giovanetti and Mosse (1980). The rate of mycorrhization was estimated by two parameters of arbuscular mycorrhizal infection described by Trouvelot et al. (1986), namely: (i) mycorrhization frequency (F), which reflects the degree of infection of the root system, and (ii) mycorrhization intensity or absolute mycorrhization intensity (m), which expresses the portion of the colonized cortex in relation to the entire root system, calculated according to Equations 2, 3, respectively.

$$F(\%) = \frac{(N - n_o)}{N} \quad (2)$$

where

N is the number of fragments observed and

n<sub>o</sub> is the number of fragments with no trace of mycorrhization;

$$m(\%) = \frac{95n_5 + 70n_4 + 30n_3 + 5n_2 + n_1}{N - n_o} \quad (3)$$

In Equation 3, n<sub>5</sub>, n<sub>4</sub>, n<sub>3</sub>, n<sub>2</sub>, and n<sub>1</sub> are the numbers of fragments, respectively, noted in the five classes of infection marking the importance of mycorrhization, namely, 5: more than 95%; 4: from 50 to 95%; 3: 30 to 50%; 2: 1 to 30%; 1: 1% of the cortex.

## Statistical Analysis

Data per site for all parameters assessed were collected. Mixed-effect linear models on longitudinal data were fitted to evaluate the effect of treatments and area on plant growth parameters (height and crown diameter). In each model, treatments and zones were considered fixed factors, and time was considered random. Adjusted averages were also calculated to represent trends in each growth parameter at the treatment and zone level. These analyses were performed using the nlme (for model fitting), and it means (for the calculation of adjusted averages) packages. Descriptive statistics were calculated for each growth parameter measured.

In order to assess the effect of treatments and zone on yield and leaf area, it was evaluated using a two-criteria analysis of variance (treatment and zone). The Shapiro-Wilk and Levene tests (Glèlè Kakaï et al., 2006) were performed to verify the conditions of normality and homoscedasticity of the data required for ANOVA. As the experimental design was balanced, the ANOVA type II test was adopted. Once the ANOVA test was significant, a pairwise comparison *post-hoc* test using the Tukey's *post-hoc* test (Douglas and Fligner, 1991) was performed to assess the statistical differences in the means. In addition, descriptive statistics were calculated for each measured parameter. These analyses required the use of the dplyr and DescTools packages for the calculation of descriptive statistics, the ggplot2, and ggpubr packages for the mustache boxes, the stats package for the Shapiro test and Levene test, the car package for the ANOVA, and the multcomp package for the pairwise comparison *post-hoc* test.

The significance threshold retained is 5%, and all the different tests were performed in R 4.0.2 software (R Core Team, 2020).

## RESULTS

### Chemical Characteristics of the Soil

The soil chemistry characteristics of the R&D Sites are presented in **Table 1**. The soil water pH in Zouzouvou (pH = 5.6), Ouénou (pH = 5.5), and Minifi (pH = 6.2) is acidic. Organic matter varies between 0.95 and 1.17%, while assimilable phosphorus has a respective value of 2 ppm at Zouzouvou, 11.7 ppm at Minifi,

and 6.9 ppm at Ouénou. Exchangeable bases vary between 1.82 and 7.84 meq/100 g of soil.

### Effect of the Mycorrhizal Fungus *Rhizophagus intradices* on the Growth of Maize Seedlings

The results of the analysis of variance show that the height variations observed at the plant level depend only on the area ( $p$ -value < 0.0001) and over time (Table 2).

The evolution of these average plant heights over time and by treatment are presented graphically by zone (Figure 2). From the figure, it appears that whatever the zone, the growth in height of the plants reaches its maximum around 60 days after sowing. The highest maize plant heights were observed in the south regardless of treatment. However, in the Center and North, the best heights were observed, respectively, at the level of plants treated with Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2) and N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3), (Figure 2).

The results of the analysis of variance show that the variations in collar diameter observed at the plant level depend on the treatments ( $p$ -value < 0.001) and on the zone ( $p$ -value = 0.016). However, these variations over time do not depend on the treatments ( $p$ -value = 0.751) but rather on the zone ( $p$ -value < 0.001), (Table 2).

The curves of the evolution of the diameter at the collar of the plants over time and by treatment show these variations by zone (Figure 3). Thus, in general, and whatever the zone, the growth in diameter of the plants reaches its maximum around 60 days after sowing. The treatment Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2) followed by N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) induced the largest collar diameters (Figure 3) in the Center, while the largest collar diameter values were recorded with treatments Farmer's practice (T1) and N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) in the South and North, respectively.

From the analysis in Figure 4, it appears that the plants in the southern zone perform best for most treatments. The results of the analysis of variance show a significant difference in the effects of the interaction between treatment and zone (Df = 8,  $p$ -value = <0.001) on the leaf area of the plants. It can be deduced that the variation in plant leaf area per treatment depends on the experimental area. The Tuckey's test performed (Figure 4) shows the difference in performance between the treatments according to each zone. Thus, the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) in the South zone gives the best performance. Next comes the treatment Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2) and treatment Farmer's practice (T1) in the southern zone, followed by the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) in the northern zone. Treatments Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2), and Farmer's practice (T1) in the northern zone have similar performances as the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) in the central zone. The treatment Farmer's practice (T1) in the central zone, gives a low performance.

### Effect of the Mycorrhizal Fungus *Rhizophagus intradices* on Maize Grain Yield

The analysis in Figure 5 shows that the plants in the Southern and Central zone perform best for most treatments. The results of the analysis of variance show a significant difference in the

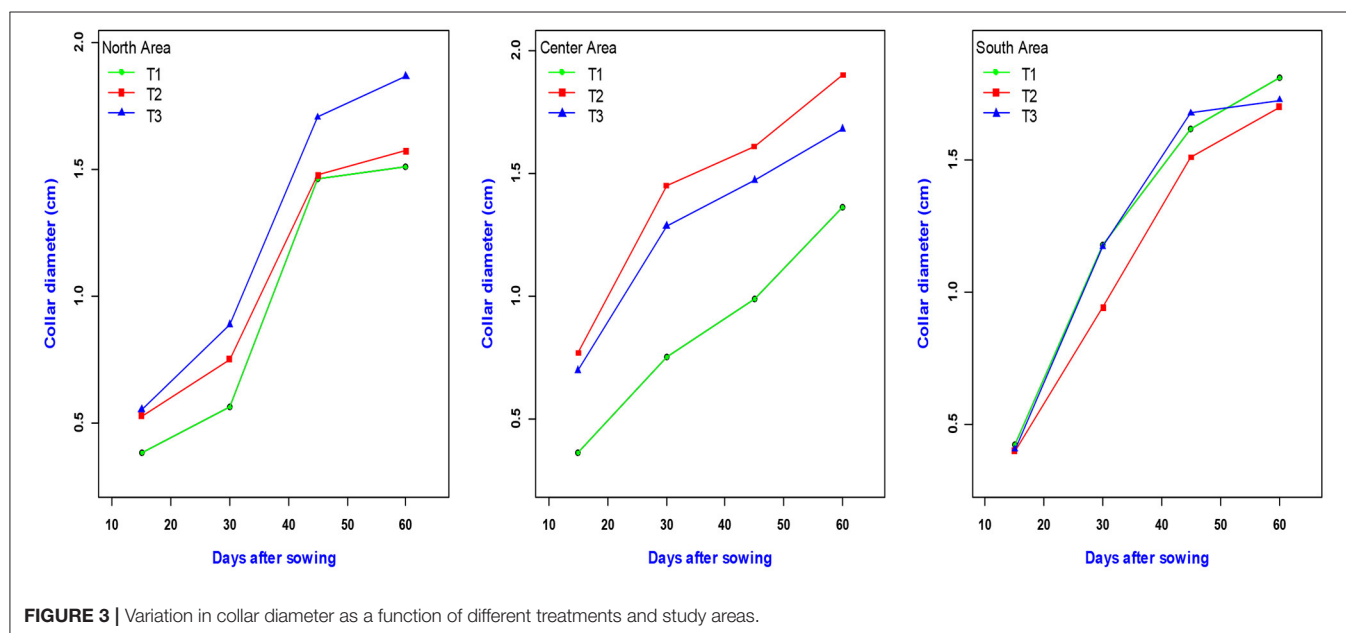
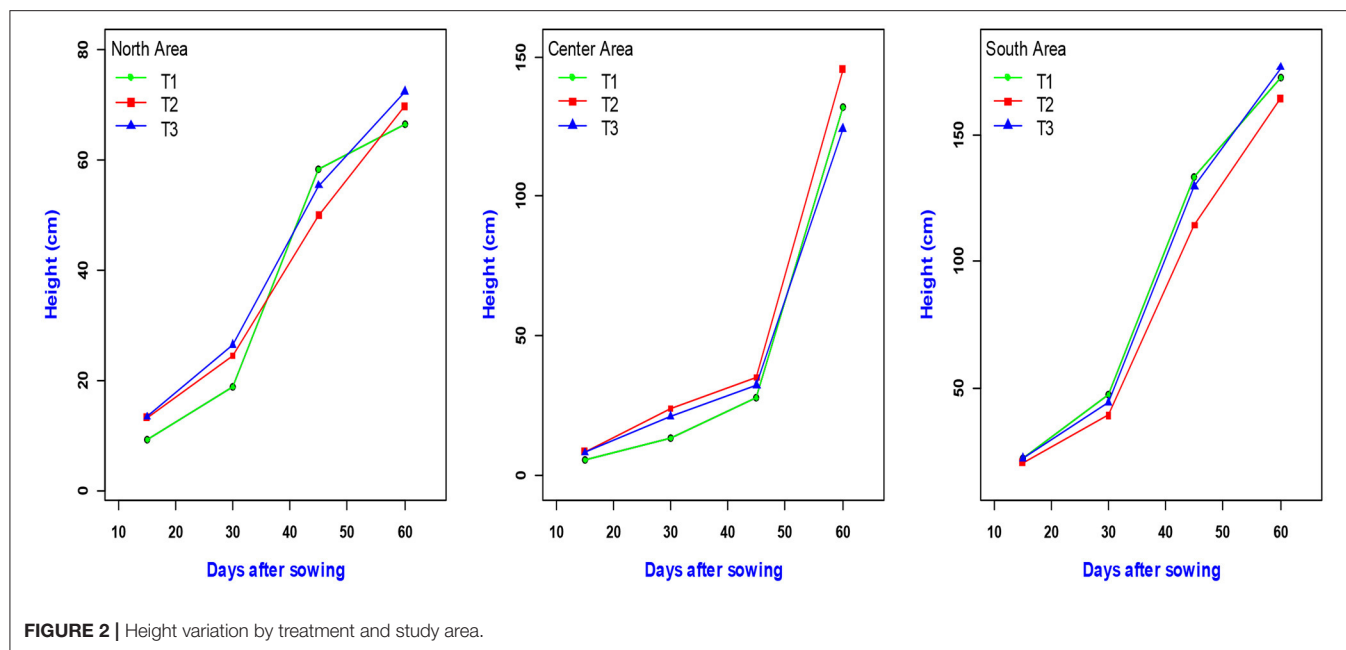
effects of the interaction between treatment and zone (Df = 8,  $p$ -value = <0.001) on plant grain yield. This suggests that the variation in plant grain yield per treatment depends on the experimental area. The Tuckey's test performed (Figure 5) shows the difference in performance between the treatments according to each zone. Thus, the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3) in the South and Central zone gives the best performance (3.3 t ha<sup>-1</sup>). Next comes the treatment Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2), (2.8 t ha<sup>-1</sup>) and the treatment Farmer's practice (T1) in the South and Central zones. The treatment Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2) in the North zone has similar performances as the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3). Of all the zones, the treatment Farmer's practice (T1) in the North zone gives the lowest performance (1.6 t ha<sup>-1</sup>).

### Effect of the Mycorrhizal Fungus *Rhizophagus intradices* on the Mycorrhization of Maize Plant Roots

Mycorrhization frequencies in plants treated with the treatment Ri½ N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T2) vary between 37.44 and 51.67% in the three zones (Figure 6). Mycorrhization intensity varies between 6.19 and 27.02%. It should be noted, however, that we did not observe mycorrhization at the root level of the plants treated with the treatment Farmer's practice (T1) and the plants treated with the treatment N<sub>15</sub>P<sub>15</sub>K<sub>15</sub>\_Urea (T3). The results of the analysis of variance of mycorrhization intensity and frequency in the three study areas revealed a significant difference ( $p$  < 0.001) in mycorrhization intensity between the study areas, while the frequency of mycorrhization was similar in the different study areas. The contact of the line with a given arc represents the mean value, while the horizontal line indicates the median. Thus, the high intensity of mycorrhization is observed in the South of Benin. The intensities of mycorrhization in the Center and in the North are similar.

## DISCUSSION

The chemical characteristics of our study soils show that organic matter varies between 0.95 and 1.17%, while assimilable phosphorus had a respective value of 2 ppm in the South, 47.5 ppm in the Center, and 6.7 ppm in the North. Exchangeable bases varied between 1.82 and 7.84 meq/100 g of soil. The chemical characterizations show that the experimental soils in the South, Center, and North were acidic and moderately poor (Sys et al., 1993). Moreover, soil contents in organic matter, phosphorus, and exchangeable bases are good for an expression of the effects of NPK mineral fertilizers (Igué et al., 2015). The water pH of the study soils varies between 5.5 and 6.20, which shows that our study soils are acidic. According to Davet (1996), AMF are preponderant in acidic soils. The pH influences the activity of soil microorganisms that participate in the mineralization of organic matter as well as that of mycorrhizal fungi (Parent and Gagné, 2010). Coughlan et al. (2000) demonstrated a positive correlation of pH with the quality and quantity of intra-root colonization. They stated that mycorrhizal colonization is high at pH levels between 5 and 7, but low at pH levels around 4,

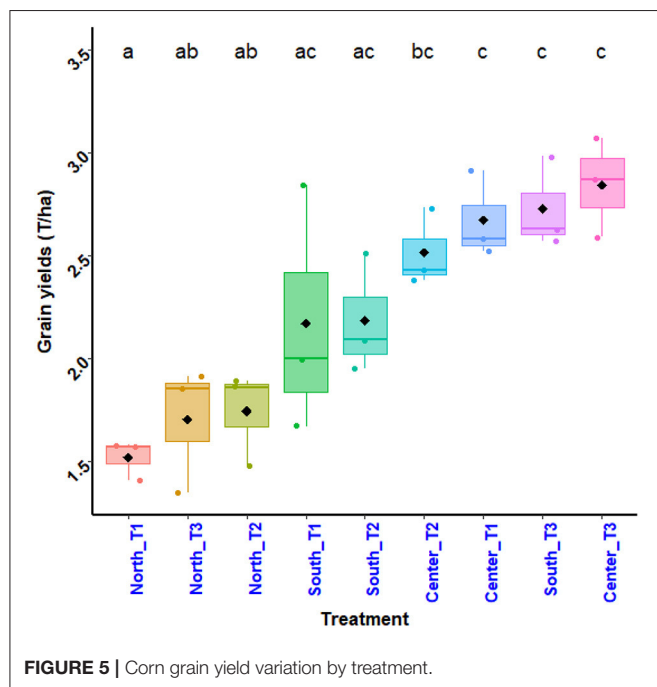
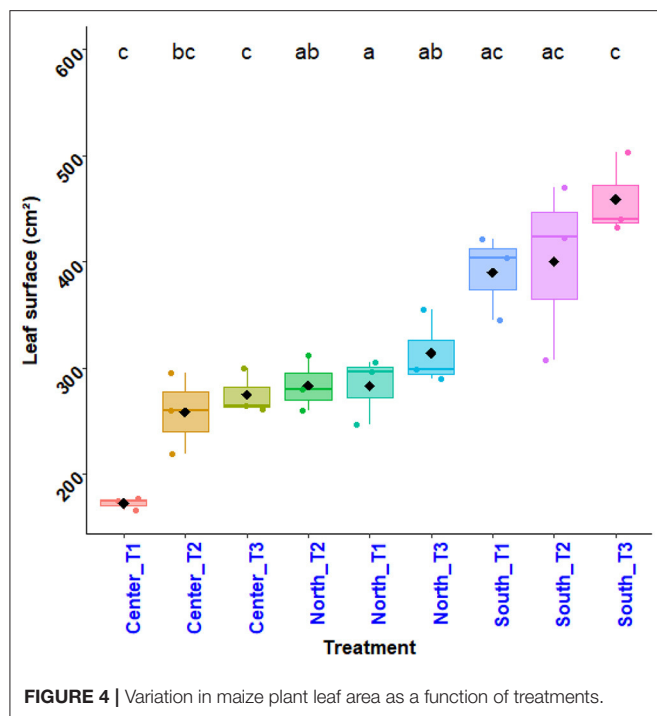


as the adaptation of fungi to different pH levels varies between species. In soils with pH between 7 and 7.6 *R. intraradices* colonizes more the roots of plants to stimulate their growth.

The contribution of arbuscular mycorrhizal fungi had a significant effect on maize plant growth. Indeed, the application of  $Ri\frac{1}{2} N_{15}P_{15}K_{15}$ \_Urea induced better growth of maize plants compared with the Farmer's practice and those receiving  $N_{15}P_{15}K_{15}$ \_Urea in Southern Benin, while in Central and Northern Benin, it was the  $Ri\frac{1}{2} N_{15}P_{15}K_{15}$ \_Urea and  $N_{15}P_{15}K_{15}$ \_Urea treatments that generated the greatest growth variables. These results are in agreement with those obtained by

Ndoye et al. (2016) in Senegal who revealed that inoculation with arbuscular mycorrhizal fungi (*Glomus manihotis*) significantly improved the growth (+28.5%) of fonio (*Digitaria exilis* Stapf) under semi-controlled conditions. Also, (Sánchez-Roque et al., 2016) also observed a positive effect of AMF inoculation on three pepper varieties.

As for corn grain yield, the results of the analysis of the means revealed a significant difference.  $Ri\frac{1}{2} N_{15}P_{15}K_{15}$ \_Urea had generated the highest yields in the Center, while  $N_{15}P_{15}K_{15}$ \_Urea had induced the highest yields in the South. However, there was no significant difference between the three treatments in the North with low performance. Indeed, grain yields



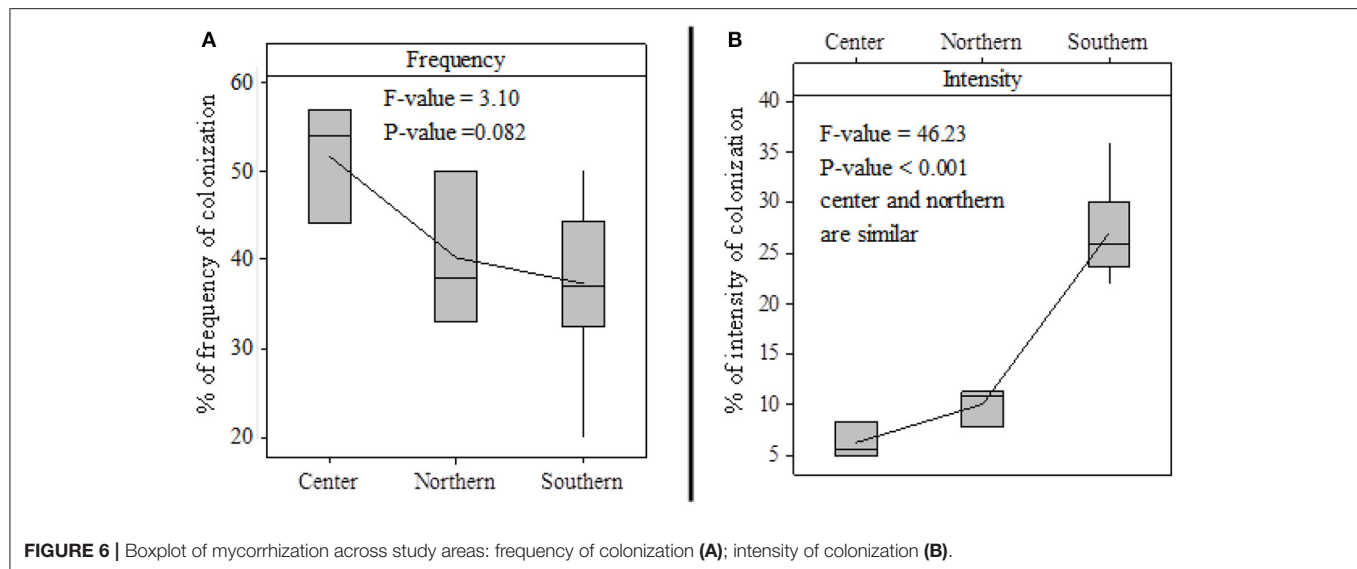
obtained in the South was  $1.95 \text{ t ha}^{-1}$  with the contribution of  $\text{Ri}\frac{1}{2} \text{ N}_{15}\text{P}_{15}\text{K}_{15}\text{-Urea}$ . These grain yield values are 28% higher than the grain yield obtained from the plants treated with Farmers' practices (without AMF, with  $\text{N}_{15}\text{P}_{15}\text{K}_{15}\text{-Urea}$ ). Plants that received  $\text{N}_{15}\text{P}_{15}\text{K}_{15}\text{-Urea}$  in Central Benin had an average yield of  $2.5 \text{ t ha}^{-1}$ , and those that received

$\text{Ri}\frac{1}{2} \text{ N}_{15}\text{P}_{15}\text{K}_{15}\text{-Urea}$  yielded around  $3.4 \text{ t ha}^{-1}$ . In North Benin, there was no significant difference between the three treatments. These differences in yield are due to several factors such as the functional diversity of AMF and environmental conditions as so well-noted by (Walder and Van Der Heijden, 2015).

Grain yield was higher on ferruginous soil in the Center compared with yields in the North and South. This is explained by a good level of assimilable phosphorus ( $11.75 \text{ ppm}$ ), which was higher than in the ferruginous soils of the North ( $6.91 \text{ ppm}$ ) and ferralitic soils of the South ( $2 \text{ ppm}$ ) in this study. In addition, the soils of Central Benin have an organic matter rate of  $1.16\%$  and a pH in water ( $6.2$ ) allowing a good expression of *R. intraradices*, which shows a good colonization of plant roots in soils with a pH between  $7$  and  $7.6$ . Rivera et al. (2003) in Cuba, Assogba et al. (2017), and Aguégué et al. (2021) in Benin also observed a  $35\text{--}50\%$  increase in maize yields compared with the control (without AMF or mineral fertilizers) following the application of  $\text{N}_{15}\text{P}_{15}\text{K}_{15}\text{-Urea}$ , which created a commercial strain. It should be noted that other factors such as temperature and pest attacks may explain the differences in yield observed from one area to another. According to the work of Hasanuzzaman et al. (2013), high temperatures in the northern zone of the country affected plant growth and development through mechanisms described by delayed growth rate, drop in biomass production, leaf and reproductive organ burning, leaf abscission and senescence, fruit damage and, in turn, yield reduction and cell death. Through symbiosis, AMF established mycorrhiza with their hosts (Nadeem et al., 2014; Zhang et al., 2017) and reduced drought-related consequences (Yooyongwech et al., 2016; Moradtalab et al., 2019). Thus, hosts benefited more often from increased access to nutrients with improved growth and yield (Hart et al., 2014; Liu et al., 2016; Chen et al., 2017). Also, the release of nitrogen from mineral fertilizers increased yield and its components (Torbert et al., 2001; Nyembo et al., 2012). Bakonyi and Csitári (2018) made the same observations, showing that AMF inoculation increased wheat grain yield from  $7.52$  to  $8.17 \text{ t ha}^{-1}$  in the same way as mineral fertilization ( $7.38$  to  $8.31 \text{ t ha}^{-1}$ ).

The frequency of mycorrhization of maize roots in this study was low. Root colonization of maize plants ranged from  $6.19$  to  $27.02\%$ . These values are low in comparison with the work of Rivera et al. (2003) and Tian et al. (2013), which showed a  $76\text{--}80\%$  colonization of maize roots as a result of the combination of arbuscular mycorrhizal fungi with the recommended half-dose of mineral fertilizer during and after moderate states of hydric stress. Ndoye et al. (2016) observed the highest mycorrhization frequencies and intensities of fonio root mycorrhization with *G. aggregatum* and *R. irregularis*. On the other hand, Incesu et al. (2015) observed higher rates of root colonization of *Diospyros virginiana* with *R. irregularis* and *G. caledonium* compared with other AMF species (*G. etunicatum*, *Funneliformis mosseae*, and *G. clarium*). However, it is important to note that above  $12\%$  mycorrhization intensity, the benefits derived by the plant symbiote are not negligible (Diagne et al., 2013).





## CONCLUSION

The results of this study showed that  $Ri\frac{1}{2} N_{15}P_{15}K_{15}$ -Urea had a positive impact on all the variables of grain growth and yield while reducing by half the use of mineral fertilizers at the different Research and Development Sites of Ouénou, Miniffi, and Zouzouvou. These results show that mycorrhization of maize could play an important role in improving the growth and yield of maize plants and, thus, contribute to the development of methods that are environmentally friendly and guarantee sustainable agriculture in Benin. Further work is needed to better understand the behavior of AMF on maize growth and productivity, and soil fertility management in a large number of producers in order to make recommendations on the use of these fungus-based biofertilizers.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

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## AUTHOR CONTRIBUTIONS

This work was carried out in collaboration with all authors. RA, SA, HSa, AK, NAg, and OA conducted the trial set-up, data collection, and harvesting. RA wrote the first draft of the manuscript and managed the bibliographical research. KS with RGK performed the statistical analysis. NAh, HSi, GD, AA, and LB-M wrote the protocol, managed the study analyses, and supervised the various activities. All authors read and approved the final manuscript.

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