



PLANT ROOT INTERACTION WITH ASSOCIATED MICROBIOMES TO IMPROVE PLANT RESILIENCY AND CROP BIODIVERSITY

EDITED BY: Nikolay Vassilev, Eligio Malusà, Davide Neri and Xiangming Xu
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PLANT ROOT INTERACTION WITH ASSOCIATED MICROBIOMES TO IMPROVE PLANT RESILIENCY AND CROP BIODIVERSITY

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Editorial: Plant Root Interaction With Associated Microbiomes to Improve Plant Resiliency and Crop Biodiversity

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Plant Root Interaction With Associated Microbiomes to Improve Plant Resiliency and Crop Biodiversity

Plant beneficial microorganisms are now accepted as potential alternatives to chemical fertilizers and pesticides in agriculture. However, despite the enormous research efforts, there is still much to be learnt about the underlying processes that affect the efficacy of biocontrol and biofertilizer products in crop systems. A deeper ecological understanding of complex interactions among introduced beneficial strains and resident microbiota under changing environment is essential (Xu and Jeger, 2020). These complex interactions are greatly influenced by the formulation of beneficial microbial strains (Vassilev and de Oliveira Mendes, 2018) as well as the growing substrate characteristics (Ponzio et al., 2013) and commercial agricultural practices (Malusà et al., 2016). This Research Topic presents 33 articles that describe the recent research progresses on the exploitation of beneficial microbes in agriculture.

The influence of management practices on plant-associated and soil microbiome was explored on a number of crop species, including pea and tomato. Accumulation of several fungal root pathogens, reduction of specific potential beneficial bacteria, and gradual decrease of soil pH value in soils are identified as possible causes contributing to reduced productivities of American ginseng associated with continuous monocropping (Zhang et al.). Removal of pruned branches from tea orchards can prevent accumulation of allelochemicals and avoid soil health issues in monocropping through the changes in the soil microbiota as influenced by pruning wastes on the orchard ground (Arafat et al.). Fungal communities in growing substrate were greatly affected by crop rotation of tomato with cabbage, bean, or celery in greenhouse (Lyu et al.). Soil management, crop residue management and tillage treatments all affected the bacterial community structure in both bulk soil and pea rhizosphere, but the plant itself was the dominating factor shaping the bacterial communities (Chaudhari et al.).

Plant genotypes have the ability of driving the composition of microbial species in their rhizosphere. Thus, different blueberry species, although sharing a common core rhizobiome, considerably shaped rhizosphere community structures of both prokaryotic and eukaryotic microorganisms (Li et al.). In addition to specific genotypes, phenotypes can also be associated with phytobiomes. For instance, large differences in cotton rhizosphere microorganisms and root endophytes were associated with resistance to *Verticillium dahliae* (Wei et al.). The large differences in the rhizosphere fungal community structure were related to the differing tolerance of soybean genotypes to aluminum (Shi et al.). Thus, identifying the genes regulating host/microbe

associations/interactions is expected to provide valuable targets for breeding new varieties capable of assembling a healthier microbiota (Corbin et al.).

The impact of **abiotic factors** on phytobiome was demonstrated for diverse environments. Karray et al. showed the prominence of the climatic aridity gradient on the microbial community composition in the rhizosphere and endosphere of prickly pear, a typical crop of arid environments. Temperature significantly affected the transmission frequency, mycelial biomass and alkaloid production of an endophyte (*Epichloë* sp.) that was isolated from a wild grass and inoculated into perennial ryegrass to improve its resistance to herbivores (Freitas et al.). The molecular mechanisms underpinning the ability of an actinobacterial strain (*Microbacterium* sp.) to improve pepper plant tolerance to water stress were related to the differential expression of osmoprotectant producing proteins, reactive oxygen species scavengers, plant-hormones, structural proteins and signaling proteins (García-Fontana et al.). Moist conditions and the presence of an endophyte (*E. gansuensis*) influenced both root-associated and rhizospheric soil bacteria communities of a needlegrass plant (*Achnatherum inebrians*) (Ju et al.). Dark septate endophytes (DSE) and their habitat of origin were associated with the plant response to drought conditions (He et al.). Otlewska et al. reviewed the microbial ecology and interactions between endophytic communities and host plants occurring in saline agricultural soils and discussed on the potential of using PGPR to increase plant's salt stress tolerance. Such an approach was demonstrated to be useful in wheat where inoculation of wheat plants with a nitrogen-fixing *E. cloacae* HG-1 strain not only resulted in increased plant growth and salt stress tolerance, but also affected microbial community structure (Ji et al.).

Plants are often symbiotically related with beneficial microorganisms in soils often poor in nutrients. However, the genetic and molecular phenomena behind such symbiotic relationships are still unclear. Several articles dealt specifically with the **nitrogen fixation**. Both the soybean nodulation phenotype and the nitrogen level affected the rhizosphere bacteria community, but the nodulation phenotype contributed more than the N-supply (Wang et al.). Using a *GmcA* mutant strain of *Rhizobium leguminosarum* with a decreased antioxidative capacity, Zou et al. showed that *GmcA* oxidoreductase is crucial to both the establishment of the symbiosis and the nitrogen fixation capacity. Several nuclear genes and the symbiotic plasmid gene present in *Bradyrhizobium* strains also affected the symbiosis incompatibility found for Type II strains in comparison to Type I strains when a host plant not belonging to the common host species was inoculated (Wu et al.).

Several articles dealt with the exploitation of soil microorganisms for **biocontrol of plant diseases**. Exploiting compost microbiomes for biocontrol of soil-borne diseases through isolation of biocontrol and PGPR strains and functional assays with genomics approaches were used as tools to improve the biocontrol efficacy of composts (Lutz et al.). The suppressiveness of compost was, indeed, largely influenced by its microbiota composition and the applied dosage, as shown in

the *Phytophthora capsici*—*Cucurbita pepo* pathosystem (Bellini et al.); furthermore the ability to shape microbial composition in the rhizosphere appeared to be an important attributes of suppressive composts. An inundate introduction of a single *Pseudomonas chlororaphis* strain could also increase relative abundance of few prokaryotic families in the soil, leading to improved biocontrol of *R. necatrix* in avocado (Tienda et al.).

Plant roots exudates and particularly allelochemicals are believed to have played significant roles on protecting plants from biotic and abiotic stresses through their influences on soil microbial communities. A higher concentration of phenolic acid exudated from rice roots, due to overexpression of one gene from the *PAL* family, resulted in an increased population of *Myxococcus* sp. in the rhizosphere soil and led to improved allelopathic inhibition of weeds (Li et al.). Similarly, the types and concentrations of phenolic acid root exudates in a maize-soybean intercropping system were crucially important for suppression of *Phytophthora* diseases (Zhang et al.). On the other hand, biocontrol efficacy could be reduced due to the interaction of biocontrol strains with resident microbiota. For instance, quorum sensing disruption mediated by specific root exudates and by an increased quorum quenching bacterial community may have contributed to reduced antagonistic effects of *Pseudomonas* spp., as observed in the *Rehmannia glutinosa* rhizosphere under long-term monoculture (Li et al.). Interestingly, plant growth promoting effects observed after application of only the fermentation broth of a beneficial microbe was related to a modification of the rhizosphere bacterial community (Lu et al.).

One current research focus is to better understand the biochemical and molecular mechanisms involved in plant–microbe–soil interactions and the **multifunctionality of soil microorganisms**. Razinger et al. reported multifaceted functions (i.e., plant protection and growth promotion) of entomopathogenic fungi, but the observed effects depended on either specific fungal strains, plant species or genotypes. One difficulty in exploiting multifunctional strains in commercial agriculture is, however, related to regulatory hurdles, which may vary greatly among regions (Kowalska et al.). These regulatory difficulties may significantly impair a wider use of beneficial microorganisms despite our improved understanding of their interactions with plants and native microbiota as well as their specific pre-, pro-, and post-biotic functions (Vassileva et al.). DNA-based techniques may, however, support the regulatory process specifically in the detection and quantification of detrimental effects on soil microorganisms and non-target organisms at sub-lethal doses (Vischetti et al.), which is a subject under consideration by regulators world-wide in view of the UN Sustainable Development Goals. Moreover, Qui et al. demonstrated that the DNA-based analysis of fungal/bacterial community composition was robust to preservation methods of plant samples and the primer sets used, suggesting that simultaneous analyses of plant and soil microbiomes are possible.

Production, formulation and application of biofertilizers are the final step for exploitation of specific beneficial strains in agriculture. Encapsulation technology for microorganisms may provide additional benefits for formulations containing

different additives, including phyto-stimulants (Vassilev et al.). The opportunities and bottlenecks for new bioproducts based on endophytic microorganisms (Chitnis et al.) or on synthetic microbial consortia tailored with microorganisms possessing traits for robust colonization, long persistence and specific beneficial functions (de Souza et al.) were reviewed, particularly in terms of mitigating abiotic and biotic stresses.

In conclusion, the aim of this Research Topic was to improve our knowledge on processes and their biological mechanisms that affect the efficacy of biocontrol and biofertilization in crop systems. These included plant genotypic effects, rhizosphere and soil microbiome composition, endophytes, formulation, and production of bioinocula and commercial agronomic practices. The articles included in this e-book are addressing all these aspects, pointing to new research areas that can improve our understanding of the mechanisms of plant-microorganisms interactions in order to support and expand practical application of bioinocula in agriculture, which will reduce the negative environmental and social impact of synthetic chemicals in

agriculture, and support the new Green Revolution we all aspire to.

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

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Cultivar-Dependent Variation of the Cotton Rhizosphere and Endosphere Microbiome Under Field Conditions

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Verticillium wilt caused by *Verticillium dahliae* is a common soil-borne disease worldwide, affecting many economically important crop species. Soil microbes can influence plant disease development. We investigated rhizosphere and endosphere microbiomes in relation to cotton cultivars with differential susceptibility to *Verticillium* wilt. Soil samples from nine cotton cultivars were assessed for the density of *V. dahliae* microsclerotia; plants were assessed for disease development. We used amplicon sequencing to profile both bacterial and fungal communities. Unlike wilt severity, wilt inoculum density did not differ significantly among resistant and susceptible cultivars. Overall, there were no significant association of alpha diversity indices with wilt susceptibility. In contrast, there were clear differences in the overall rhizosphere and endosphere microbial communities, particularly bacteria, between resistant and susceptible cultivars. Many rhizosphere and endosphere microbial groups differed in their relative abundance between resistant and susceptible cultivars. These operational taxonomic units included several well-known taxonomy groups containing beneficial microbes, such as Bacillales, Pseudomonadales, Rhizobiales, and *Trichoderma*, which were higher in their relative abundance in resistant cultivars. Greenhouse studies with sterilized soil supported that beneficial microbes in the rhizosphere contribute to reduced wilt development. These findings suggested that specific rhizosphere and endosphere microbes may contribute to cotton resistance to *V. dahliae*.

Keywords: cotton, *Verticillium dahliae*, wilt resistance, rhizosphere microbiome, root endosphere

INTRODUCTION

The plant microbiome, referred to as the host's second genome, comprises diverse microbial classes. Plant root-associated microbiomes represent a huge reservoir of biological diversity, in the order of tens of thousands of species (Berendsen et al., 2012). Plants depend upon beneficial interactions between roots and microbes for nutrient uptake, and improved tolerance to biotic and abiotic stress (Mendes et al., 2011; Berendsen et al., 2012; Zhang et al., 2019). Beneficial soil microbes contribute to pathogen resistance (Mendes et al., 2011; Berendsen et al., 2012), drought tolerance (Lau and

Lennon, 2012), and promoting plant growth (Pii et al., 2015). In return, plants secrete up to 20% of their fixed carbon and 15% of their nitrogen into the rhizosphere, thus supporting microbial communities (Sasse et al., 2018).

Maintenance of a diverse population of soil microorganisms is crucial in achieving sustainable agriculture. Interactions between microbiota and their host plants have recently received much attention, mainly due to advances in sequencing technology. Microbial composition has been investigated in various plant species, such as *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppli et al., 2014; Duran et al., 2018), *Populus* (Gottel et al., 2011; Beckers et al., 2017), maize (Peiffer et al., 2013), and rice (Edwards et al., 2015; Zhang et al., 2019). Characterization of the core root microbiome of *Arabidopsis* showed that the dominant phyla in the endosphere were much less diverse than in the rhizosphere (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppli et al., 2014). In field conditions, geographical location was the factor explaining most of the variability in the root-associated microbiomes of maize (Peiffer et al., 2013), poplar (Shakya et al., 2013), and rice (Edwards et al., 2015), whilst plant genotypes accounted for a smaller but still significant proportion of the variability (Peiffer et al., 2013). It has been suggested that plants may assemble their microbiomes in two steps; (1) a general recruitment of microbes to the vicinity of the root, and (2) genetic filtering processes that allows specific microbes into roots (Bulgarelli et al., 2013).

Recent evidence suggests that variability in plant genotypes, even at a single gene locus, can have a significant impact on rhizosphere microbiomes (Stringlis et al., 2018; Zhang et al., 2019). Weinert et al. (2011) detected 2432 bacterial operational taxonomic units (OTUs) in the potato rhizosphere, of which 40% had a site-specific abundance, 9% had a cultivar-dependent abundance at the one or the other field site, and 4% at both sites. Interestingly, OTUs which differed in relative abundance among three studied potato cultivars mainly belonged to groups known to contain isolates with biocontrol potential, such as the Pseudomonales, Streptomycetaceae, and Micromonosporaceae (Weinert et al., 2011). Thus, specific plant genotypes may recruit beneficial microorganisms that help them defend against pathogens. Specific microbial species in soil are associated with soil suppressiveness of pathogens. For instance, soil suppression of wheat soil-borne pathogens was ascribed to the differences in the ability of wheat cultivars to accumulate naturally occurring DAPG-producing *Pseudomonas* spp. (Meyer et al., 2010). Similarly, the amount of antibiotics produced by specific microbial biocontrol strains in the rhizosphere differed between wheat cultivars (Okubara and Bonsall, 2008) and cultivar-specific differences in the ability to accumulate naturally occurring specific biocontrol bacteria in the rhizosphere were found in Swiss winter wheat (Meyer et al., 2010).

Cotton (*Gossypium hirsutum* L.) is an important commercial crop grown worldwide. *Verticillium* wilt, caused by the soil-borne fungal pathogen *Verticillium dahliae* Kleb., is a major disease of cotton. The primary inoculum of *V. dahliae* is microsclerotia, fungal resting structures, in dead plant tissues

and in soil. Microsclerotia may survive in soil for more than 10 years in the absence of a host (Pegg and Brady, 2002). Chemical fumigation has been an indispensable tool for controlling soil-borne pathogens; however, several fumigants have already been banned or face an uncertain future due to legislation (Martin, 2003). Cultivars differ in their susceptibility to *V. dahliae*. Threshold values of 4.0 and 7.0 *V. dahliae* CFU g⁻¹ soil are needed for infecting susceptible and resistant cultivars, respectively (Wei et al., 2015). It is unclear whether and, if so, how cultivar resistance against *V. dahliae* is related to rhizosphere microbes and root endophytes.

In the present study, we used amplicon sequencing to characterize endosphere and rhizosphere microbial communities of nine cotton cultivars with differing resistance against wilt in a designed field experiment. We established an association of specific endosphere and rhizosphere microbes with wilt resistance and to test this association we then carried out a greenhouse study in which the nine cultivars were inoculated with *V. dahliae* in sterilized field soils.

MATERIALS AND METHODS

Field Experiment Design

A field experiment was conducted at the Institute of Cotton Research of Chinese Academy of Sciences (Anyang, China) to assess whether rhizosphere or endosphere microbial communities and specific microbial groups are associated with cultivar wilt resistance. The soil at the experimental site is classified as cambisol type soil (FAO, 1998). A completely randomized block design with three blocks was used. Nine cultivars were included: NXC1208, SNM9, ZM9421, LMY21, GXM25, BM16, JK10, KM50, and JM11. Each block consisted of nine plots, each was 5 m long with two rows (0.8 m between two rows); neighbouring plots were separated by 0.8 m. In each plot, six soil cores (three randomly selected points in each row, 2.5 cm in diameter, from just below the surface to a depth of 15 cm) were collected just before planting, and bulked into a single sample per plot for assessing wilt inoculum level. In April 2016, seeds were sown with a within-row plant-to-plant distance of 25 to 30 cm. During late August (at the boll-forming stage), approximately 16 weeks after sowing, wilt severity on all individual plants was recorded on a scale of 0 to 4: 0 = no symptoms, 1 = ≤33%, 2 = >33% and ≤66%, 3 = >66% and ≤99%, and 4 = 100% leaves with wilt symptoms. An overall disease index (DI) was calculated for each plot:

$$DI = \frac{0 \cdot n_0 + 1 \cdot n_1 + 2 \cdot n_2 + 3 \cdot n_3 + 4 \cdot n_4}{4 \cdot n} \times 100 \%$$

Where n_0 – n_4 was the number of plants with the corresponding disease ratings (0–4), and n was the total number of plants assessed in each plot.

In order to ascertain that wilt development of individual plants (hence cultivar resistance) was not mainly due to the level of *V. dahliae* inoculum, we estimated the density of *V. dahliae* inoculum using a wet sieving and plating method (Wei et al.,

2015). Then ANOVA was applied to assess when differences in inoculum densities could largely account for cultivar differences in the observed wilt severities.

Sample Collection of Rhizosphere and Endosphere Fractions

Rhizosphere Samples Collection

At the same time as wilt assessment, three plants from each plot were randomly selected and carefully removed from the soil using a spade. Root systems of the three plants from each plot were first vigorously shaken to remove loosely adhering soil particles, then the root systems were combined as a single composite sample. Plant fine roots were cut into pieces of approximately 2 cm length using sterile scissors. Rhizosphere samples were harvested in aliquots of 20 g roots in 500 ml screw-cap bottles. Each bottle was filled up to 300 ml with 1:50 TE buffer (1 M Tris, 500 mM EDTA, and 1.2% Triton diluted in sterile distilled water) and shaken at 270 rpm for 1 h (room temperature). The root-washing suspension was filtered with sterile cheesecloth and centrifuged ($4,000 \times g$) at 4°C for 20 min (Wei et al., 2016a). The supernatant was discarded by pipetting. This step was repeated several times before the pellets were re-suspended in remaining solution, transferred to a 2 ml Eppendorf tube and centrifuged at $14,000 \times g$ for 20 min. The pellets were immediately frozen and stored at -80°C before DNA extraction.

Endosphere Samples Collection

After washing, clean roots were moved to a new bottle and surface sterilized as described in Li et al. (2010). Cotton root samples that were not contaminated as determined by a culture-dependent disinfection test (Li et al., 2010) were used for subsequent analyses. Roots were cut into pieces of approximately 1 cm in length using sterile scissors and homogenized with a soft-headed hammer as described earlier by Hardoim et al. (2011) in a sterile polythene bag to release endophytes. The residues were shaken again with glass beads in 300 ml 1:50 TE buffer for 3 h at room temperature to detach microorganisms (Kadivar and Stapleton, 2003). The washing suspension was filtered with sterile cheesecloth. To collect root endosphere microbes, the suspension was centrifuged ($4,000 \times g$) at 4°C for 20 min and the supernatant discarded. This step was repeated several times and the pellets were re-suspended in the remaining solution, transferred to a 2 ml Eppendorf tube and centrifuged at $14,000 \times g$ for 20 min. The pellets were immediately frozen and stored at -80°C until DNA extraction.

DNA Extraction and Next-Generation Sequencing

Extraction of DNA and next-generation sequencing for rhizosphere and endosphere samples followed the same procedure.

Cells (250 mg) were re-suspended in 500 μl MoBio PowerSoil bead solution, and DNA was extracted using the MoBio PowerSoil DNA Kit (MoBio Laboratories, Carlsbad, CA, USA) following the manufacturer's protocol; extractions were carried

out in triplicate for each sample, pooled after extraction, and quantified using a NanoDrop ND-2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

For bacteria, the V5-V7 16S rRNA gene region was amplified in triplicates for each sample using the 799F (Chelius and Triplett, 2001) and 1193R primers (Bodenhausen et al., 2013) with the barcodes. For fungi, primers ITS5 and ITS2 (White et al., 1990) with the barcodes were used to amplify the ITS1 region. For amplification, the 30 μl reaction mixtures contained 15 μl of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, and 10 ng template DNA. PCR amplification was performed using a Bio-Rad T100™ thermal cycler (Hercules, CA, USA) with the following amplification cycles: 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s; Finally 72°C for 5 min. Negative and positive controls were included in all amplifications.

The PCR products were mixed with the same volume of 1 \times loading buffer containing SYBR green (Takara Biotechnology Co., Ltd) and electrophoresed on 2% agarose gel for confirmation. PCR products from three technical replicates were mixed in equidensity ratios. Then, the mixed PCR products were purified with the GeneJET Gel Extraction Kit (Thermo Scientific, Fermentas, USA). Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit 2.0 Fluorometer (Life Technologies, USA) and Agilent Bioanalyzer 2100 system. Finally, the library was sequenced on an Illumina HiSeq 2,500 and 250 nucleotide paired-end reads were generated. All samples were sequenced in one run: total 108 samples—54 samples (nine cotton cultivars \times three replicates \times two niches) for 16S rRNA gene sequences and 54 samples (nine cotton cultivars \times three replicates \times two niches) for ITS sequences.

Sequence Processing

High-quality sequences were obtained for rhizosphere and endosphere samples by quality control and filtering of sequence quality with stringent criteria following our previous publication (Tilston et al., 2018) and was carried out separately for the four type of data sets (16S and ITS for endosphere and rhizosphere). High quality sequences were first dereplicated and unique sequences with only one read were discarded. Then, all unique sequence reads were sorted by their respective frequencies and clustered into operational taxonomic units (OTUs) at 97% similarity with a representative sequence generated for each OTU. All OTU processing was carried out with the UPARSE pipeline (Version 10.0) (Edgar, 2013) unless specified otherwise. The clustering algorithm also removed chimeras. The SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) then assigned each OTU representative sequence to taxonomic ranks by alignment with the gene sequences against two reference databases: Unite V7 fungal database (Köljalg et al., 2013) and RDP training set 15 bacterial database (Cole et al., 2014). Finally, an OTU table

(a sample-by-observation contingency table) was generated by aligning all sequences filtered with far less stringent criteria with the OTU representative sequences as described by Deakin et al. (2018).

Statistical Analysis of Sequence Data

Alpha diversity were calculated by analysing the observed OTUs, Chao1, Shannon, and Simpson indices using the R vegan 2.3-1 package (Dixon, 2003). The rank of alpha diversity indices were subjected to ANOVA to assess the differences between wilt susceptible and resistant cultivars *via* a permutation of significance.

To assess differences in the overall microbial communities among cultivars and between wilt resistant/susceptible cultivars (i.e. beta diversity), we used two approaches. First, UniFrac distances between samples were calculated, subjected to non-dimensional scaling analysis, and analyzed with permutation multivariate ANOVA (PERMANOVA). In this analysis, library size normalization was performed using the median-of-ratios method implemented in DESeq2 (Anders and Huber, 2010; Love et al., 2014). Second, principal component analysis (PCA) were applied to the library size normalized reads using the DESeq2 variance stabilization transformation (VST). ANOVA was then performed to assess the difference between wilt susceptible and resistant cultivars as well as between all cultivars on the first four PC scores.

Once we had tested whether there was an overall association of both endosphere and rhizosphere microbial communities with cultivar wilt resistance, we then conducted further analysis to identify specific (or core) microbes that differed significantly in their relative abundances between wilt susceptible and resistant cultivars. For this purpose, DESeq2 was applied to normalized OTU count data without rarefaction (McMurdie and Holmes, 2013). DESeq2 also implements an algorithm for the automatic filtering of OTUs before differential abundance analysis using several criteria, including variance in abundance across samples and overall abundance level. To correct for the false discovery rate associated with multiple testing, the Benjamini-Hochberg (BH) adjustment was used with DESeq2 (Benjamin and Aikman, 1995). For tree view graphs, OTU abundances were aggregated at each taxonomic rank (at the SINTAX confidence of 0.8) and these aggregated count values were tested for differential abundance between wilt susceptible and tolerant cultivars with DESeq2 as above.

Greenhouse Trials

To exclude the effects of rhizosphere microbes on wilt development, the nine cultivars were assessed for wilt in sterilized soil in a greenhouse. Before planting, soil to a depth of 20 cm from the field experimental site was collected autoclaved at 121°C and 115 kPa twice, each for 45 min. A sterility check using plating method (Trevors, 1996) was implemented to ensure that the sterilization process was successful. Cultures of *V. dahliae* Vd076 isolate in the maize-sand (V/V = 1:1) medium were ground into particles, size range between 1 and 2 mm and mixed with the sterilized soil (V/V = 0.006:1). For each cultivar, there were six pots (diameter 39 cm and height 30 cm), each with five seedlings; all individual pots were located in randomized positions in the greenhouse trial area. Disease severity on individual plants was recorded eight weeks after planting using the same wilt assessment and disease indices as for the field experiment. The experiment was repeated twice. Analysis of variance (ANOVA) was applied to the disease indices; no transformation was needed to satisfy analysis assumptions. For the field data, CFU data were also included as a covariate.

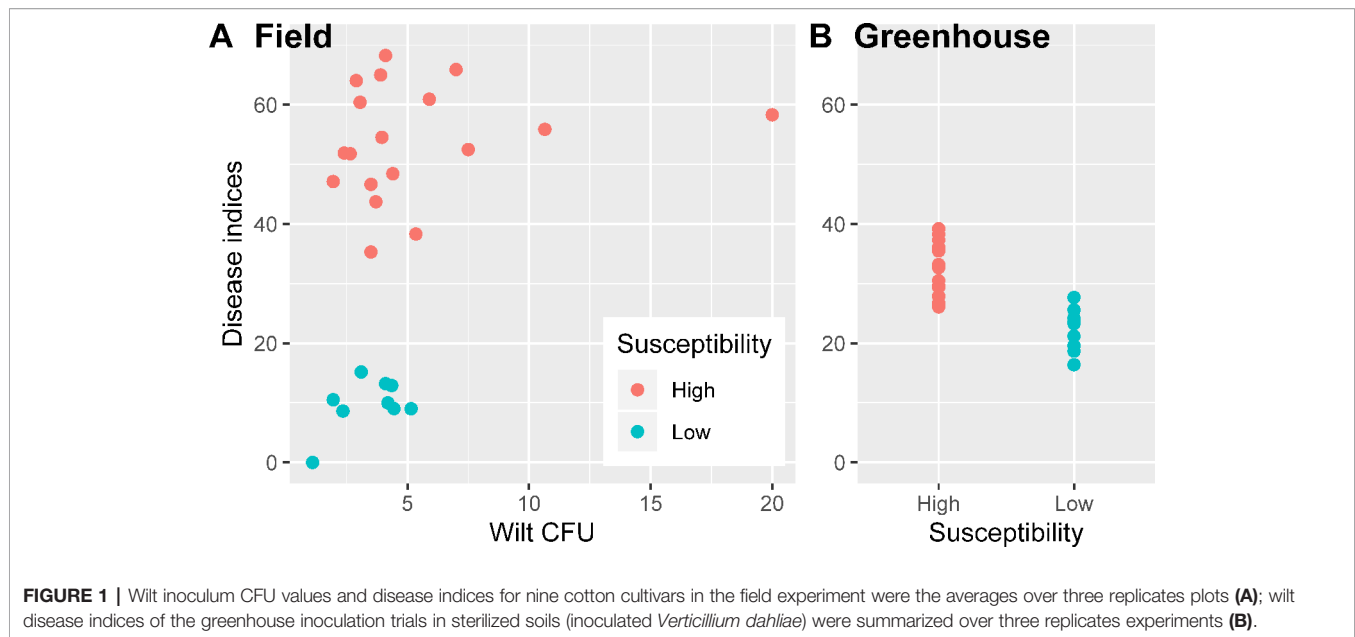
RESULTS

Field Disease Development

Average wilt index ranged from 7.9 (cv. NXC1208) to 60.6 (cv. JM11) in the field and from 19.8 (cv. NXC1208) to 36.9 (cv. JM11) in the greenhouse trials (Table 1). Three cultivars (NXC1208, SNM9, and ZM9421) had very low wilt indices in the field trial and were classified as wilt resistant cultivars; the other six cultivars (LMY21, GXM25, BM16, JK10, KM50, and JM11) were classified as susceptible to *V. dahliae*. There were no significant differences in the *V. dahliae* CFU per gram of dried soil between the wilt resistant and susceptible cultivars in the field trial (Figure 1A). The high average CFU value for A59 is due to one extreme high count of 20, compared to the next highest value of 10.7. When used as a covariate in the ANOVA of individual plant data, CFU was positively related ($P < 0.001$) to wilt indices but only accounted for 7.7% of the total variance in the wilt index. In contrast, cultivar differences in the field trial accounted for 81.5% of the total variability, most (93.0%) of

TABLE 1 | *Verticillium* wilt summary of those nine cultivars included in the study; wilt inoculum CFU values and disease indices in the field experiment were the averages over three replicates plots; wilt disease indices of the greenhouse inoculation trials in sterilized soils (inoculated *Verticillium dahliae*) were summarized over three replicates experiments.

Cultivar		Field CFU (g ⁻¹ dry soil)	Wilt disease indices	
Name	Code		Field trial	Greenhouse trial
NXC1208	B13	2.18	7.9	19.8
SNM9	B9	3.77	9.8	21.4
ZM9421	B6	4.30	11.7	25.5
LMY21	LM21	4.48	43.5	30.4
GXM25	B7	5.37	46.1	29.6
BM16	A23	3.78	55.4	32.8
JK10	A59	8.43	58.1	33.7
KM50	A60	4.23	59.4	32.0
JM11	JM11	5.83	60.6	36.9



which were due to the differences between susceptible and resistant cultivars.

General Sequence Data

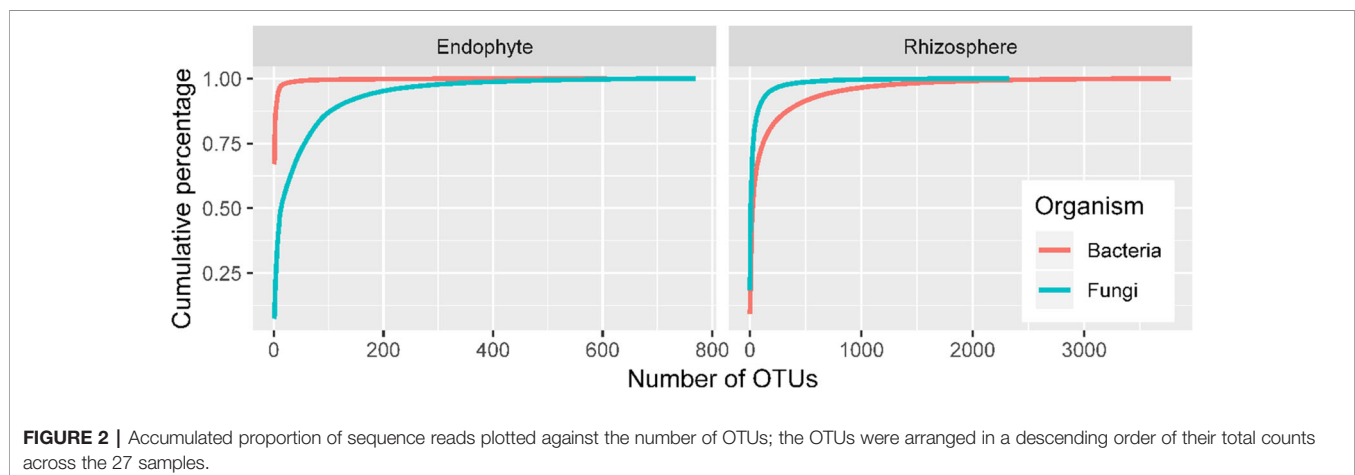
There were high numbers of raw sequence reads for 27 samples: ranging from 62,524 to 99,932 for 16S endophytes, from 81,614 to 99,262 for bacteria rhizosphere, from 90,406 to 108,661 for ITS endosphere, and from 78,035 to 191,678 for ITS rhizosphere. The corresponding values for the number of sequence reads included in the OTUs are from 43,518 to 81,844, from 43,839 to 67,025, from 57,702 to 96,798, and from 56,211 to 102,000. Sequencing depth is sufficient for all four combinations of ITS/16S and sample site (endosphere/rhizosphere) as shown by the rarefaction curves (Figure 2). Overall, a limited number of OTUs account for most sequence reads, particularly for bacterial endophytes and rhizosphere fungi (Figure 2). For instance, the first five bacterial OTUs in endosphere samples accounted for 90% of the total reads with the first one accounting for two thirds

of the reads. The top 93 OTUs accounted for 90% of the total reads in the rhizosphere fungi.

Taxonomy Information

Almost all 16S OTU sequences (99.9%) could be reliably assigned to the phylum level. Overall, there were more diverse bacteria in the rhizosphere than in endosphere (Figure 3A). Based on the DESeq2 normalized sequence data, nearly all endosphere bacterial sequences belonged to Proteobacteria (99.7%); whereas the three most abundant rhizosphere bacterial phyla were Proteobacteria (74.5%), Acidobacteria (11.4%), and Firmicutes (9.6%) (Figure 3A).

There is considerable numbers of fungal sequences that could not be reliably classified at the phylum level: 29.1% and 17.5% for endosphere and rhizosphere samples, respectively (Figure 3B). In addition to the unidentified sequences, the three most abundant endophyte fungal phyla were Ascomycota (76.9%), Basidiomycota (4.1%), and Zygomycota (1.5%); the same was



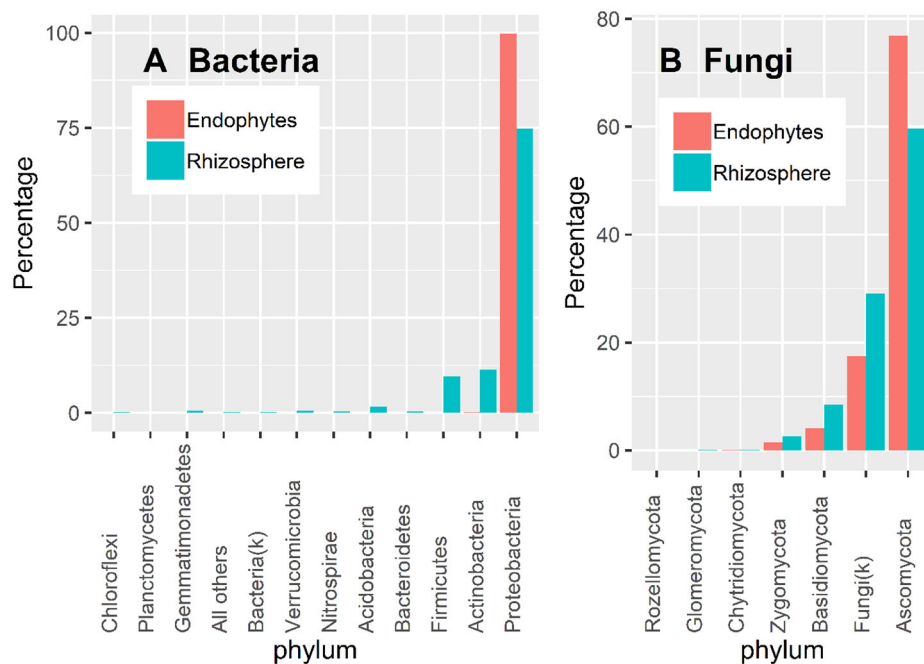


FIGURE 3 | Proportion of DESeq2 normalized sequence reads assigned to different phyla at $\geq 90\%$ confidence for bacteria (A) and fungi (B).

true for rhizosphere samples with the corresponding values of 59.6%, 8.4% and 2.6% (**Figure 3B**). Glomeromycota only accounted for 0.01% and 0.06% of the total endosphere and rhizosphere sequence reads.

Alpha Diversity

The number of observed bacterial OTUs was much greater for the rhizosphere samples (1,850–2,300 per sample) than for the endosphere samples (80–500 per sample). Both Simpson and Shannon indices were much higher for the rhizosphere than for the endosphere samples (**Supplementary Figures S1 and S2**). Of the six alpha diversity indices (Chao1, Simpson, and Shannon for endosphere and rhizosphere), only the Chao1 for endosphere was greater ($P < 0.01$) for the resistant than for the susceptible cultivars (**Supplementary Figure S2**). Similarly, the number of observed bacterial endosphere OTUs was much higher for the resistant (ca. 350) than for the susceptible (ca. 200) cultivars (**Supplementary Figure S2**).

As for bacteria, within-sample fungal population was more diverse for the rhizosphere than for endosphere samples (**Supplementary Figures S3 and S4**) but the differences were less than for bacteria. There were no significant differences in all alpha diversity indices between the wilt resistant and susceptible cultivars.

Sample-To-Sample (Beta Diversity) Differences and Variation in Individual OTU Abundances

Both UniFrac and PCA analyses resulted in similar results and hence only PCA results are presented. For both bacteria and fungi, the proportion of sequence reads in each phylum was very

similar in the samples from the wilt resistant and susceptible cultivars. **Table 3** presents the summary results from DESeq2 analysis, assessing statistical significance of the differences in the relative abundance of individual endosphere and rhizosphere bacterial and fungal OTUs between the wilt resistant and susceptible cotton cultivars. Many OTUs were automatically filtered out before DESeq2 analysis. For example, only 1,650 of 3,768 rhizosphere bacterial OTUs were compared by DESeq2.

Endosphere Bacteria

There were significant differences between cultivars in PC1 and most of the cultivar differences in PC1 were due to the differences between the wilt resistant and susceptible cultivars (**Table 2**). The difference between the wilt resistant and susceptible was also significant for PC2. However, the wilt resistant samples were separated only along the PC1 axis (**Figure 4**) with lower PC1 scores for samples from resistant cultivars. DESeq2 analysis was applied to 313 OTUs. Wilt resistant cultivars differed in the relative abundance from susceptible cultivars for 80 OTUs; for 77 of these OTUs, resistant cultivars had higher relative abundance than susceptible cultivars (**Figure 5, Table 3**). Most of these OTUs cannot be assigned to the taxonomic rank below Order with confidence (**Supplementary Table S1**) and these OTUs spread across a number of bacterial classes (**Figure 6**). These 80 OTUs included one from *Streptomyces*, one from *Nitrospira*, eight from Bacillales (five from *Bacillus*), three from Rhodocyclaceae (one is from *Azoarcus*), one from *Brevundimonas*, one from *Rhodobacter*, one from *Lysobacter*, and six from Rhizobiales. Overall, average sequence counts were low except for a few OTUs (>30 , **Figure 5**): two from Acidimicrobiales, one *Bacillus*, and two Rhizobiales. For one of

TABLE 2 | Percent variance in the first four principal components accounted by cultivars and the comparison between susceptible and resistant cultivars.

	Endosphere bacteria			Rhizosphere bacteria			Endosphere fungi			Rhizosphere fungi		
	%Var	Cul [*]	Sus [§]	%Var	Cul	Sus	%Var	Cul	Sus	%Var	Cul	Sus
PC1	27.1	39.8**	37.3**	11.3	50.3**	38.5**	13.9	35.3	29.0**	11.9	42.3	13.4**
PC2	11.1	26.1	12.9	8.6	41.4	0.7*	11.1	50.3	7.0*	8.3	38.8	0.1
PC3	6.4	56.7**	0.2	7.2	65.5**	4.8	7.8	49.2**	0.0	6.5	59.2**	7.7*
PC4	5.6	27.9*	7.2	6.2	59.8*	3.1	7.5	67.7**	6.5*	5.8	57.2**	27.1**
All PCs		40.4	18.8		39.0	8.6		41.9	10.1		37.5	6.2

^{*}Between the nine cultivars; [§]between the wilt resistant and susceptible cultivars.

*, **: P values < 0.05 and 0.01, respectively.

TABLE 3 | Summary of DESeq2 analysis results, comparing the relative abundance of individual endosphere and rhizosphere bacterial and fungal OTUs between the wilt resistant and susceptible cotton cultivars.

Organisms	Number of OTUs			
	Total	After DESeq2 filtering	Significantly different	Resistant > susceptible
Endosphere bacteria	607	313	80	77
Rhizosphere bacteria	3,768	1,650	136	52
Endosphere fungi	743	709	83	29
Rhizosphere fungi	2,286	688	54	16

the two Rhizobiales OTUs, susceptible cultivars had greater relative abundance than resistant cultivars.

Rhizosphere Bacteria

There were significant differences between cultivars in PC1 and nearly 80% of such differences were due to the differences between the wilt resistant and susceptible cultivars (**Table 2**). Although cultivar differences were significant for PC3 and PC4 as well, there were very little differences in PC2-PC4 scores between the wilt resistant and susceptible cultivars. As for bacterial endosphere, the wilt resistant samples were separated only along the PC1 axis (**Figure 4**) with lower PC1 scores for samples from resistant cultivars. DESeq2 analysis was applied to 1,650 OTUs. For 136 OTUs, there were significant differences in the relative abundance between wilt resistant and susceptible cultivars; for 52 of these OTUs, resistant cultivars had higher relative abundance than susceptible cultivars (**Figure 5**, **Table 3**). As for rhizosphere bacteria, most of these OTUs cannot be assigned to the taxonomic rank below the order with confidence (**Supplementary Table S2**). Those OTUs with higher relative abundance in wilt tolerant cultivars were clustered within Bacilli, Actinobacteria, and Chloroflexi whereas those OTUs with lower abundance in wilt-tolerant cultivars were more spread among a number of bacterial taxa groups (**Figure 7**). Noticeable OTUs included three from Nitrospira (all Log2FoldChange < 0), two from Planctomycetaceae (all Log2FoldChange > 0), one Pseudomonadales (Log2FoldChange > 0), eight from Bacillales (seven with Log2FoldChange > 0, three from Bacillus), one from Rhodocyclaceae (Log2FoldChange < 0), seven from Rhizobiales (six with Log2FoldChange > 0), and nine Xanthomonadales (Log2FoldChange < 0). Four OTUs had very large average sequence counts (**Figure 5**): 1,561, 1,013, 843, and 784 for *Ilumatobacter*, *Bcaillus*, Burkholderiales, and Steroidobacter, respectively (of these four OTUs, only for *Bacillus* Log2FoldChange > 0).

Endosphere Fungi

Cultivars differed significantly ($P < 0.01$) in PC1 with the most (ca. 80%) of the differences attributable to the differences between the wilt resistant and susceptible cultivars (**Table 2**). The difference between the wilt resistant and susceptible was also significant for PC2 and PC4, but accounted for very low variability (**Table 2**). Samples from wilt resistant cultivars were separated from susceptible cultivars along the PC1 axis (**Figure 4**) with higher PC1 scores for resistant cultivars. DESeq2 analysis was applied to 709 OTUs. For 83 OTUs, there were significant differences in the relative abundance between wilt resistant and susceptible cultivars; for 29 of these OTUs, wilt resistant cultivars had higher relative abundance than susceptible cultivars (**Figure 5**, **Supplementary Table S3**). These 83 OTUs appear not to cluster around particular taxa groups except for several OTUs (with Log2FoldChange > 0) from Agaricomycetes (**Figure 8**). Of the 83 OTUs, 48 cannot be assigned to the taxonomic rank of phylum, and only 24 can be signed to the order rank (**Supplementary Table S3**). These 83 OTUs included *Alternaria solani*, *Aspergillus aculeatus*, *Penicillium*, *Verticillium longisporum*, and *Choanephora*; Log2FoldChange < 0 for all five OTUs except *Penicillium*. Five OTUs had very large average sequence counts (>750, **Figure 5**) but only one of them can be assigned to a rank below Kingdom, in the Pleosporaceae family (Log2FoldChange < 0).

Rhizosphere Fungi

Cultivar differences were only significant ($P < 0.01$) for PC3 and PC4; whereas the differences between the wilt resistant cultivars and susceptible cultivars were significant for PC1, PC3, and PC4 they did not account for much variability (**Table 2**). Samples from wilt resistant cultivars cannot be clearly separated from the susceptible cultivar samples along the PC1 or PC2 axis (**Figure 4**) although average PC1 score was lower for resistant than for susceptible cultivars. DESeq2 analysis was applied to 688 OTUs. For 54 OTUs, there were significant differences in the relative

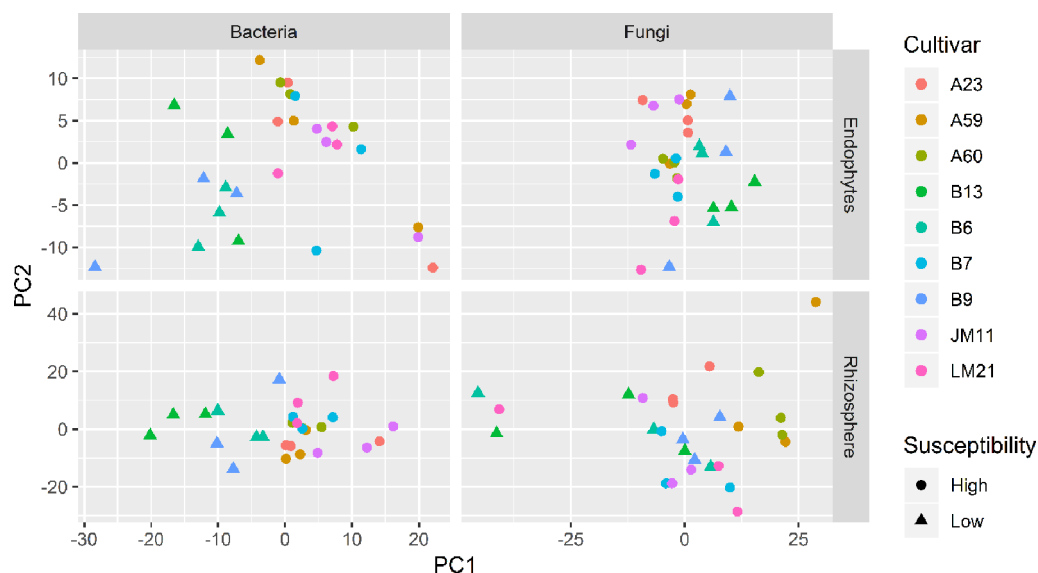


FIGURE 4 | Plots of the first two principle components of normalized OTU data for bacteria and fungi in both cotton endosphere and rhizosphere of nine cultivars with differing susceptibility to *Verticillium* wilt.

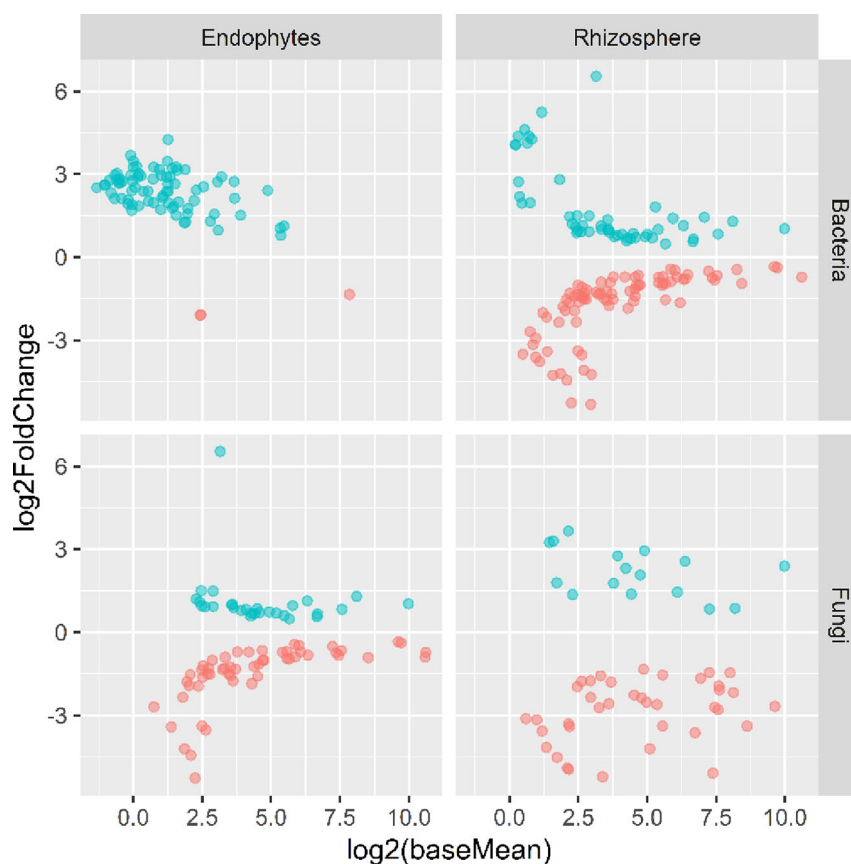


FIGURE 5 | Plots of DeSeq2 analysis results for OTUs with significant ($P < 0.05$) differences between the wilt resistant and susceptible cultivars; baseMean is the average number of sequence reads for each OTU and $\log_2\text{FoldChange}$ is the loge of the ratio in the number of sequence reads between the wilt resistant and susceptible cultivars. The blue symbols (positive $\log_2\text{FoldChange}$) indicates that the relative abundance of specific OTUs was greater in the wilt resistant cultivar samples than in susceptible cultivar samples.



FIGURE 6 | Tree views of the relative abundance between wilt-tolerant and wilt-susceptible cotton cultivars for endosphere bacteria; the relative difference in abundance is expressed as log2FoldChange with the values indicating that the relative abundance of specific OTUs was greater in the wilt tolerant cultivar samples than in susceptible cultivar samples. The size of nodes represents the abundance of endosphere bacteria at the specific taxonomic rank. Those OTUs that could not be assigned reliably to a taxonomic rank below Kingdom have been excluded from the graph. The graph is drawn with the R package—Metacoder (Foster et al., 2017).

abundance between wilt resistant and susceptible cultivars; for 16 of these OTUs, wilt resistant cultivars had higher relative abundance than susceptible cultivars (**Figure 5**, **Supplementary Table S4**). As for fungal endosphere, these 54 OTUs appear not to cluster around particular taxa groups except for several OTUs (with Log2FoldChange > 0) from Pezizomycetes (**Figure 9**). Of the 54 OTUs, 33 cannot be assigned to the taxonomic rank of phylum, and a further 16 can be assigned only to the rank of family (**Supplementary Table S4**). Of the 54 OTUs, noticeable OTUs included *Alternaria*, *Trichoderma*, *Magnaporthe grisea*, *Thielaviopsis basicola*, *Microascus brevicaulis*, two from Ceratobasidiaceae, and two from Ustilaginaceae. Of these OTUs, only for *M. brevicaulis* and *Trichoderma* was Log2FoldChange > 0. Two OTUs had very large sequence counts (>790, **Figure 5**) but only one can be assigned to the taxonomy rank of Pleosporaceae (Log2FoldChange < 0).

Wilt Development in Sterilized Soil

Although the correlation in the cultivar mean wilt indices were significant ($P < 0.001$) between field and greenhouse studies, the

actual differences in the average wilt indices among the nine cultivars were much smaller in the greenhouse trial than in the field trial (**Table 1**, **Figure 1**). Average wilt index ranged from 19.8 (cv. NXC1208) to 36.9 (cv. JM11) in the greenhouse trial (**Table 1**). Cultivar differences accounted for 77.6% of the total variability, most (87.5%) of which were due to the differences between susceptible and resistant cultivars.

DISCUSSION

As a monocyclic disease, inoculum levels of *V. dahliae* (CFU per gram of soil) in the soil at planting plays a critical role in the development of cotton wilt (Wei et al., 2015). Increasing cultivar tolerance/resistance to *V. dahliae* leads to corresponding increases in the inoculum threshold value necessary for wilt development (Wei et al., 2015). In the present study, there were no significant differences in the *V. dahliae* CFU counts between cultivars but the wilt indices differed largely between the resistant and susceptible cultivars. Only on the basis of individual plants

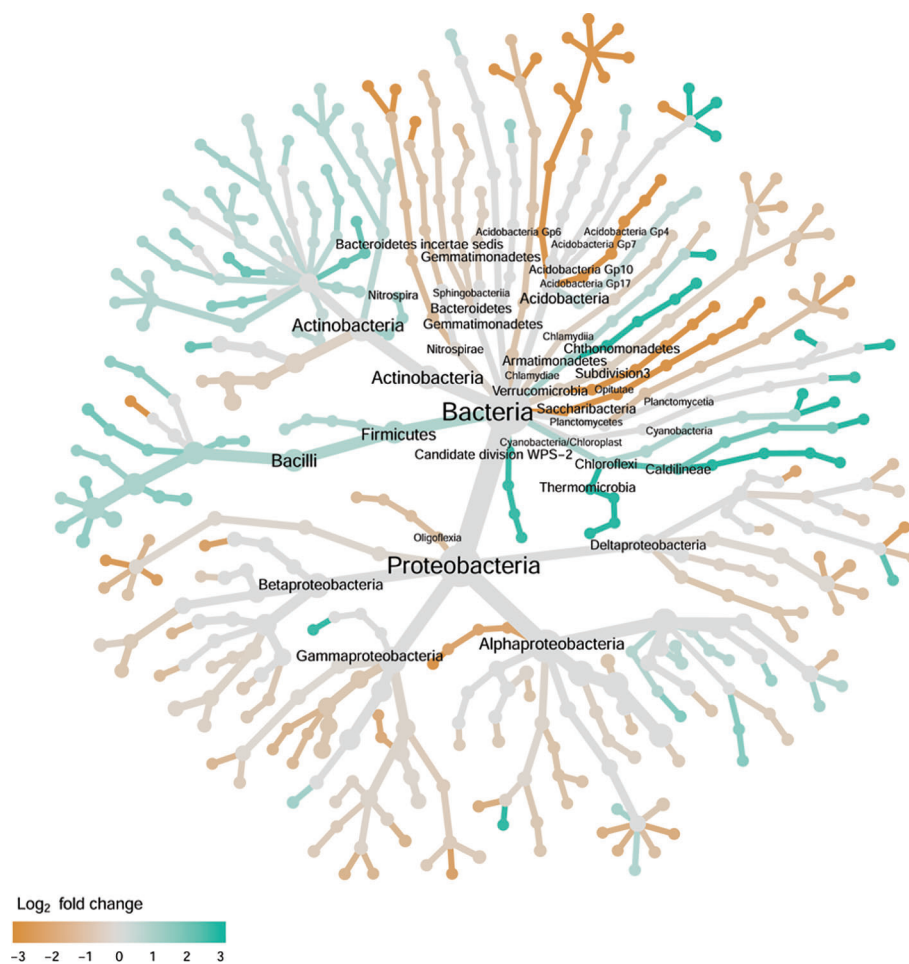


FIGURE 7 | Tree views of the relative abundance between wilt-tolerant and wilt-susceptible cotton cultivars for rhizosphere bacteria; the relative difference in abundance is expressed as log2FoldChange with the values indicating that the relative abundance of specific OTUs was greater in the wilt tolerant cultivar samples than in susceptible cultivar samples. The size of nodes represents the abundance of rhizosphere bacteria at the specific taxonomic rank. Those OTUs that could not be assigned reliably to a taxonomic rank below Kingdom have been excluded from the graph. The graph is drawn with the R package—Metacoder (Foster et al., 2017).

was the CFU positively related to observed wilt severity, but only accounted for ca. 9.0% of the total variability, compared to 81.5% by cultivars. Thus, most differences in wilt severities among cultivars in the field trial are unlikely due to the differing levels of wilt inoculum.

The most abundant bacterial rhizosphere phylum was Proteobacteria (74.5%), followed by Actinobacteria (11.4%), and Firmicutes (11.4%). Similar to studies in *Arabidopsis* (Bulgarelli et al., 2012; Lundberg et al., 2012; Schlaeppi et al., 2014) and rice (Edwards et al., 2015), the relative abundance of Proteobacteria increased in the endosphere relative to the rhizosphere, and the opposite was true for Acidobacteria and Firmicutes. With regard to fungi, Ascomycota was the most abundant phylum in both endosphere and rhizosphere. As with field strawberry plants (Wei et al., 2016b), the two most abundant fungal phyla were Ascomycota and Basidiomycota. The relative abundance of both bacterial and fungal phyla in the rhizosphere and inside roots thus appears to be similar for land plants.

Sample alpha diversity indices indicated a large reduction in the microbial diversity from rhizosphere to the root endosphere. This points to a gating role of the root surface for selective entry of bacteria and fungi into the root interior; this phenomenon has been found in other plant species, such as *Arabidopsis thaliana* (Duran et al., 2018) and rice (Edwards et al., 2015). In general, the endophytic microbiome is more specific than in rhizosphere, as fewer well-adapted bacteria are permitted to enter and survive in the plant interior (Compant et al., 2010). The complexities of specific endophyte community structures indicate that they may interact with the plant host and influence plant physiology (Gaiero et al., 2013). Specific plant factor(s) regulating endophytic communities remain little understood. A number of studies indicate that individual endophytic microbial members have antagonistic activity against pathogens (Berg et al., 2005; Procopio et al., 2009; Li et al., 2014). Such antagonistic effects may have resulted from direct effects of biologically active compounds produced by endophytes and/or indirect effects through induced resistance (Zamioudis and Pieterse, 2012).

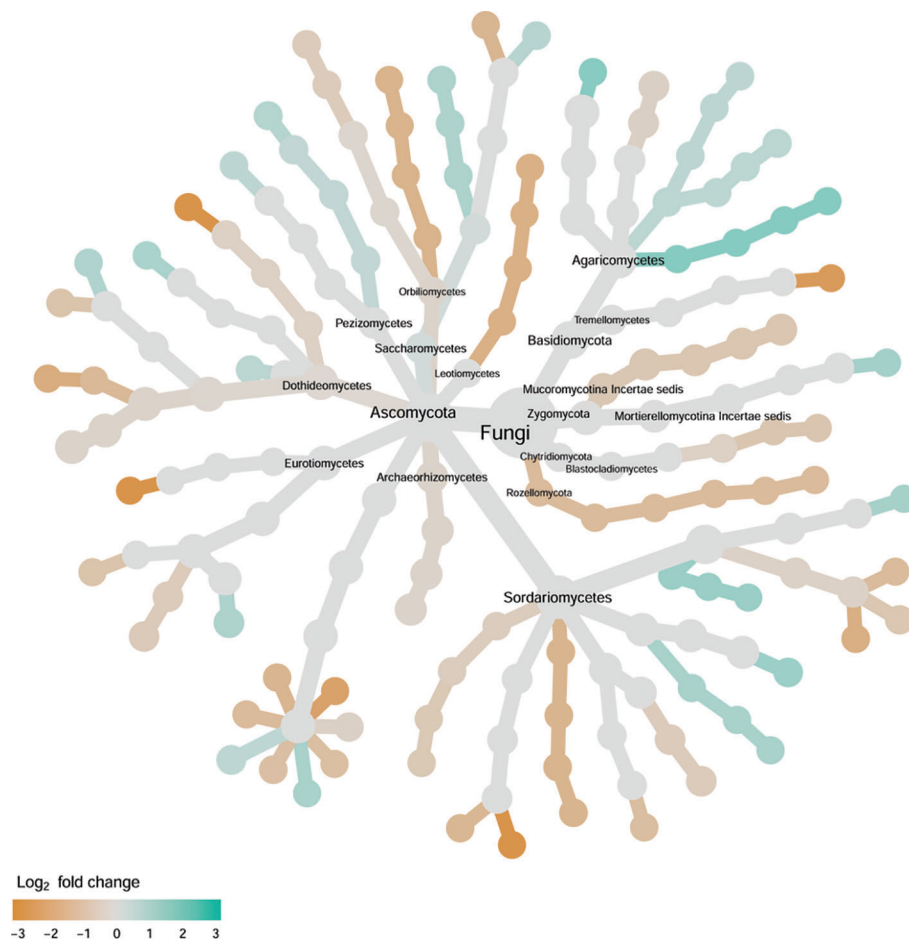


FIGURE 8 | Tree views of the relative abundance between wilt-tolerant and wilt-susceptible cotton cultivars for endosphere fungi; the relative difference in abundance is expressed as log2FoldChange with the values indicating that the relative abundance of specific OTUs was greater in the wilt tolerant cultivar samples than in susceptible cultivar samples. The size of nodes represents the abundance of endosphere fungi at the specific taxonomic rank. Those OTUs that could not be assigned reliably to a taxonomic rank below Kingdom have been excluded from the graph. The graph is drawn with the R package—Metacoder (Foster et al., 2017).

Verticillium wilt resistance is mediated by quantitative trait loci and such quantitative traits can be considerably influenced by other factors, including environmental conditions and plant-associated microbiota. The composition of plant microbiome is influenced by many factors, including genotypes, plant developmental stage, and plant health (Berg et al., 2016). For instance, endophytic bacterial communities differed among cultivars (genotypes) of potato (*Solanum tuberosum* L.) (Sessitsch et al., 2004; Manter et al., 2010; Ardanov et al., 2012) and common bean (*Phaseolus vulgaris*) (de Oliveira Costa et al., 2012). This genotypic association is usually interpreted as the consequence of recruitment of specific microbes through characteristic root exudates. Structural and functional diversity of plant-associated microbiome can also greatly be affected by soil physical properties and nutrient availability (Berg and Smalla, 2009). The host-genotypic microbiome association has not yet been specifically explored in commercial agriculture.

In the present study, within-sample (alpha) diversity of nine cotton cultivars appears to have no relationships with wilt susceptibility, except bacterial endophytes: resistant cultivars

appears to have more endophytic bacterial OTUs, and hence higher Chao1 (species richness) value. Similarly, a previous study reported that there were no significant differences in the abundance of isolated fungal endophytes between resistant cotton cultivars and susceptible cultivars (Li et al., 2014). However, the alpha diversity of endophytic bacteria and the abundance of culturable bacteria were both higher in the peach roots of a crown gall disease resistant cultivar than a susceptible cultivar, particularly after inoculation (Li et al., 2019).

Microbial diversity in the rhizosphere is crucial for suppressing soil-borne disease development and a higher abundance of rare species also seems to represent a barrier against soil-borne pathogens (Latz et al., 2012; van Elsas et al., 2012). Plant species and genotype are still significant factors determining composition of microbial communities resident to the rhizosphere and soils (Mazzola, 2004). We demonstrated that a large proportion of genotypic differences in plant-associated microbial community structures was associated with their resistance/susceptibility to *V. dahliae*. Present results suggest that apparent cultivar resistance to wilt may result partially

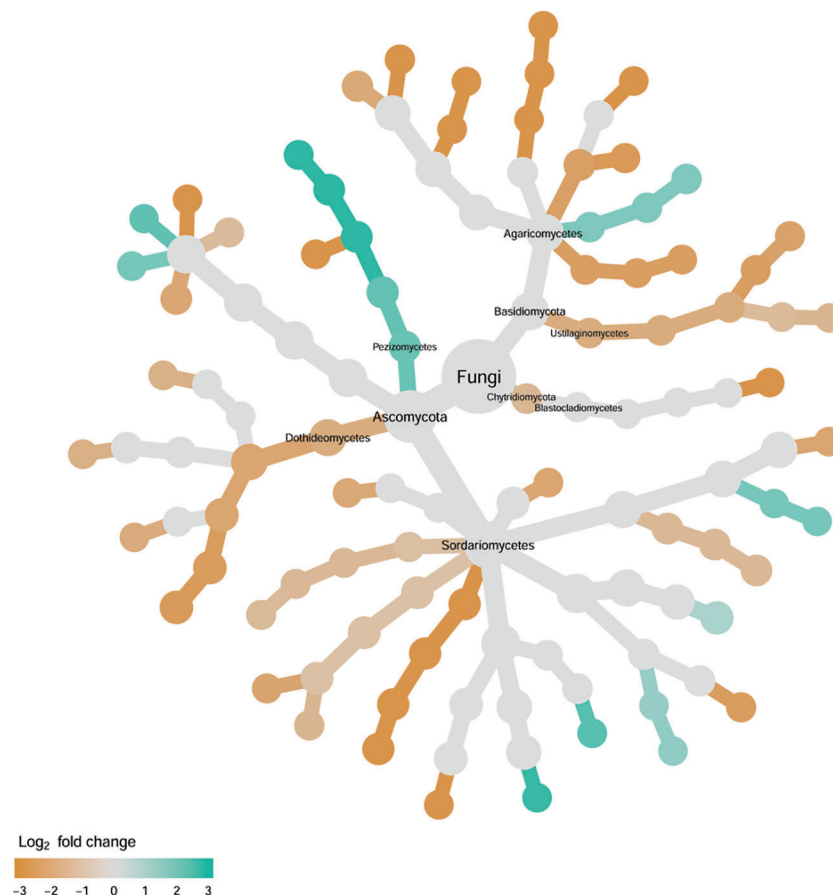


FIGURE 9 | Tree views of the relative abundance between wilt-tolerant and wilt-susceptible cotton cultivars for rhizosphere fungi; the relative difference in abundance is expressed as log2FoldChange with the values indicating that the relative abundance of specific OTUs was greater in the wilt tolerant cultivar samples than in susceptible cultivar samples. The size of nodes represents the abundance of rhizosphere fungi at the specific taxonomic rank. Those OTUs that could not be assigned reliably to a taxonomic rank below Kingdom have been excluded from the graph. The graph is drawn with the R package—Metacoder (Foster et al., 2017).

from abundant beneficial microbes in the rhizosphere. A similar finding was also obtained for the association of the rhizosphere microbial community with cucumber resistance to *Fusarium* wilt (Yao and Wu, 2010). Rhizosphere microbiome structures of tomato plant differed between resistant (resistant to *Ralstonia solanacearum*) and susceptible cultivars (Kwak et al., 2018). These results suggest that plant-microbiome may contribute to the observed host resistance/susceptibility against specific pathogens.

In addition to the differences in the overall rhizosphere and endosphere microbial communities, we identified many specific bacterial and fungal groups that have differential relative abundance between wilt resistant and susceptible cotton cultivars. Although it was not possible to classify many of those groups to the rank of genus or species, most of those identified to lower taxonomic ranks appear to have plausible biological interpretations. Thus, wilt tolerance is associated with commonly known beneficial bacteria, including *Bacillus* (Egamberdieva, 2016), *Lysobacter* (Sullivan et al., 2003), *Streptomyces* (Niu et al., 2016), Rhizobiales (Erlacher et al., 2015) and *Pseudomonas* (Thierry et al., 2004). In addition, bacterial endophytes *Azoarcus* play an important role in N₂-

fixation in natural plant ecosystems (Franché et al., 2009). All nine Xanthomonadales were enriched in susceptible cultivars; the *Xylella* and *Xanthomonas* species in Xanthomonadales cause serious diseases in more than 400 agriculturally important plant species (Naushad and Gupta, 2013). Many fungal groups had increased relative abundance in wilt susceptible cultivars, including fungal endophytes of *Alternaria solani*, *Aspergillus aculeatus*, *V. longisporum*, and *Choanephora*, and rhizosphere fungi of *Alternaria*, *Magnaporthe grisea*, *Thielaviopsis basicola*, Ceratobasidiaceae, and Ustilaginaceae. *Alternaria* spp. and *Thielaviopsis basicola* are both known pathogens of cotton (Pullman et al., 1981; Coumans et al., 2009) and *Rhizoctonia*, of the Ceratobasidiaceae family, can cause damping-off of cotton seedlings (Pullman et al., 1981). *V. longisporum* could cause wilt diseases on cruciferous hosts (Zeise and Von Tiedemann, 2002). In wilt resistant cultivars, both *Microascus brevicaulis* and *Trichoderma* had higher abundance. *Trichoderma* spp. are the most important fungal biocontrol agents for controlling a number of plant diseases, including *Verticillium* wilt (Harman et al., 2004). In addition to being a typical soil decomposer, it is well known that *M. brevicaulis* lives within the American dog

tick (*Dermacentor variabilis*); this relationship seems to be highly adapted but not as a typical host-parasite interaction. Studies have shown that *M. brevicaulis* in the form of endosymbionts exist in the host, which may provide protection against the insect-pathogenic fungus *Metarhizium anisopliae* (Yoder et al., 2008). Further research is needed to isolate and confirm which bacterial and fungal OTUs associated with *V. dahliae* resistance.

The greenhouse trial with sterilized soil strongly suggested that the beneficial microbes in the rhizosphere are partially responsible for reduced wilt development in the three 'resistant cultivars'. Without these beneficial organisms, the differences between 'resistant' and 'susceptible' cultivars (as defined based on the field results) are much smaller and average cultivar wilt index is more or less in a continuum without large differences between 'susceptible' and 'resistant' cultivars. However, it was not possible to estimate possible contributions by endophytes to observed wilt differences between cultivars.

Although the use of classical single-strain biocontrol products for the management of soil-borne disease has long been a goal in commercial agriculture, there are limited examples of successful application in commercial field crop production systems (Mazzola and Freilich, 2017). Recently, there is a growing trend of designing consortia of multiple beneficial microbes for managing soil-borne diseases (Lebeis et al., 2015) and improving nitrogen use in crops (Zhang et al., 2019). The present results support this microbial consortium approach since multiple beneficial microbes were associated with wilt resistant cultivars. We have been characterizing several microbial groups (*Pseudomonas*, *Bacillus*, *Trichoderma*, etc.) for their effects on wilt development, and will further evaluate their joint effects on wilt suppression in commercial fields. Furthermore, we may need to investigate interactions of multiple cotton pathogens on wilt development since wilt susceptible cultivars are associated with high abundance of other candidate pathogens in rhizosphere and endosphere.

CONCLUSION

The present study demonstrated that plant genotype contribute to the shaping of the plant-associated microbial community and specific groups of rhizosphere microbiota and root endophytes may associate with cotton resistance to *V. dahliae* when sampled at the boll-forming stage. Such an association is stronger for bacteria than for fungi. Many individual microbial OTUs differ in their relative abundance between wilt resistant and susceptible cultivars. These OTUs included several well-known taxonomy groups containing beneficial microbes, such as Bacillales, Pseudomonadales, Rhizobiales, and *Trichoderma*, with higher relative abundance associated with resistant cultivars.

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Greenhouse data supported that beneficial microbes in rhizosphere contribute to reduced wilt development. These findings suggested that specific rhizosphere and endosphere microbes may contribute to cotton resistance to *V. dahliae*.

DATA AVAILABILITY STATEMENT

Raw sequence data reported in this paper have been deposited (PRJEB32779) in the European Nucleotide Archive (ENA).

AUTHOR CONTRIBUTIONS

FW, XX, ZF, and HZ planned and designed the research and experiments. FW, LZ, HF, YS and ZF performed the experiments. FW, XX, and GD analyzed the data. FW and XX wrote the manuscript. FW and HZ acquired the funds for the study. 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/fpls.2019.01659/full#supplementary-material>

FIGURE S1 | Alpha diversity for rhizosphere bacteria of nine cotton cultivars, classified into wilt tolerant or susceptible groups. Permutation ANOVA was applied to assess the differences in Chao1, Simpson and Shannon indices between the wilt-tolerant and susceptible cultivars.

FIGURE S2 | Alpha diversity for endosphere bacteria of nine cotton cultivars, classified into wilt tolerant or susceptible groups. Permutation ANOVA was applied to assess the differences in Chao1, Simpson and Shannon indices between the wilt-tolerant and susceptible cultivars.

FIGURE S3 | Alpha diversity for endosphere fungi of nine cotton cultivars, classified into wilt tolerant or susceptible groups. Permutation ANOVA was applied to assess the differences in Chao1, Simpson and Shannon indices between the wilt-tolerant and susceptible cultivars.

FIGURE S4 | Alpha diversity for rhizosphere fungi of nine cotton cultivars, classified into wilt tolerant or susceptible groups. Permutation ANOVA was applied to assess the differences in Chao1, Simpson and Shannon indices between the wilt-tolerant and susceptible cultivars.

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Formulation of Microbial Inoculants by Encapsulation in Natural Polysaccharides: Focus on Beneficial Properties of Carrier Additives and Derivatives

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In the last 10–15 years, the wide application of bioformulated plant beneficial microorganisms is accepted as an effective alternative of chemical agro-products. Two main problems can be distinguished in their production and application: (a) economical competitiveness based on the overall up-stream and down-stream operational costs, and (b) development of commercial products with a high soil-plant colonization potential in controlled conditions but not able to effectively mobilize soil nutrients and/or combat plant pathogens in the field. To solve the above problems, microbe-based formulations produced by immobilization methods are gaining attention as they demonstrate a large number of advantages compared to other solid and liquid formulations. This mini-review summarizes the knowledge of additional compounds that form part of the bioformulations. The additives can exert economical, price-decreasing effects as bulking agents or direct effects improving microbial survival during storage and after introduction into soil with simultaneous beneficial effects on soil and plants. In some studies, combinations of additives are used with a complex impact, which improves the overall characteristics of the final products. Special attention is paid to polysaccharide carriers and their derivatives, which play stimulatory role on plants but are less studied. The mini-review also focuses on the potential difficulty in evaluating the effects of complex bio-formulations.

Keywords: biofertilizers, formulation, immobilization, polysaccharides, additives

INTRODUCTION

Different groups of soil microorganisms, such as root endophytic fungi, mycorrhizal fungi, plant growth-promoting rhizobacteria, rhizobia, and phosphate solubilizers affect plant growth through direct and plant-mediated mechanisms, including in stressed conditions (van der Heijden et al., 2008; Berg, 2009; Shilev et al., 2019). The application of selected plant beneficial microorganisms individually or as microbial consortia with multifunctional properties is an important tool to

promote crop health and productivity (Ahmad et al., 2018; Maron et al., 2018). The scientific literature abounds in studies on isolation and characterization of plant-beneficial microorganisms, but only few of them have reached the commercial market. Many commercial bio-inoculants do not work under field conditions with the efficiency demonstrated in greenhouse or laboratory experiments (Stephens and Rask, 2000; Vassilev et al., 2015; Arora and Mishra, 2016; Malusá et al., 2016) due to inadequate and/or poor quality formulation, including poor compatibility and stability of the carriers (Bhattacharyya and Jha, 2012; Bashan et al., 2016; Baez-Rogelio et al., 2017; Stamenkovic et al., 2018).

The main roles of the formulation of inoculants are: (i) to provide a more suitable micro-environment for the microbial strain/s, combined with physical or chemical protection over a prolonged period, in order to avoid a rapid decrease of the cells' viability during storage, (ii) to support the strain/s competition with the better-adapted native soil microflora, and (iii) to reduce losses due to the depredation by the micro-fauna after being introduced into soil. All these functions are aiming at providing a reliable source of living cells available to interact with plants and soil microbiome (Bashan, 1998; Herrmann and Lesueur, 2013; Bashan et al., 2014; Malusá and Vassilev, 2014). Indeed, a critical number of cells are essential to obtain the expected positive response from the formulated inoculum (10^6 – 10^7 cells/plant; Bashan, 1986).

Different microbial formulations have been developed using liquid or solid materials as carriers. Liquid inoculants are microbial cultures modified with water, oil or polymers (i.e., additives) that improve cell-suspension viscosity, stability and dispersion capacity (Catroux et al., 2001; Bashan et al., 2016; Malusá et al., 2016). The problem with this type of products is that the microbial population and its metabolic activity decrease rapidly after the introduction of cell suspensions into the soil, particularly if they are not containing suitable additives. A special attention has been paid in the recent years on cell-free formulations (Bashan et al., 2016) like fermentation broth filtrates (Kumar et al., 2012; Vinale et al., 2014; Vassilev et al., 2017). Since some plant beneficial microorganisms demonstrated multiple activities (Vassileva et al., 2010), their culture extracts contain various metabolic products such as antibiotics, siderophores, toxins, lytic enzymes (Thrane et al., 1997; Aydi-Ben Abdallah et al., 2014), and solubilized phosphate (Mendes et al., 2017; Vassilev et al., 2017), which positively affect the plant growth. Such type of products and the related strategy can be denominated as post-biotic.

The solid formulations are based on inorganic or organic carriers, prepared in solid, granular, or powdery forms and classified according to their particle sizes or application mode (Adholeya and Das, 2012; Malusá et al., 2012; Stamenkovic et al., 2018). The most important solid formulations are based on carriers such as peat, compost, agro-industrial wastes, vermiculite, perlite, rock phosphate, calcium sulfate, and polysaccharides (Sahu and Brahmaaprakash, 2016). In the recent years, in the field of solid formulation technologies, more attention is paid to polysaccharide-immobilized inoculants (Malusá et al., 2016) as well as to inoculants produced under

solid-state fermentation (SSF) conditions using agro-industrial wastes (Vassilev and Mendes, 2018). SSF processes offer many advantages including co-cultivation of two microorganisms, enrichment with soluble P (Mendes et al., 2015), induction of biocontrol activity (Vassilev et al., 2009), as well as the use of solid substrates alone, combined, and moistened with liquid wastes (Vassilev and Mendes, 2018). However, the gel-cell immobilized approach is the technological solution that can better assure a standardization of the formulated inoculum as well as its quality.

In this mini-review, we analyze the immobilized-cell approach underlying the possibilities for its improvement and some specific characteristics of the carrier structure and formulation, particularly the role of additional compounds introduced into the cell-gel structures and the effect of the gel-forming polysaccharides and their derivatives on plant health and growth.

CELL-IMMOBILIZATION AS A TOOL FOR INOCULANT FORMULATION

In bio-immobilization technology, water-soluble polymeric materials such as agar, methoxy-pectin, gellan gum, and mixtures of xanthan and locust bean gum, among many others, are largely used in the production of microbial-based products but alginate and carrageenan are the most used polymer-forming materials in microbial formulations to be introduced into soil-plant systems (Bashan, 1998; Vassilev et al., 2001, 2005, 2014). The most frequently applied method of microbial cells/spores encapsulation uses the technique of interfacial polymerization.

There are a number of review papers describing in details the advantages and the “know-how” of the immobilization technology applied in formulation of plant beneficial microorganisms (Vassilev et al., 2001, 2005, 2014, 2015; Malusá et al., 2012; Bashan et al., 2016; Stamenkovic et al., 2018). Despite obvious benefits of immobilized-cell formulations of plant beneficial microorganisms having a controlled cell-release, their large-scale production and field application are still limited. One of the main reasons is the relatively high production cost (Vassilev et al., 2001; Chen et al., 2013; Bashan et al., 2016), since the cost of the polymeric carrier is higher than the other solid and liquid formulation components (John et al., 2011). Furthermore, the structure of a polymer carrier (e.g., that of alginate) is characterized by a low mechanical strength, which determines an unstable, uncontrolled release of its content. Cell mortality during the drying of encapsulated cells has also been recognized as a critical point of the bioencapsulation process (Cassidy et al., 1996; Bashan et al., 2002).

A future frontier in this field and one of the solutions of the above problems is the development of *polymeric nanoparticle coatings* (nano-formulations) or microencapsulated formulations. Microcapsules formulated by Wu et al. (2011) enhanced the survival rate of *Klebsiella oxytoca* Rs-5 under salinity stress. The cells released from microcapsules reached up to 10^{10} cfu/g when immersed in physiological saline solution for 3 weeks, improving cotton growth under high salinity conditions in pot experiments. However, there is the need to fully evaluate

environmental and health safety issues before such technology could be implemented at industrial level (Kah, 2015).

Another possibility to develop a cost-effective encapsulated formulation is to find a low-cost gel carrier or gelling agent or partly replace the expensive polymer with low-cost additives. Nano-additives might enhance the stability of microbial-encapsulated products with respect to environmental conditions (e.g., desiccation, heat and UV inactivation) or provide substances needed by the inoculum and consequentially improve the shelf-life of these products or their delivery (Jampilek and Králová, 2017; Prasad et al., 2017). **Table 1** illustrates the beneficial effects of some additives.

THE ROLE OF ADDITIVES ON THE OVERALL PERFORMANCE OF IMMOBILIZED INOCULANTS

Clay Minerals

There is a wide selection of additional materials used in bio-immobilized systems, which can serve as carrier bulking agents, enhance the formulation stability, protect and feed microbial cells or spores. Since the early studies on gel-entrapped soil microbial inoculants, polysaccharides/clay minerals combinations were used to protect the immobilized/encapsulated cells and to ensure their slow release into the environment (Marshall, 1968; Jung et al., 1982; van Elsas et al., 1992; Vassileva et al., 1999; Bashan et al., 2002). *Clay minerals* such as pyrophyllite have been experimented as bulking agents (Fravel et al., 1985) and bentonite and kaolin were used as fillers in alginate-glycerol immobilized *Pantoea agglomerans* and *Trichoderma harzianum* (Zohar-Perez et al., 2003). The freeze-dried alginate-bentonite and alginate-kaolin combinations had a considerable positive effect on the bead's average wall thickness and significantly increased

microbial survival reducing UV transmission compared to free-cell and cells immobilized in alginate-glycerol without fillers.

The addition of bentonite to alginate-based formulation was found to increase the solid content and the porosity of alginate polymer used as a carrier of *Raoultella planticola* (He et al., 2015). Without bentonite, the release of the immobilized microbial cells was rapid in the first 3-day period followed by a constant cell release, while the presence of the additive regulated the continuous flow of the microorganism to the soil. *Pseudomonas putida* Rs-198 microencapsulated with a mix of alginate, bentonite and starch was reported to increase cotton biomass, soluble protein content, and chlorophylls a, b and carotenoid concentrations of cotton grown under saline conditions (He et al., 2017).

Liffourrena and Lucchesi (2018) applied perlite as filler of alginate microbeads formed in CaCl₂ – paraffin emulsion mixture to formulate *P. putida* biostimulant. The number of cells reached 10⁸ CFU/g micro-beads and the increase in cell-gel mechanical stability was proportional to perlite concentration. This amount was sufficient to colonize *Arabidopsis thaliana* rhizosphere, with an increase in colonization over time from 2.1 × 10⁴ to 9.2 × 10⁵ CFU/g soil after 21 days.

Skim Milk

Skim milk is another additive widely used in bioformulations to enhance cell viability after storage (Yu et al., 2001). Bashan et al. (2002) found that the addition of skim milk powder to alginate-encapsulated *Azospirillum brasilense* significantly increased the cell number within the cell-bead structure. These beads degraded faster in soil than beads without skim milk thus releasing rapidly the entrapped cells into the soil-plant system. Alginate carrier with 10% skim milk significantly increased the numbers of *Pseudomonas fluorescens* cells released into the soil compared to combinations with soil extract and control beads

TABLE 1 | Examples of beneficial effect of additives on inoculant gel-based formulations.

Microorganism	Additive	Beneficial effect	References
<i>Pseudomonas cepacia</i> ; <i>Talaromyces flavus</i> ; <i>Penicillium oxalicum</i> ; <i>Gliocladium virens</i> ; <i>Trichoderma viride</i>	Pyrophyllite	Bulking agent	Fravel et al., 1985
<i>Pantoea agglomerans</i> ; <i>Trichoderma harzianum</i>	Chitin, kaolin or bentonite	Reduced UV transmission	Zohar-Perez et al., 2003
<i>Raoultella planticola</i>	Bentonite	Continuous cell release	He et al., 2015
<i>P. putida</i>	Perlite	Cell-gel stability	Liffourrena and Lucchesi, 2018
<i>Azospirillum brasilense</i>	Skim milk	Increased cell number in beads	Bashan et al., 2002
<i>P. fluorescens</i>	Skim milk	Increased cell number and soil; enhanced cell viability	Power et al., 2011
<i>Enterobacter sp.</i>	Skim milk	Better mycorrhization	Vassileva et al., 1999
	Skim milk and montmorillonite	Higher cell survival rate	Vassilev et al., 1997
<i>Pseudomonas aeruginosa</i>	Skim milk and clay minerals	Higher plant growth promotion	Cassidy et al., 1995
<i>Fusarium oxysporum</i>	Starch	High cell viability, shelf life, and soil colonization	Bailey et al., 1998
<i>A. brasilense</i>	Starch	Extended shelf life	Ivanova et al., 2005
<i>Penicillium janthinellum</i>	Chitin and dry dry olive wastes	Chitinase synthesis; biocontrol activity	Vassilev et al., 2008
<i>Bacillus subtilis</i>	Humic acids	Higher survival rate	Young et al., 2006
<i>Raoultella terrigena</i>	Trehalose	Desiccation protection	Schoebitz et al., 2013
<i>Bacillus salmalaya</i>	Protein hydrolysate	High encapsulation index	Vejan et al., 2018

(Power et al., 2011). After 250 days of storage, 100% recovery of viable cells was obtained from skim milk-alginate encapsulated *P. fluorescens*. *Enterobacter* sp. encapsulated in alginate gel enriched with 3% skim milk stimulated plant mycorrhization and demonstrated better bacterial establishment and phosphate-solubilizing activity in soil (Vassileva et al., 1999). This resulted in both higher growth of *Lactuca sativa* and higher number of cells released in the soil in comparison with plants inoculated with formulations without skim milk.

Designing complex formulations containing both skim milk and clay materials can be a strategy to increase the inoculum efficacy in comparison to single additives. Bentonite clay (3% w/v) was found to increase the positive effect of skim milk powder (3% w/v) on the survival rate of *P. fluorescens* R2f encapsulated in alginate (van Elsas et al., 1992). In another study, with *lac-lux* marked *P. aeruginosa*, a 1% κ -carrageenan amended with skim milk and bentonite:montmorillonite (60:40%) was more effective compared to alginate-skim milk formulation particularly after a 3-month storage of the dried beads (Cassidy et al., 1995). Vassilev et al. (1997) used skimmed milk and clay as additives to enhance metabolic activity in fermentation and soil conditions as well as the survival rate of the P-solubilizing *Enterobacter* sp. entrapped in agar beads.

Starch

Starch has been well studied in various biotechnological schemes with dried beads or liquid core capsules (Jankowski et al., 1997; Kim et al., 2005). It has successfully been used as a carrier or additive in formulations of plant beneficial microorganisms. In the bioencapsulation matrix, starch reduced the physical stress to microbial cells and significantly improved their survival (Bashan et al., 2002). *Bacillus thuringiensis* was entrapped in gelatinized corn starch (Shasha et al., 1984), which coupled with broad-band UV screens such as Congo red provided protection from solar radiation (Dunkle and Shasha, 1989). The effectiveness of this system in conidia formation per gram of mycelium was confirmed with the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana* (Pereira and Roberts, 1991). Complex formulations based on alginate-starch were used to formulate myco-herbicidal strains of *Fusarium oxysporum* (Bailey et al., 1998), which showed high viability/shelf-life and rhizosphere colonization rate. Two endophytic fungi (*Muscador albus* and *Muscador roseus*) producing volatile myco-fumigants were formulated in a mixture of water-absorbent starch, corn oil, sucrose, and fumed silica (Stinson et al., 2003). The produced formulations reduced the disease incidence of soilborne pathogens but plant growth reduction was observed probably due to the growth of deleterious rhizobacteria on some components of the complex carrier.

The protective effect of starch on the microbial cells under stress conditions is based on the cell adhesion to the starch. This process depends on the strain and the relationship between the adhesion to the starch and its use as a substrate (Crittenden et al., 2001). Furthermore, Tal et al. (1999) reported that the strength of an alginate-starch bead is directly proportional to its starch content and the distribution of starch granules within the beads is homogeneous at higher starch concentration. Even though the

porosity of the beads' structure decreases with an increase in their starch content, the opposite tendency is observed after a period of storage when the porosity increases as the immobilized cells utilize the starch. The later phenomenon resulted in bacterial population levels of up to 10^9 CFU/bead in dry alginate-starch beads (Ivanova et al., 2005).

Chitin and Chitosan

Chitin and chitosan are oligosaccharides used in formulations as fillers or coating material, respectively. Chitosan is a bioactive polymer with a wide variety of functional properties such as antibacterial activity, non-toxicity, ease of modification, and biodegradability (Muxika et al., 2017). Addition of chitin or chitin-containing materials improved the multiplication of *Bacillus subtilis* and its fungicidal activity to control Fusarium wilt (Manjula and Podile, 2001). Chitin and dry olive wastes (DOW) were mixed with alginate to encapsulate *Penicillium janthinellum* (Vassilev et al., 2008). The fungus showed higher chitinase synthesis compared to alginate-entrapped mycelium, even when this was added singly with DOW or chitin. The three-component formulation induced P-solubilizing fungal activity while alginate-chitin formulation exhibited biocontrol activity suppressing the soil-borne pathogen *F. oxysporum*. The use of chitin/chitosan to encapsulate microbes can also ease the storing and application on farms, which has been one of the major restriction to the use of biopesticides in recent times (John et al., 2011).

Chitosan is an excellent chelating agent, well known for its biocontrol activity against pathogens (Goy et al., 2009; Franco and Peter, 2011; Berger et al., 2014) and as elicitor enhances stress tolerance, antioxidant activity, and production of osmoregulators in plants (Dar et al., 2015). As a coating material, chitosan can lower the formulation cost, making the final product multifunctional due to its biocontrol and plant strengthening activities. The formulations can be produced by dropping alginate in a chitosan-CaCl₂ solution or introducing already formed alginate beads into chitosan solution (Wittaya-Areekul et al., 2006).

Chitosan can also be an excellent carrier for plant beneficial microorganisms (Chanratana et al., 2018). Applying the methodology used for the development of a controlled-release fertilizer (Perez and Francois, 2016), where starch was added to a chitosan-based formulation as a filler, using a sodium tripolyphosphate aqueous solution as the crosslinking agent, *A. brasilense* and *P. fluorescens* were encapsulated in chitosan-starch formulation (Perez et al., 2018). The formulated bacteria survived at least 12 months at room temperature and humidity, maintaining a high viability (10^9 CFU of *A. brasilense*/g and 10^8 CFU of *P. fluorescens*/g). When introduced in soil, the bacterial cell number increased progressively during the first 20 days and then decreased.

Humic Acids

Humic products are known to promote or decrease the populations or activities of specific microbiome species (Pukalchik et al., 2019). Encapsulation of *B. subtilis* in alginate beads supplemented with humic acids ensured high viability of the immobilized biostimulant (Young et al., 2006). The

immobilized gel-humic acid-cell system demonstrated excellent survival rate after storage for 5 months and slow cell release at various levels of pH, providing also successful plant growth promotion by the encapsulated bacteria. The positive effect of this additive on plant growth can be explained considering its role as stimulant of the microbial growth and activity (Rekha et al., 2007) as well as for its effects on the physiology of plants (Nardi et al., 2002). The addition of humic acids in bacteria formulations could be also useful to promote root colonization by native mycorrhizal fungi (Gryndler et al., 2005).

Sugars

Sugars, such as sucrose, trehalose or glucose, are widely used to preserve microorganisms from changes in the osmotic pressure and can contribute to their conservation and maintenance particularly after drying (Morgan et al., 2006). However, combinations between sugars and sugars-additives have not been studied before and after the formulation of microbial-based products although various types of sugars and other additives (see previous paragraphs) usually improve the overall encapsulation efficacy. The addition of trehalose to the growth medium increased the survival of *Raoultella terrigena* during the drying process much more effectively protecting against desiccation than adding it to the matrix solution just prior to drying (Schoebitz et al., 2013). The complex formulation of *B. bassiana* based on the use of skimmed milk powder, polyvinylpyrrolidone K-90, and glucose was reported to achieve 100% conidial germination and 78% conidial viability, even after storage for 12 months at 30°C (Mishra et al., 2013).

Protein Hydrolysates

Protein hydrolysates derived from animal wastes and plant biomass after chemical, thermal and enzymatic hydrolysis, have been shown to enhance both nutrient uptake by plants and soil microbial activity (Colla et al., 2017; Casadesús et al., 2019). The latter was suggested to be the result of the stimulating presence of the organic molecules in protein hydrolysates, which serve as nutrients for the rhizospheric and phyllospheric microorganisms (Colla et al., 2017). Vegetable protein hydrolysates received more research interest particularly as co-polymers in microcapsules in the food, pharmaceutical and cosmetics industries (Nesterenko et al., 2013) but also in biostimulant production (Colla et al., 2015). In a recent work, *B. salmalaya* was encapsulated in chitosan-alginate-protein (brown rice) capsules, formulated in slurry or powder achieving an encapsulation index of 99.7 and 89.3%, respectively (Vejan et al., 2018). Such result underlines the importance of additives based on vegetable proteins in future studies on formulation of biostimulants by gel-encapsulation.

Glycerol, Silicon, Poly-Lactic Acid, and Strigolactones

Some compounds with well-manifested functions advantageous to microorganisms could also be considered as potential additives. In a recent article we have analyzed the potential of *glycerol*, a trihydroxyalcohol widely used as a cell viability protector in strains' maintenance practice, in this respect and

suggested the need for more studies on its application in formulation techniques (Vassilev et al., 2017). Similarly, *silicon* has not found wide applications as biostimulant to plants yet, particularly in encapsulated-cell formulations, although its benefits were widely reviewed (Savvas and Ntatsi, 2015). Trials using hydrophobic silica nanoparticles to the water-in-oil emulsion have shown an improvement in the delivery of the product, as well as an enhancement in shelf life by reduction of desiccation (Kaushik and Djiwanti, 2017). The use of new polymer-forming materials such as *poly-lactic acid* (PLA) could also open new possibilities to develop encapsulated inocula that would benefit of the physical characteristics of these compounds (Lai et al., 2009). *Strigolactones* (synthetic analogs), which communicate with the plant-microbiota systems, have been suggested as potential active additives (Vassilev et al., 2015) but as they demonstrate stimulating signals to parasitic plants and microorganisms (De Cuyper and Goormachtig, 2017), it would be challenging to develop an effective complex gel-based biostimulants.

POTENTIAL EFFECT OF THE GEL-FORMING POLYSACCHARIDES ON PLANT HEALTH AND GROWTH

Encapsulation of inoculant cells in polymers of polysaccharides such as alginate and carrageenan has been proposed long time ago as a technique to ensure controlled release of plant beneficial microorganisms into soil (Dommergues et al., 1979; Bashan, 1986). Surprisingly, few studies have examined simultaneously the fate of the gels in soil and the effect of the cell-free carriers on plants and rhizosphere microbiota. The positive effect on plants of seaweed crude extracts, the raw material from which several polysaccharides used for encapsulation technology derive, is based on the synergic action of growth regulators, osmolytes, polysaccharides and other algal compounds (Battacharyya et al., 2015). Seaweeds are known for their action as bioelicitors and particularly laminarin, carrageenan, and alginate, have been studied for their plant defense stimulating effects (Chandía et al., 2004; Khan et al., 2009; El Modafar et al., 2012; Vera et al., 2012; Zhang et al., 2015; Abouraicha et al., 2017; Ben Salah et al., 2018). There are strong evidences that polysaccharides play an important role in the mechanisms of abiotic stress protection for microorganisms (Vassilev et al., 2012). The production of alginate as exopolysaccharide increased in bacteria growing under drought conditions (Sa et al., 2019) creating a hydrated microenvironment contributing to biofilm architecture (Chang et al., 2007).

Particularly attractive for increasing plant growth and health are oligosaccharides derived from natural polysaccharides as they play the role of signal molecules regulating plant development and defense (Larskaya and Gorshkova, 2015). They can be obtained by enzymatic (Murata et al., 1993) and acidic depolymerization (Haug et al., 1966), and thermal polysaccharide treatment (Aida et al., 2010). Oligosaccharides produced by different methods demonstrated different physiological activities in animal cells (Iwamoto et al., 2005), but this phenomenon

has not been widely studied on soil-plant-microbiota systems. A great part of studies on the plant growth promoting effect of oligosaccharides are performed after γ -irradiation of polysaccharides such as chitosan, κ -carrageenan and alginate. In this latter case, the effect of the resulting products on plants was higher when the irradiation was performed in solid-state compared to liquid solution (Hien et al., 2012). Oligochitosan, obtained after gamma-irradiation, was defined as a growth stimulator and anti-microbial agent for various plant systems (Muley et al., 2019a) including under conditions of drought stress (Muley et al., 2019b). Oligosaccharides (galacto-, isomalto-, fructo-, and xylo-) used as a part of alginate gel beads were reported to enhance cell viability of oral *Lactobacillus fermentum* and carrier stability when exposed to the specific environmental conditions (Liao et al., 2019). Similar inclusion of oligosaccharides should be expected in the near future in formulations of plant biostimulants. Other studies should be carried out on the behavior of the polysaccharide carriers in soil-plant systems, particularly to unravel in more details their degradation processes in soil by plants and/or microorganisms producing polysaccharide-cleaving enzymes. Such studies could help addressing the question on how the soil microbiota and plants are affected by the polysaccharide derivatives, including oligosaccharides, composing the formulation of microbial-based products.

CONCLUSION

Significant progress has been made in developing formulations of plant beneficial microorganisms by entrapment in natural water-soluble polymer-based carriers and their application as biostimulants. However, published reports often do not consider or discuss the changes in the carrier characteristics by the entrapped cells or additives. There are indications that changed properties of polymers in presence of additives positively affect their ability to maintain and protect the microorganisms. Moreover, in many cases the additives potentially affect plant growth and health and simultaneously

induce microorganisms to release metabolites thus provoking changes in the typical gel structure and integrity. The resulting microbial and gel side-products might stimulate plant growth and exert biocontrol activity. In some cases, it has been shown that additives are exerting a negative effect on the whole cell encapsulation system. Mannitol was reported to decrease germination of gel-encapsulated spores (Liu et al., 2015). Vivekanandan and Jauhri (2000) found that charcoal-soil, mixed with alginate, adversely affected the loading and survival of phosphate-solubilizing bacteria. Therefore, a deeper analysis of the relationship carriers-additives-microorganisms-soil-plant systems can provide important information that is essential to understand the functional characteristics of immobilized biostimulants and determine strategies for their application. Research efforts should also be oriented toward development of micro-environmental conditions to facilitate the growth and functional activity of the bioformulates, including in the carriers specific prebiotic compounds. As we repeatedly pointed out, research scientists working with immobilization methods should use techniques already proven in other biotechnological fields. Further improvement of immobilized cell methodologies should be based on multidisciplinary research of wide number of experts in microbiology, plant physiology/pathology, formulation specialists and agricultural engineers in order to provide efficient, safe, economically acceptable, and easy to apply complex biotechnological products for plant growth and health.

AUTHOR CONTRIBUTIONS

NV and MV designed and drafted the work. EM, LG, VM, JK, and BT contributed to the revision of the manuscript.

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Comparative Analysis of Rhizosphere Microbiomes of Southern Highbush Blueberry (*Vaccinium corymbosum* L.), Darrow's Blueberry (*V. darrowii* Camp), and Rabbiteye Blueberry (*V. virgatum* Aiton)

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Plants are inhabited by millions of parasitic, commensal, and mutualistic microorganisms that coexist in complex ecological communities, and profoundly affect the plant's productivity, health, and capacity to cope with environmental stress. Therefore, a better understanding of the rhizosphere microbiome may open a yet untapped avenue for the rational exploitation of beneficial plant-microbe interactions in modern agriculture. Blueberries encompass several wild and cultivated species of shrubs of the genus *Vaccinium* that are native to North America. They are grown commercially for the production of fruits, which are considered a health food due to the rich content of minerals, trace elements, and phenolic compounds with antioxidant, antitumor, and anti-inflammatory properties. Despite a long history of breeding and extensive commercial use, remarkably little is known about the composition and function of the blueberry root microbiome. To address this gap, we employed molecular approaches to characterize and compare microbial communities inhabiting the roots of rabbiteye blueberry (*Vaccinium virgatum*), Darrow's blueberry (*Vaccinium darrowii*), and southern highbush blueberry (SHB; an interspecific hybrid of *Vaccinium corymbosum* and *V. darrowii*). Our results revealed that these plant species share a common core rhizobiome, but at the same time differ significantly in the diversity, relative abundance, richness, and evenness of multiple groups of prokaryotic and eukaryotic microorganisms. Although the host signature effects were especially pronounced at the plant species level, we also observed genotype-level variations in the distribution of specific microbial taxa, which suggests that the assembly of the blueberry microbiome is shaped by the plant genotype and modifications associated with the domestication and breeding of

members of the *Vaccinium* genus. We also demonstrated that the studied *Vaccinium* species differ in the abundance of beneficial rhizobacteria and ericoid mycorrhizal fungi, which play a vital role in their adaptation to soils with low pH and slow turnover of organic matter.

Keywords: *Vaccinium*, blueberry, rhizosphere, microbiome, 16S, ITS, 18S

INTRODUCTION

Plants are meta-organisms that are inhabited by millions of parasitic, commensal, and mutualistic microorganisms that coexist in complex ecological communities (Vandenkoornhuyse et al., 2015). The plant microbiome profoundly affects the development, health, productivity, and capacity of plants to cope with abiotic and biotic stresses and represents an area of active ongoing research. Most plant-associated microbes are found in contact with, or in immediate proximity to plant roots, where they form part of an extended food web driven by the release of plant rhizodeposits, or exudates (Sørensen and Sessitsch, 2006). Plants and their root-associated microbiome (rhizobiome) have both evolved to use their close association for the mutual benefit. The host plant provides rhizosphere microorganisms with a carbon-rich niche formed by the secretion of exometabolites into the soil that directly surrounds roots (Bulgarelli et al., 2012, 2015; Badri et al., 2013). In return, members of the rhizosphere and root microbiota supply plants with macro- and micronutrients, stimulate organ development, suppress pathogens, and modulate levels of stress phytohormones (Bulgarelli et al., 2015).

The makeup and function of the rhizobiome are strongly influenced by soil properties and by the presence and composition of rhizodeposits (Peiffer et al., 2013; Canarini et al., 2019). Root exudates contain a complex mixture of high- (lysates, mucilages, proteins), and low-molecular-weight (carbohydrates, amino acids, organic and fatty acids, phenolics, sterols) metabolites that supply rhizobacteria with carbon, nitrogen, and energy for growth. The amounts and patterns of rhizodeposition change depending on the plant growth stage, environmental factors, and abiotic stressors (Sasse et al., 2018). The profiles of exudation also vary substantially within and between plant species and it is thought that these differences drive the recruitment of specific taxa from the microbial seed bank of the soil (Lareen et al., 2016; Beattie, 2018). Consistent with the idea that plants actively select and shape their root microbiota, comparative analysis revealed significant variations in the composition of root-associated microbial communities across 30 species of angiosperm plants (Fitzpatrick et al., 2018). Interestingly, the structure and function of the rhizobiome are also significantly affected by the process of domestication, and commercial varieties of barley, maize, lettuce, beet, and agave harbor rhizosphere communities that differ substantially from their counterparts in the closely related species of wild plant (Zachow et al., 2014; Bulgarelli et al., 2015; Cardinale et al., 2015; Szoboszlay et al., 2015; Coleman-Derr et al., 2016). Although the available information is limited, several studies reported that domestication changes the rhizomicrobial diversity and affects

the association with beneficial mycorrhizal fungi and nitrogen-fixing rhizobia (Perez-Jaramillo et al., 2016, 2018). Therefore, a better understanding of the impact of plant breeding on the composition and function of the rhizosphere microbiome may open a yet untapped avenue for the rational exploitation of beneficial plant-microbe interactions in modern agriculture.

Blueberries encompass several wild and cultivated species of shrubs of the genus *Vaccinium* L. that are native to eastern North America (Camp, 1945). They are grown commercially for the production of fruits, which are considered a health food due to the rich content of minerals, trace elements, and phenolic compounds with antioxidant, antitumor, and anti-inflammatory properties (Wang et al., 1997; Lobos and Hancock, 2015; Massarotto et al., 2016). The breeding and selection of blueberries began in the early 1900s (Coville, 1937), and three species, Tetraploid lowbush *Vaccinium angustifolium* Aiton ($2n = 4 \times = 24$), tetraploid highbush *Vaccinium corymbosum* L. ($2n = 4 \times = 48$), and hexaploid rabbiteye *Vaccinium virgatum* Aiton ($2n = 6 \times = 72$), constitute the backbone of the current commercial cultivars (Chavez and Lyrene, 2009). Over the past 70 years, the acreage of the highbush varieties has expanded dramatically due to the introduction of the southern highbush blueberry (SHB) with lower chilling requirements (Retamales and Hancock, 2018). The development of SHB cultivars was initiated by crosses between the tetraploid NHB *V. corymbosum* L. to Florida's native diploid blueberry species *Vaccinium darrowii* Camp, but later, native *V. angustifolium* and *V. virgatum* were introduced into breeding programs (Sharpe and Darrow, 1959). These efforts helped to introduce novel adaptation genes and led to the release of several commercial cultivars with improved tolerance to higher soil pH and drought (Finn et al., 1993; Nunez et al., 2015). However, despite significant progress, the expansion of SHB in the Gulf Coast region of the United States is still challenged by fluctuations in temperature, rainfall patterns, UV levels, elevated soil pH, and drought (Lobos and Hancock, 2015).

The ability of the rhizosphere microorganisms to influence plant susceptibility to diseases and fitness in response to water stress, salinization, and soil pollution prompted detailed microbiome studies in numerous crop species (Raaijmakers et al., 2009; Edwards et al., 2015; Mahoney et al., 2017; Pfeiffer et al., 2017). In contrast, most relevant studies in blueberries employed traditional culture-based approaches and focused primarily on the association of *Vaccinium* spp. with ericoid mycorrhizae. The only comprehensive microbiome study focused on the effect of cultural practices on the structure of rhizosphere microbial communities of wild blueberry *V. angustifolium* grown in managed and forest sites in Nova Scotia, Canada (Yurgel et al., 2017, 2018). Hence, despite a long history of breeding and extensive commercial use, remarkably little is known about

the composition and function of blueberry rhizobiome. We hypothesized that the domestication and long history of breeding introduced changes in the structure of blueberry rhizobiome. We tested this hypothesis by comparing the diversity and abundance of bacteria, fungi, and eukaryotic organisms in microbiomes associated with roots of two genotypes each of the SHB (*V. corymbosum* Camp), Darrow's blueberry (*V. darrowii* Camp), and rabbiteye blueberry (*V. virgatum* Aiton).

MATERIALS AND METHODS

Plant Growth Conditions

The study employed three different *Vaccinium* species, including *V. virgatum* Aiton (Vg) ($2n = 6 \times = 72$), *V. corymbosum* L. (SHB) ($2n = 4 \times = 48$), and *V. darrowii* Camp (Vd) ($2n = 2 \times = 24$) (Table 1). The *V. virgatum* species was represented by breeding selections MS 1089 and MS 1408, while *V. corymbosum* was represented by MS 2337 and MS 2276. *V. darrowii* was represented by the wild-type clone B0008 and breeding selection MS 2230. Plants were grown in a greenhouse in a mixture of pine bark mulch and sand (1:1, v/v) with pH 5.2. Briefly, rooted cuttings of all genotypes were transplanted into 1-gallon pots filled with the potting mix and maintained in a greenhouse under 16 h photoperiod at 22/18°C day/night temperature and drip-irrigated daily with 300 mL of water per pot. Every 15 weeks the pots were fertilized with 1 g of the Osmocote® 14-14-14 slow-release fertilizer (ICL Specialty Fertilizers—North America, Dublin, OH, United States). After about 48 weeks of growth, the plants were transferred in a laboratory, carefully uprooted, and processed for the isolation of rhizosphere soil DNA. The pH of potting mix was monitored with a B-213 Twin pH meter (Horiba Instruments, Irvine, CA, United States).

Extraction of Rhizosphere Soil DNA and Sequencing of 16S rRNA, ITS, and 18S rRNA Amplicons

Partial root systems were cut from blueberry plants ($n = 6$ per *Vaccinium* genotype), excess soil was removed, and 1 g of excised roots was placed into 50 mL Falcon tubes. Each tube was filled with 20 mL of sterile water and rhizosphere soil was dislodged by a combination of vortexing and treatment in an ultrasonic bath. The resultant soil suspensions were used for the extraction of rhizosphere soil DNA with a DNeasy PowerSoil kit (Qiagen, Germantown, MD, United States). The concentration of the purified DNA was measured using a DNA Quantification kit (Bio-Rad, Hercules, CA, United States) by measuring fluorescence at 460 nm with a Synergy 2 microplate reader (BioTek Instruments, Winooski, VT, United States). The absence of PCR inhibitors in the extracted DNA was verified by PCR with the DreamTaq DNA polymerase (Thermo Fisher Scientific, Waltham, MA, United States) and 16S rRNA-specific universal eubacterial primers 8F and 1492R (Weisburg et al., 1991). The amplification was performed as described by Mavrodi et al. (2018). The samples of purified rhizosphere DNA were shipped for analysis to the Center for Comparative Genomics

and Evolutionary Bioinformatics at Dalhousie University¹. Barcoded amplicons were generated by PCR with the high-fidelity Phusion DNA polymerase (Thermo Fisher Scientific) and primers targeting the V6–V8 region of bacterial 16S rRNA (forward primer B969F: ACGCGHNRAACCTTACC; reverse primer BA1406R: ACGGGCRGTGWGTRCAA) (Comeau et al., 2011), the internal transcribed spacer (ITS) 2 region of fungi [forward primer ITS86(F): GTGAATCATCGAATCTTTGAA; reverse primer ITS4(R): TCCTCCGCTTATTGATATGC] (Op De Beeck et al., 2014), and the V4 region of eukaryotic 18S rRNA (forward primer: CYGCGGTAATTCCAGCTC; reverse primer: AYGGTATCTRATCCTCTTYG). The purification of amplicons, library preparation, and its sequencing on a MiSeq instrument (Illumina, San Diego, CA, United States) using the MiSeq v3 chemistry (2×300 bp) were performed as described by Comeau et al. (2017).

Bioinformatics Analyses

The Illumina sequence data were processed using the Microbiome Helper pipeline (Comeau et al., 2017). The sequence quality was confirmed with the FastQC toolkit², after which the forward and reverse reads were merged with PEAR v 0.9.10 (Zhang et al., 2014) followed by filtered out the low-quality and chimeric reads with FASTX-Toolkit v 0.0.14³ and USEARCH v 6.1 (Edgar et al., 2011), respectively. Operational taxonomic units (OTUs) were characterized by matching to the RDP 16S rRNA database (11 release) (Cole et al., 2014) and the UNITE database (12_11 release) of the fungal ITS sequences (Abarenkov et al., 2010), and SILVA dataset of 18S rRNA sequences (Yilmaz et al., 2014). Reads were subsequently mapped back to OTUs to determine the OTU abundance for each sample, and differences in the community composition/structure in each sample were analyzed with the Quantitative Insights Into Microbial Ecology (QIIME) package (Caporaso et al., 2010). Any OTUs that constituted less than 0.1% of the total OTUs were removed, and the data were normalized by the total sum normalization (TSS) with square root transformation. Differences in alpha diversity between the species of *Vaccinium* were determined by Duncan's new multiple range test ($P < 0.05$). Differences in the diversity and OTU abundance between genotypes of the same *Vaccinium* species were determined by the two-sample *t*-test ($P < 0.05$) or by the Wilcoxon rank-sum test ($P < 0.05$) and visualized in the heat tree format using Metacoder (Foster et al., 2017). The R package Phyloseq (McMurdie and Holmes, 2013) was used to generate non-metric multidimensional scaling (NMDS) ordination ($K = 2$) based on Bray–Curtis dissimilarity matrices to visualize the most abundant phyla from different *Vaccinium* species and genotypes. The amount of variation in the composition of bacterial, fungal, and eukaryotic communities that could be explained by *Vaccinium* species and genotypes was estimated by the permutational multivariate analysis of variance (PERMANOVA) following the calculation of a Bray–Curtis dissimilarity matrix with 999 permutations. Network analyses

¹<http://cgeb-imr.ca/>

²<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>

³http://hannonlab.cshl.edu/fastx_toolkit/download.html

TABLE 1 | Description of species and cultivars of *Vaccinium* used in the study.

Treatment	<i>Vaccinium</i> species	Sub-treatment	<i>Vaccinium</i> genotypes (breeding selections)	Pedigree	Blueberry type
Vg	<i>V. virgatum</i> (2n = 6x = 72)	Vg1	MS 1089	Baldwin/96-6	Rabbiteye
		Vg2	MS 1408	T366/MS 635	
SHB	<i>V. corymbosum</i> (interspecific hybrid, 2n = 4x = 48)	SHB1	MS 2337	Pearl/MS 1387	Southern highbush
		SHB2	MS 2276	MS 771/Abundance	
Vd	<i>V. darrowii</i> (2n = 2x = 24)	Vd1	Clone B0008	Selection from the wild in Florida	Darrow's
		Vd2	MS 2230	Aromi Sunshine/NJ8810-13	

and identification of differentially enriched microbial taxa using the linear discriminant analysis effect size (LEfSe) method were carried out in Calypso (Zakrzewski et al., 2017).

Community Level Physiological Profiling (CLPP)

In order to perform the CLPP analysis, 1–3 g of plant roots were gently shaken to remove excess soil and placed into 50 mL conical centrifuge tubes. Nine parts of phosphate-buffered saline (PBS) were added to each tube, and root-associated bacteria were dislodged by vortexing and treatment in a sonicating bath (1 min each). All root washes were further diluted 10-fold with PBS and inoculated into EcoPlates (100 μ L per well) containing 31 different carbon sources (Biolog, Hayward, CA, United States). The inoculated EcoPlates were incubated for a week at 23°C in the dark, and patterns of the C source utilization were scored daily by measuring absorbance at 590 nm with a Synergy 2 microplate reader (BioTek Instruments). Functional diversity and CLPP similarity indices were calculated based on readings obtained on the sixth day of incubation. The entire experiment was repeated twice.

Data Availability

Sequences generated in this project were deposited in the NCBI sequence read archive under accession numbers PRJNA577971 and PRJNA578171.

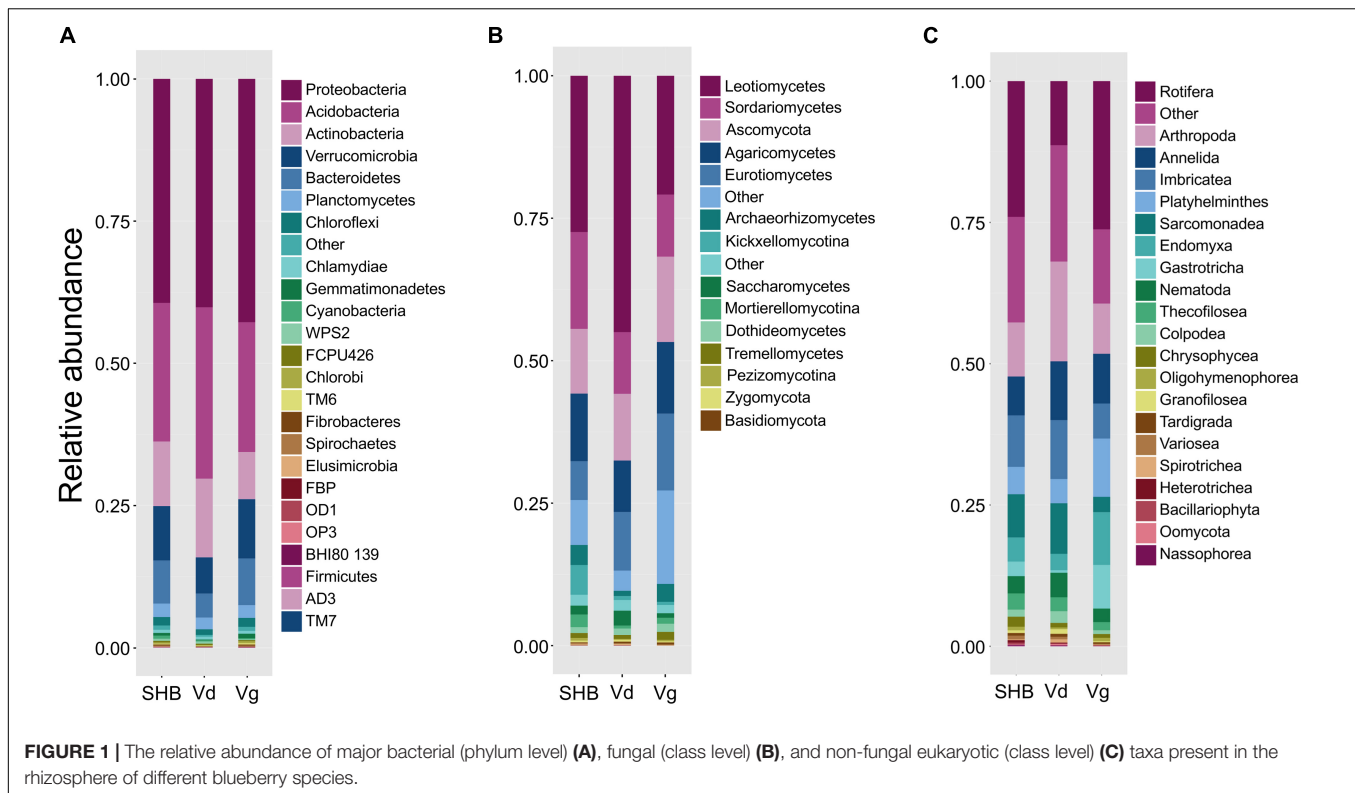
RESULTS

Composition of Rhizobiomes Associated With *V. virgatum*, *V. corymbosum*, and *V. darrowii*

The rhizosphere communities were characterized via high-throughput sequencing of 16S, ITS2, and 18S amplicons generated using DNA extracted from the rhizosphere of two genotypes each of *V. virgatum* (Vg), *V. corymbosum* (SHB), and *V. darrowii* (Vd) species of blueberry (Table 1). In order to profile the bacterial part of the rhizobiome, pools of 16S amplicons were processed to remove low-quality reads, chimeras, and samples with a low depth of coverage. The resultant dataset of 2,096,766 high-quality reads (median reads per sample = 60,990) was rarified to an even depth of 21,000 reads and binned into OTUs at 97% sequence identity. We observed OTUs

from 15 bacterial phyla with an average relative abundance of above 0.1%, and seven of these phyla (*Proteobacteria*, *Acidobacteria*, *Actinobacteria*, *Verrucomicrobia*, *Bacteroidetes*, *Planctomycetes*, and *Chloroflexi*) collectively accounted for 96.7% of all sequencing reads (Figure 1A). Despite the fact that *Vaccinium* species harbored overall similar rhizobacterial communities, we observed several differences in the abundance of individual phyla. For example, samples from SHB were characterized by higher levels of *Cyanobacteria*, whereas Vd had a higher abundance of *Actinobacteria*, *Acidobacteria*, and *Gammaproteobacteria* (Kruskal–Wallis, $P_{FDR} < 0.05$). In contrast, Vg had a higher abundance of *Beta-* and *Deltaproteobacteria*, *Gemmatimonadetes*, *Chlorobi*, *Bacteroidetes*, *Verrucomicrobia*, *Fibrobacteres*, and *Spirochaetes* (Kruskal–Wallis, $P_{FDR} < 0.05$). Genotypes of the same *Vaccinium* species were not significantly different ($P_{FDR} > 0.05$) in the relative abundance of prevalent bacterial phyla (data not shown). Core microbiome analysis with the detection threshold of 70% sample prevalence and minimum relative abundance of 0.01% identified several bacterial families, including *Hyphomicrobiaceae*, *Acetobacteriaceae*, *Rhodospirillaceae*, *Koribacteriaceae*, *Sinobacteriaceae*, *Acidobacteriaceae*, *Chitinophagaceae*, *Solibacteriaceae*, and *Opitutaceae* (Supplementary Figure S1A). Many of these dominant core microbiome families belonged to *Alphaproteobacteria* (especially *Rhizobiales*), as well as to *Acidobacteria* and *Verrucomicrobia*.

The analysis of the fungal communities was performed using a dataset of 1,770,655 high-quality ITS reads (median reads per sample = 50,066), which was obtained after the removal of low-quality and chimeric sequences, as well as samples with the low depth of coverage. The ITS dataset was further rarified to an even depth of 8,000 reads and binned into OTUs at 97% sequence identity. At the phylum level, most of the OTUs were classified as *Ascomycota* (72.4%) and *Basidiomycota* (12.4%), whereas at the class level the communities were dominated by *Leotiomycetes* (30.6%), *Sordariomycetes* (12.9%), *Agaricomycetes* (11.2%), *Eurotiomycetes* (10.4%), *Archaeorhizomycetes* (2.5%), *Saccharomycetes* (1.6%), *Dothideomycetes* (1.2%), and *Tremellomycetes* (1.0%) (Figure 1B). Several groups of these fungi varied significantly between the studied blueberry species, including *Zygomycota*, *Rozellomycota*, *Kickxellomycotina*, and *Archaeorhizomycetes*, which were more abundant in the rhizosphere of *V. corymbosum* (SHB) (Kruskal–Wallis, $P_{FDR} < 0.05$). In contrast, *V. darrowii* (Vd) had higher abundance of *Ascomycota*, *Leotiomycetes*, and *Saccharomycetes*,



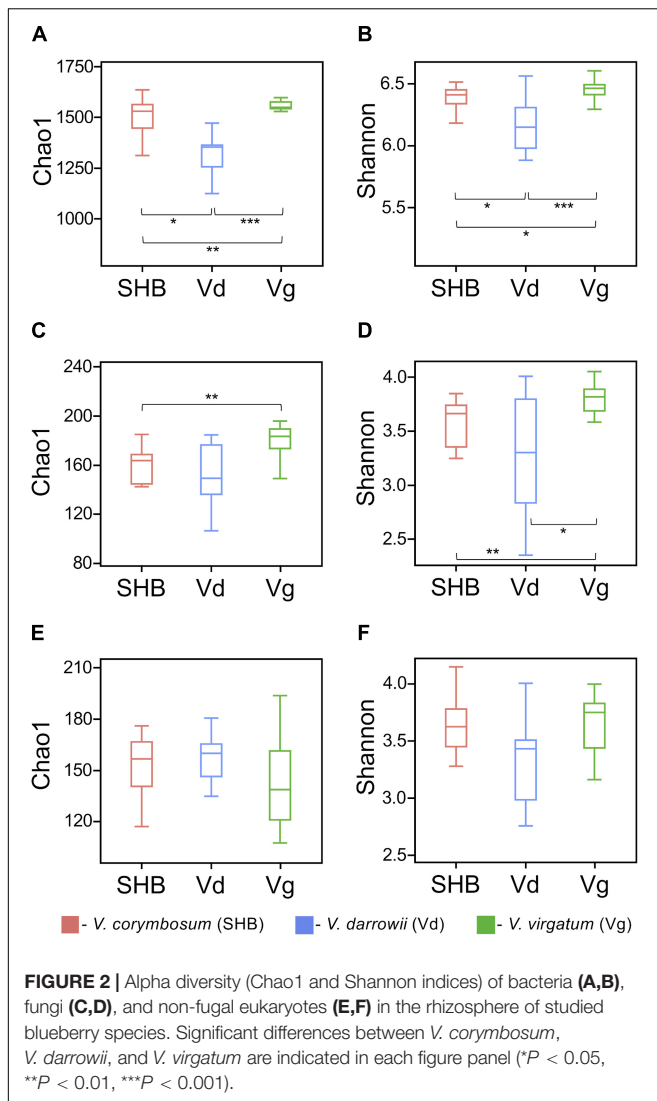
whereas samples from *V. virgatum* (Vg) had higher abundance of *Lecanoromycetes* (Kruskal–Wallis, $P_{FDR} < 0.05$). The core microbiome analysis identified *Leotiaceae*, *Trichomaceae*, *Hypocreaceae*, *Hyaloscyphaceae*, *Herpotrichiellaceae*, *Archaeorhizomycetaceae*, *Saccharomycetaceae*, *Mortierellaceae*, *Cryptococcaceae*, *Cordycipitaceae*, and *Amphisphaeriaceae* as the dominant fungal families of the *Vaccinium* rhizobiome (Supplementary Figure S1B). Interestingly, the first and third most abundant fungal genera of the core microbiome were *Pezoloma* and *Hyaloscypha*, which are prominent ericoid mycorrhizal symbionts of the *Ericaceae* plants, including species of the genus *Vaccinium*.

As with the 16S and ITS amplicons, the 18S-based analysis of eukaryotic communities was initiated by the removal of low-quality sequences, chimeras, and low-coverage samples followed by binning into OTUs at 97% identity. The resultant dataset of 443,016 high-quality reads (median reads per sample = 13,262) was processed further to filter out the unassigned OTUs and plant-derived *Archaeplastida* sequences and then normalized to a depth of 1,459 reads. A largest proportion of 18S OTUs belonged to fungi (54.9%), *Metazoa* (26.1%), *Cercozoa* (11%), and *Alveolata* (1.0%) (data not shown). In agreement with results of the ITS profiling, most fungal reads were belonged to *Ascomycota*, followed distantly by *Basidiomycota*, *Kickxellomycotina*, and *Chytridiomycota*. The non-fungal OTUs collectively accounted for 42.8% of the 18S reads and were dominated by *Rotifera* (9.4%), *Arthropoda* (6.6%), *Cryptomycota* (5.3%), *Platyhelminthes* (3.5%), *Imbricatea* (3.3%), *Annelida* (3.2%), *Endomyxa* (3.0%), *Sarcomonadea* (2.5%),

Gastrotricha (2.0%), and *Nematoda* (1.3%) (Figure 1C). The core part of the eukaryotic rhizobiome was dominated by several families of *Cercozoa*, including *Trinematidae*, *Euglyphidae*, *Leptophryidae*, *Vampyrellidae*, *Sandonidae*, *Allapsidae*, and *Cercomonadidae* (Supplementary Figure S1C). Other dominant core microbiome groups included arthropods (*Arachnida* and *Maxillopoda*), chrysophytes, and catenulid flatworms. Although the studied *Vaccinium* species harbored similar eukaryotic rhizosphere communities, we observed significantly higher levels of *Sarcomonadea* in *V. darrowii* (Vd), while *Rotifera*, *Endomyxa*, and *Gastrotricha* were more abundant in Vg samples (Kruskal–Wallis, $P_{FDR} < 0.05$).

Diversity of Rhizosphere Microbial Communities

The alpha diversity of rhizosphere microbial communities was assessed by calculating Chao1 and Shannon indices. The Chao1 index is as an abundance-based coverage estimator of richness, whereas the Shannon index takes into account both richness and evenness. For rhizobacteria, both metrics indicated significant differences between the studied *Vaccinium* species, with SHB and Vg harboring communities of significantly higher diversity compared to Vd (Kruskal–Wallis; $P < 0.05$) (Figures 2A,B). The comparison of Chao1 and Shannon indices also revealed significant variation in the richness and evenness of fungal communities, which were higher in *V. virgatum* (Vg) compared to *V. corymbosum* (SHB) and *V. darrowii* (Vd) (Figures 2C,D). In contrast, the eukaryotic alpha diversity



estimated using Chao1 and Shannon metrics showed that differences between the *Vaccinium* species were not significant (Kruskal–Wallis; $P < 0.05$) (Figures 2E,F). Similarly, no significant differences in the alpha diversity of rhizosphere-dwelling bacteria, fungi, and eukaryotes were detected between different genotypes of the same *Vaccinium* species (Wilcoxon rank-sum test, $P > 0.05$).

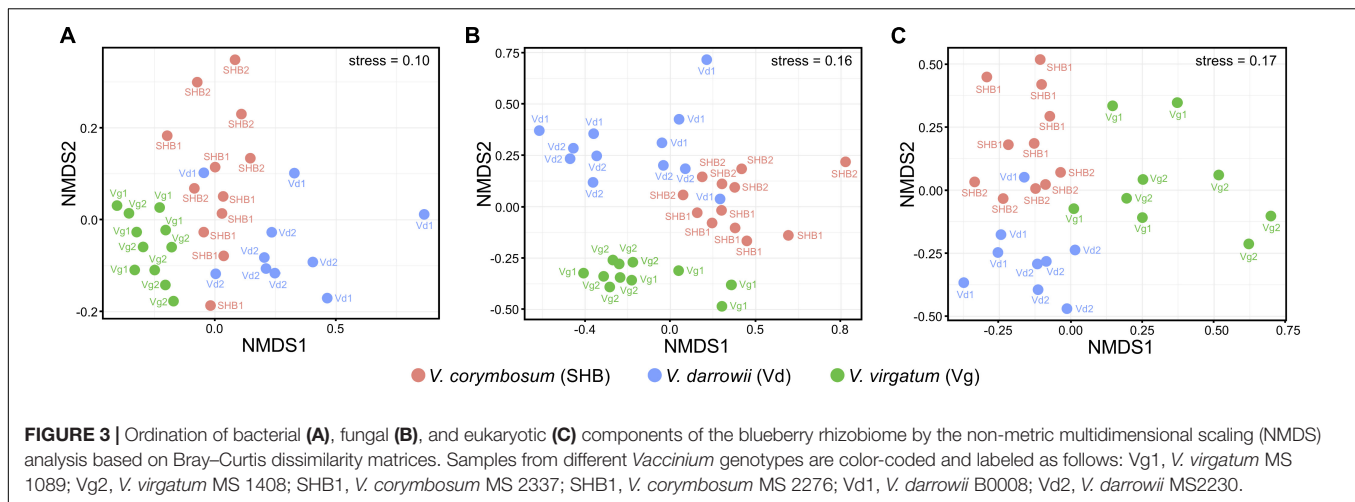
The NMDS analysis based on Bray–Curtis dissimilarities revealed that bacterial and fungal communities of the studied *Vaccinium* species clustered distinctively in the ordination space (Figure 3). These results were confirmed by the analysis of similarity (ANOSIM) test, which showed significant differences in the composition of bacterial ($R = 0.605$, $P = 0.001$), fungal ($R = 0.613$, $P = 0.001$), as well as eukaryotic ($R = 0.548$, $P = 0.001$) communities across the three *Vaccinium* species (Supplementary Table S1). Finally, the permutational multivariate ANOVA (Adonis) also demonstrated that bacterial ($R^2 = 0.33$, $P < 0.001$), fungal ($R^2 = 0.28$, $P < 0.001$), and eukaryotic ($R^2 = 0.22$, $P < 0.001$) components of the rhizobiome were significantly

differentiated by *Vaccinium* species. Interestingly, some variation in the composition of rhizobacterial and fungal communities was also significantly associated with genotypes of *Vaccinium* species (Adonis test: bacteria $R^2 = 0.16$, $P < 0.001$; fungi $R^2 = 0.26$, $P < 0.001$; eukaryotes $R^2 = 0.42$, $P < 0.001$). A particularly pronounced genotype effect was observed in *V. darrowii*, where the Vd1 and Vd2 bacterial and fungal communities were clearly separated by results of the ANOSIM test (bacteria $R = 0.70$, $P = 0.008$; fungi $R = 0.96$, $P = 0.004$; eukaryotes $R = 0.69$, $P = 0.014$) (Supplementary Table S1).

Finally, we compared the catabolic potential of rhizosphere microbial communities based on the utilization of 31 different carbon substrates present in Biolog EcoPlates. Results of the principal component analysis (PCA) readily distinguished catabolic profiles of the three *Vaccinium* rhizobiomes, with the first two principal components (PC1 and PC2) accounting for 45 and 19% of the total variance (Supplementary Figure S2A). The hierarchical clustering analysis of absorbance values suggested a broader range of metabolic activity in Vg and SHB samples, which resulted in the utilization ($OD_{590} \geq 0.1$) of 27 different carbon sources (Supplementary Figure S2B). In contrast, only 21 substrates were metabolized in EcoPlates inoculated with the Vd root washes. Rhizosphere microorganisms from the three species of *Vaccinium* actively consumed several carbohydrates (D-galacturonic acid, N-acetyl-D-glucosamine, D-mannitol, D-cellobiose, D-glucosaminic acid), L-asparagine, L-phenylalanine, pyruvic acid methyl ester, γ -hydroxybutyric acid, Tween 40, and Tween 80. On the other hand, the studied microbiomes differed in the capacity to metabolize glycogen, β -methyl-D-glucoside, α -D-lactose, 2-hydroxybenzoic acid, α -cyclodextrin, and itaconic acid.

Differences Between Rhizobiomes of *V. virgatum*, *V. corymbosum*, and *V. darrowii*

Differences in the composition of rhizobiomes of different *Vaccinium* species were evaluated at the family and genus level by calculating LEfSe scores that indicate the degree of consistent difference in relative abundance between treatments (Segata et al., 2011). Overall, our results revealed both plant species-level and genotype-level differences in the composition of bacterial, fungal, and eukaryotic communities associated with blueberry roots. A total of 25 distinct bacterial biomarkers were identified using the LDA threshold score of ≥ 3.0 , most of which were Proteobacteria (especially *Alphaproteobacteria*) and *Actinobacteria* (Figure 4A). The SHB-enriched phylotypes belonged to the proteobacterial and actinobacterial families *Ectothiorhodospiraceae*, *Sphingomonadaceae*, *Steroidobacter*, *Gaiellaceae*, *Nocardiaceae*, and *Streptomyetaceae*, as well to the *Cytophagaceae* (*Bacteroidetes*) and *Nostocaceae* (*Cyanobacteria*). The Vd rhizobiome was characterized by the abundance of several *Actinobacteria* (*Conexibacter*, *Actinospicaceae*, *Mycobacterium*, *Salinispora*, and *Patulibacteraceae*) and *Proteobacteria* (*Sinobacteraceae*, *Acetobacteraceae*, *Beijerinckiacaceae*, and *Aquicella*), and by the presence of *Acidobacteriaceae* (*Acidobacteria*) and *Sphingobacteriaceae*



(*Bacteroidetes*). The identified Vg-specific phylotypes were taxonomically diverse and included members of *Proteobacteria* (*Hyphomonadaceae*, *Syntrophobacteraceae*, *Hyphomicrobium*), *Verrucomicrobia* (*Opitut*), *Spirochaetes*, and *Chloroflexi* (*Ktedonobacteraceae*). We also observed genotype-level differences in the distribution of individual families and genera associated with roots of *V. corymbosum* (SHB), *V. darrowii* (Vd), and *V. virgatum* (Vg) (**Supplementary Figure S3A**).

The analysis of fungal communities revealed 32 distinct biomarkers that were differentially distributed among rhizobiomes of the studied blueberry species (**Figure 4B**). The rhizobiome of *V. corymbosum* (SHB) was enriched in diverse ascomycetes (*Trichoderma*, *Archaeorhizomyces*, *Rasamsonia*, *Byssoschlamys*, and *Teratosphaeriaceae*) and basidiomycetes of the orders *Agaricales* (*Clavaria*, *Gymnopilus*) and *Filobasidiales* (*Piskurozymaceae*). In contrast, the *V. virgatum* (Vg)-specific fungi included an abundance of ascomycetes from the orders *Helotiales*, *Ostropales*, and *Chaetothyriales*, and were particularly enriched in ericoid mycorrhizae (EM) (*Phialocephala*, *Melinomyces*, *Hyaloscypha*) and endophytic taxa (*Cystodendron*, *Corniculariella*) (**Figure 4D**). Some closely related EM fungi (i.e., *Pezoloma*, *Oidiodendron*, and *Myxotrichaceae*) were also specifically associated with roots of *V. darrowii* (Vd) along with the members of *Eurotiales* (*Penicillium*, *Talaromyces*), *Hypocreales* (*Acremonium*, *Simplicillium*), and *Candida* yeast (*Saccharomycetales*). We identified multiple fungal taxa that were differentially distributed between genotypes of three *Vaccinium* spp., including several species of EM fungi and plant-beneficial endophytes *Verruconis*, *Gongronella*, *Pestalotiopsis*, and *Cladophialophora* (**Supplementary Figure S3B**).

The LEfSe analysis revealed 19 eukaryotic biomarkers that were differentially enriched in the rhizosphere of *V. corymbosum*, *V. virgatum*, and *V. darrowii* (**Figure 4C**). Over half of these taxa were represented by amoeboids and flagellates of the phylum *Cercozoa*, which were specifically associated with roots of SHB (*Rhagostoma*, *Euglypha*, *Sandona*, *Limnophilidae*, *Mesofilidae*), Vd (*Allapsidae*, *Spongomonas*), or Vg (*Thalassomyxa*, *Leptophryidae*). Other differentially

enriched groups of protists included ciliates (*Blepharisma*, *Scuticociliatia*, *Cyrtolophosis*) and amoebas (*Parvamoeba*, *Acanthamoeba*), which were present in *V. corymbosum* and *V. darrowii*. Finally, 18S-based profiling also identified certain gastrotrichs (*Chaetonotus*, *Lepidochaetus*), microscopic nematodes (*Eumonhystera*, *Aphelenchoides*), and flatworms (*Catenula*) as specific biomarkers associated with SHB and Vg. Although there were fewer genotype-level differences in the distribution of eukaryotic biomarkers compared to bacteria or fungi, we identified several genera of ciliates, filose amoebas, and flagellates that differed in abundance between the two genotypes of *V. corymbosum* (**Supplementary Figure S3C**). Collectively, the LEfSe results were in agreement with differences in the relative abundance of bacteria, fungi, and eukaryotes estimated by the non-parametric Wilcoxon rank-sum test and visualized in the form of differential heat trees (**Supplementary Figure S4**). The family-level analysis of rhizosphere microbial communities also revealed the presence of complex co-occurrence networks. The bacterial network contained two distinct clusters of co-occurring taxa (**Figure 5A**). The first cluster contained some *Actinobacteria* (*Mycobacteriaceae*, *Conexibacteraceae*), *Acidobacteria* (*Acidobacteriaceae*, *Koribacteraceae*), *Alpha*-(*Sinobacteraceae*, *Beijerinckiaceae*), and *Gammaproteobacteria* (*Acetobacteraceae*) associated predominantly with *V. darrowii*. The second cluster was associated with *V. virgatum* and was comprised of *Alphaproteobacteria* (*Rhizobiaceae*), *Bacteroidetes* (*Chitinophagaceae*, *Cytophagaceae*), *Deltaproteobacteria* (*Syntrophobacteraceae*, *Haliangiaceae*), and *Verrucomicrobia* (*Opitutaceae*). Although the fungal network was overall more diffuse, it still contained a distinct group of co-occurring taxa (*Boletaceae*, *Hyaloscyphaceae*, *Plectosphaerellaceae*, *Dermateaceae*, *Herpotrichiellaceae*, *Chaetosphaeriaceae*, *Helotiaceae*) found in the rhizosphere of *V. virgatum* (**Figure 5B**). Finally, the eukaryotic network included a large group of positively correlated taxa that were associated with roots of *V. corymbosum* and *V. darrowii*, as well as a much smaller cluster of *Rotifera*, *Gastrotricha*, and *Leptophryidae* that were associated with *V. virgatum* (**Figure 5C**).

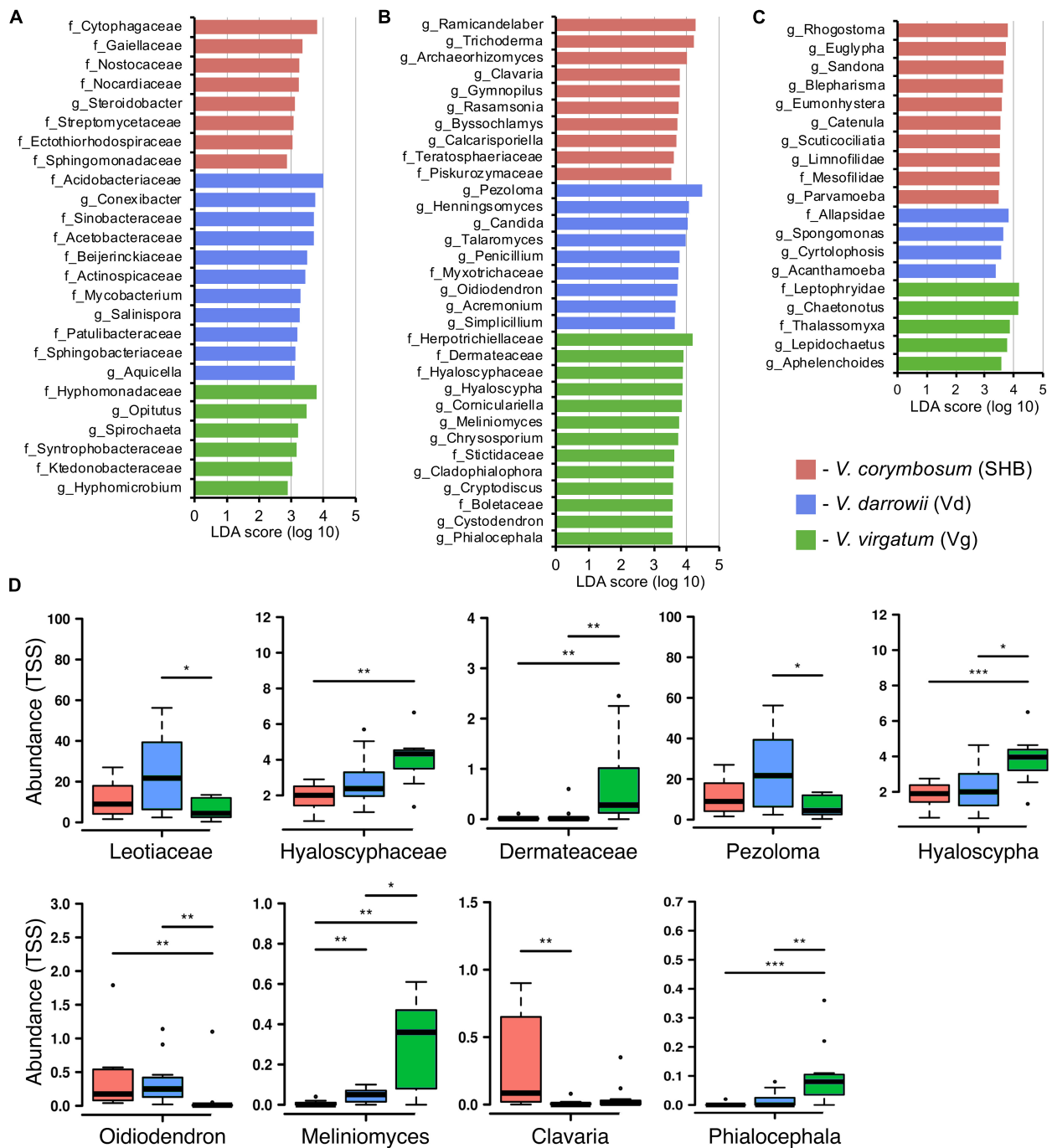


FIGURE 4 | LEfSe analysis of differentially abundant (LDA threshold score ≥ 3.0) families and genera of bacteria (A), fungi (B), and eukaryotes (C) between *Vaccinium* species. (D) Ericoid mycorrhizal fungi with significant differences ($*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$) in the total sum normalized (TSS) transformed abundance.

DISCUSSION

To our knowledge, this is the first study that compares side-by-side rhizosphere microbial communities of SHB (*V. corymbosum* interspecific hybrid), Darrow's blueberry (*V. darrowii*), and rabbiteye blueberry (*V. virgatum*). Our results revealed that

these species share a common core rhizobiome, which is similar in composition to that of the lowbush blueberry *V. angustifolium* (Yurgel et al., 2017). At the same time, the rhizosphere communities of SHB, Vd, and Vg differed significantly in the diversity, relative abundance, richness, and evenness of multiple groups of prokaryotic and eukaryotic

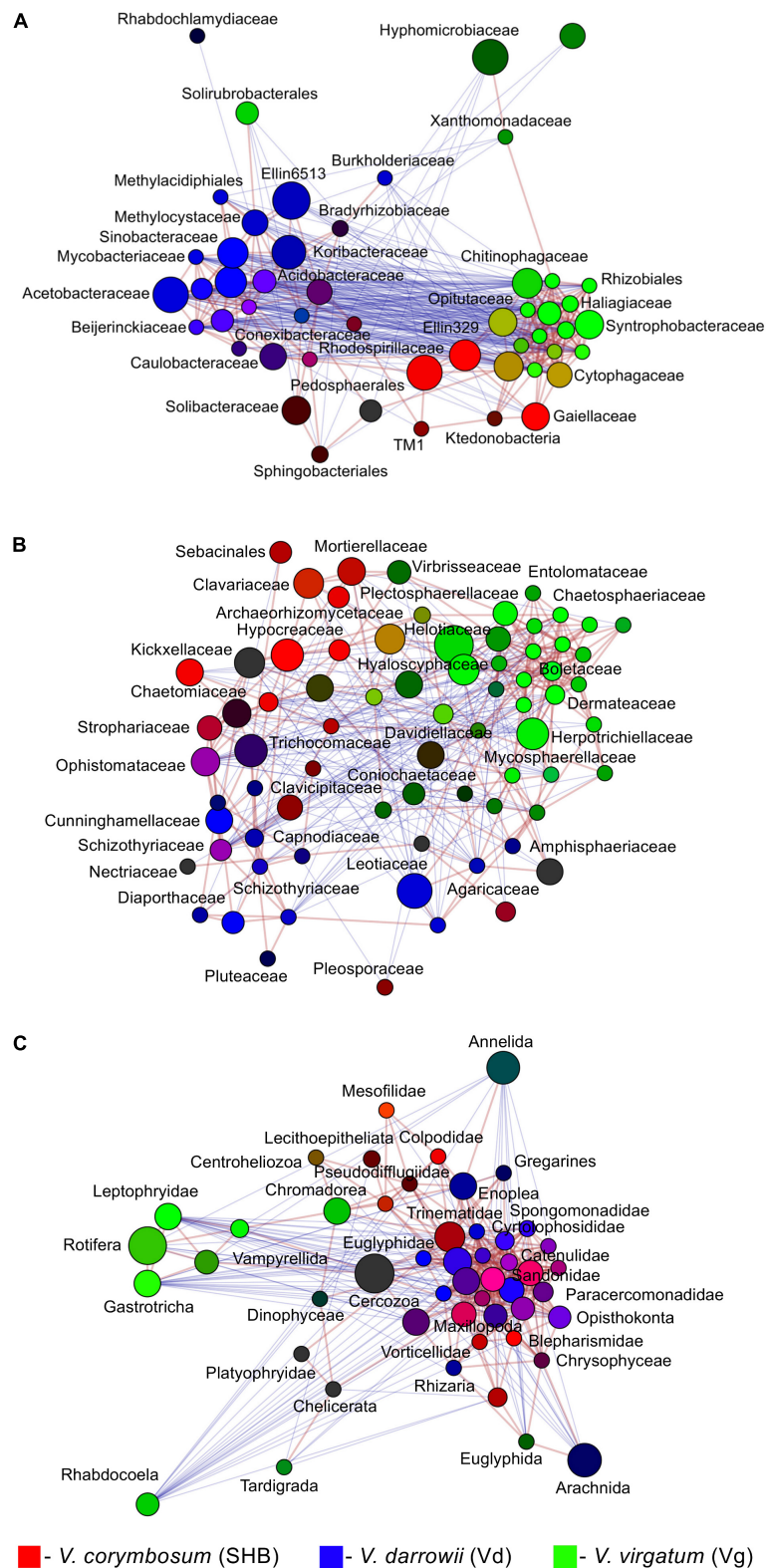


FIGURE 5 | Network analysis illustrating significant co-occurrence patterns among bacterial (A), fungal (B), and eukaryotic (C) taxa and *Vaccinium* species. The analysis was performed using Spearman correlations. Network edges represent statistically significant positive (red) and negative (blue) correlations (FDR < 0.05). Taxa are represented by nodes colored based on their association with the studied blueberry species [red, *V. corymbosum* (SHB), blue, *V. darrowii* (Vd), green, *V. virgatum* (Vg)]. The relative brightness (i.e., the color gradient) of each node reflects the significance level of the association.

microorganisms (Figure 2 and Supplementary Figure S4). Although the host signature effects were especially pronounced at the plant species level, we also observed genotype-level variations in the distribution of specific microbial taxa, which suggests that the assembly of the blueberry microbiome is shaped by the plant genotype and modifications associated with the domestication and breeding of members of the *Vaccinium* genus. Overall, *V. corymbosum* (SHB) and *V. darrowii* (Vd) harbored more similar microbial communities compared to *V. virgatum* (Vg), a finding that aligns well with the fact that southern highbush blueberries were developed by hybridizing the northern highbush blueberry *V. corymbosum* with several other *Vaccinium* species, including *V. darrowii* (Hancock, 2006). Although the impact of plant domestication on the root-associated microorganisms is poorly understood, several recent studies reported that the domestication is associated with compositional changes in the rhizosphere microbiome (Kiers et al., 2007; Zachow et al., 2014; Bulgarelli et al., 2015; Cardinale et al., 2015; Perez-Jaramillo et al., 2016, 2017). Interestingly, a meta-analysis of microbiome studies in barley, lettuce, common bean, and Arabidopsis revealed that members of the *Bacteroidetes* were more abundant in roots of wild plant relatives, while *Proteobacteria* and *Actinobacteria* were consistently associated with the rhizosphere of corresponding domesticated accessions (Perez-Jaramillo et al., 2018). We also identified these phyla among differentially distributed microbial groups and demonstrated that *Bacteroidetes* were associated with *V. virgatum*, whereas *Actinobacteria* were more abundant in *V. darrowii*. However, plants used in this study were grown under controlled conditions. It will be interesting to address the effect of domestication on the blueberry rhizobiome by including in future studies field-grown plants of wild accessions of *V. angustifolium*, *V. darrowii*, *V. arboreum*, *V. myrsinites*, and *V. myrtilloides*.

The analysis of differentially distributed taxa revealed several genera of ericoid mycorrhizal (EM) fungi, which are important mutualistic endosymbionts of blueberries and other plants of the *Ericaceae* family (Grelet et al., 2017). Ericoid mycorrhizae enhance the fitness and productivity of the *Ericaceae* plants and play a vital role in their adaptation to soils with low pH and slow turnover of organic matter. Mycorrhizal fungi supply their host plants with nitrogen, phosphate, and other nutrients, which are mobilized via the breakdown of complex organic compounds by fungal exoenzymes (Kariman et al., 2018). Several studies also reported that EM colonization has positive effects on the tolerance of plants to heavy metals (Daghino et al., 2016). Ericoid mycorrhizae interact with their plant hosts by forming hyphal coils in the epidermal cells of specialized hair roots (Read, 1996). The EM fungi are closely related to Dark Septate Endophytes, a group of fungi with melanized hyphae that form in the root tissue loose loops instead of dense ericoid coils (Vohnik and Albrechtova, 2011). While earlier studies limited the capacity to form ericoid mycorrhizae to a small number of fungi, the advent of molecular methods revealed that hair roots colonized by EM harbor diverse assemblages of ascomycetes of the orders *Helotiales*, *Erysiphales* (*Oidiodendron* spp.), and some

basidiomycetes, such as members of the *Sebacinales* and *Clavaria* spp. (Leopold, 2016).

Our results revealed an overall enrichment of the blueberry rhizobiome in various EM fungi, as well as the differential abundance of *Hyaloscyphaceae* (*Hyaloscypha*) and dark septate endophytes (*Meliniomyces*, *Phialocephala*) on roots of *V. virgatum*. We also observed higher levels of *Leotiaceae* (*Pezoloma*) and *Oidiodendron* in *V. darrowii* and an abundance of the EM basidiomycete *Clavaria* in the rhizosphere of *V. corymbosum*. The differential distribution of several EM taxa and plant-beneficial endophytes was also observed at the genotype level. Although the host preference/specificity effects in the interaction of the *Ericaceae* plants with EM fungi are poorly understood, Scagel and Yang (2005) demonstrated that highbush blueberry cultivars that fruit early in the season had higher levels of mycorrhizal infection than cultivars fruiting later in the growing season. Similarly, Sun et al. (2011) demonstrated that communities of EM fungi in *Rhododendron decorum* varied with the host genotype, which was in contrast to non-EM communities that were shaped by geography. That study concluded that genetic make-up of the host plant represents a significant “driving force” shaping communities of ericoid mycorrhizae. Our findings agree with earlier studies that demonstrated the genotype-specific variation in the sensitivity of blueberries to ericoid mycorrhizae and hint at the possibility that the genetics of blueberry plants plays an important role in the recruitment of certain EM fungi from the seed bank of soil microorganisms.

The comparison of rhizosphere communities of SHB, Vd, and Vg revealed several groups of bacteria that may exert beneficial effects on the health and productivity of blueberries. The three compared rhizobiomes had an abundance of free-living diazotrophs from *Bradyrhizobiaceae*, *Methylocystaceae*, *Burkholderiaceae*, and *Frankiaceae*, which may represent another adaptation to poor soils typical of natural habitats of blueberries and other *Ericaceae* plants. The microbiome of *V. darrowii* also had higher levels of nitrogen-fixing *Beijerinckiaceae*, which are often found in the plant rhizosphere where they exchange ammonium for the photosynthetically fixed carbon. We further identified several groups of bacteria that in other crop systems had been implicated in the suppression of soilborne pathogens. For example, the rhizobiome of *V. virgatum* had an increased abundance of *Opitutus*, and the decline of *Opitutaceae* in soil was implicated in the increased susceptibility of cotton to infection with *Fusarium oxysporum* f. sp. *vasinfectum* (Li et al., 2015). The rhizosphere communities of *V. corymbosum* and *V. darrowii* had higher levels of *Sphingobacteriaceae*, *Sphingomonadaceae*, *Cytophagaceae*, *Gaiellaceae*, and several other *Actinobacteria* (Figure 4). *Gaiellaceae* is a recently discovered family that belongs to a deep lineage of the class *Actinobacteria* and consists of a single genus with only one cultured species, *Gaiella occulta* (Albuquerque et al., 2011). These aerobic chemoorganotrophs have been found worldwide in the rhizosphere of corn, rice, soybean, hemp, and sudex (Eo et al., 2015; Sun et al., 2018; Wang et al., 2019). Members of *Gaiellaceae*, together with *Sphingomonadaceae*, and *Streptomyetaceae* were associated with the soil suppressiveness

to *F. oxysporum* f. sp. *cubense*, a causative agent of the Panama disease of banana (Xue et al., 2015). The differentially abundant SHB and Vd taxa were also identified among key microbial groups in the Dutch soil suppressive to the plant pathogen *Rhizoctonia solani* AG2-2IIIB (Chapelle et al., 2016). That study employed metagenomic and metatranscriptomic techniques to characterize the transcriptional changes in the rhizobiome of sugar beet plants grown in the presence of the fungal pathogen in a *Rhizoctonia*-suppressive soil. The analysis identified members of the *Sphingobacteriaceae*, *Sphingomonadaceae*, and *Cytophagaceae* among rhizobacteria that responded to the presence of pathogens by upregulation of stress-related genes. A follow-up study by van der Voort et al. (2016) used heat treatment of soil to examine the contribution of different rhizobacterial taxa to the suppression of *R. solani*. The authors suggested that different *Actinobacteria*, including members of the *Streptomycetaceae* and *Mycobacteriaceae*, may contribute to the disease protection since their decrease coincided with the loss of pathogen suppression. Interestingly, *Actinobacteria* were also identified as hub taxa in the rhizosphere microbiome of lowbush blueberry *V. angustifolium*, suggesting their crucial role in the structure of microbial communities of blueberries (Yurgel et al., 2018).

The assembly of rhizosphere microbiome is influenced by the interplay between soil properties (structure, pH, moisture, salinity, organic matter), climate, and human practices (Cordovez et al., 2019). For example, the mycorrhizal colonization in blueberries is modulated by the application of fertilizers, and increased N fertilization can reduce the colonization of roots by ericoid mycorrhizae (Sadowsky et al., 2012). However, given that our study was performed under controlled conditions, it is likely that the observed differences in the structure of rhizosphere microbiomes of the studied *Vaccinium* species are driven by a combination of host effects and microbial interactions. The recruitment of soil microorganisms by plant species and genotypes is mediated by the root morphology and secretion of exudates, which serve as nutrients and signals for the rhizosphere microorganisms (Sasse et al., 2018). A recent elegant study by Zhalnina et al. (2018) demonstrated that the rhizosphere microbiome of wild oat (*Avena barbata*) is recruited via the metabolic synchronization of the root exudation and microbial substrate utilization traits.

Although currently unknown, it is plausible that similar processes govern the assembly of distinct microbial communities in the rhizosphere of different species of blueberry. The observed differences in the composition of microbial communities of *V. corymbosum*, *V. darrowii*, and *V. virgatum* are also likely shaped by mutualistic and competitive interactions between different members of the rhizobiome. The co-occurrence analysis of bacteria, fungi, and protozoa revealed complex networks with numerous positive and negative correlations, similar to those described in the rhizosphere of wild and managed *V. angustifolium* (Yurgel et al., 2018). Interestingly, our analysis also revealed that microbial communities of SHB, Vd, and Vg differ in the abundance of various predatory protists, which feed on bacteria and fungi and have recently emerged as a key factor that shapes the rhizosphere

microbiome and selects for plant-beneficial functional traits (Gao et al., 2019).

CONCLUSION

Our study is the first to compare the rhizosphere microbial communities of the SHB, Darrow's blueberry, and rabbiteye blueberry using the modern culture-independent approaches. Our results revealed an extensive diversity of pro- and eukaryotic microorganisms inhabiting the blueberry rhizosphere and demonstrated that the studied species of *Vaccinium* differ in the abundance of beneficial rhizobacteria and EM fungi. Cultivated blueberries were initially selected by crossing several wild relatives to improve the yield and fruit quality. However, the rising popularity of this commodity has expanded the production into areas that present a challenging environment for blueberry plants (Retamales and Hancock, 2018). Blueberries, like other members of the *Ericaceae* family, rely on their microbiomes for the protection against abiotic stresses and survival in soils that are low in nutrients. In particular, mycorrhizal symbiotic partners improve the uptake of soil nutrients, efficacy of fertilizers, and protect plants from the metal toxicity in acidic soils (Scagel, 2005; Scagel and Yang, 2005; Vega et al., 2009; Caspersen et al., 2016). Therefore, we suggest that species- and genotype-specific differences in the structure and function of rhizobiome represent an important facet in the adaptation of blueberry cultivars to soil and climate conditions.

The rhizosphere microbiome is shaped by exudates and root morphology, both of which are influenced by plant genotype (Sasse et al., 2018). There is a growing consensus that the traditional plant breeding should be expanded to include plant genotype \times environment \times microbiome interactions (Wei and Jousset, 2017). Such microbiome-supported breeding should be performed in the absence of excessive fertilizers and pesticides and complemented by the microbial community profiling and screening for metabolites that mediate interactions with key pathogenic or beneficial species (Wille et al., 2018). We reason that similar experimental approaches can be employed in blueberries to harness microbial communities that improve the disease resistance, tolerance to heat and drought, and vigor to thrive in soils with low organic matter content.

DATA AVAILABILITY STATEMENT

Sequences generated in this project were deposited in the NCBI sequence read archive under accession numbers PRJNA577971 and PRJNA578171.

AUTHOR CONTRIBUTIONS

DM, OM, and EB conceived the research project. EB provided blueberry plants. OM and JL extracted soil DNA. OM and CB conducted the Biolog EcoPlate profiling. DM, JL, OM, and JH conducted the microbiome analysis. DM, JL, OM,

and EB wrote the manuscript. All authors contributed to the manuscript revision.

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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 GMC Oxidoreductase GmcA Is Required for Symbiotic Nitrogen Fixation in *Rhizobium leguminosarum* bv. *viciae*

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GmcA is a FAD-containing enzyme belonging to the GMC (glucose-methanol-choline oxidase) family of oxidoreductases. A mutation in the *Rhizobium leguminosarum* *gmcA* gene was generated by homologous recombination. The mutation in *gmcA* did not affect the growth of *R. leguminosarum*, but it displayed decreased antioxidative capacity at H₂O₂ conditions higher than 5 mM. The *gmcA* mutant strain displayed no difference of glutathione reductase activity, but significantly lower level of the glutathione peroxidase activity than the wild type. Although the *gmcA* mutant was able to induce the formation of nodules, the symbiotic ability was severely impaired, which led to an abnormal nodulation phenotype coupled to a 30% reduction in the nitrogen fixation capacity. The observation on ultrastructure of 4-week pea nodules showed that the mutant bacteroids tended to start senescence earlier and accumulate poly-β-hydroxybutyrate (PHB) granules. In addition, the *gmcA* mutant was severely impaired in rhizosphere colonization. Real-time quantitative PCR showed that the *gmcA* gene expression was significantly up-regulated in all the detected stages of nodule development, and statistically significant decreases in the expression of the redoxin genes *katG*, *katE*, and *ohrB* were found in *gmcA* mutant bacteroids. LC-MS/MS analysis quantitative proteomics techniques were employed to compare differential *gmcA* mutant root bacteroids in response to the wild type infection. Sixty differentially expressed proteins were identified including 33 up-regulated and 27 down-regulated proteins. By sorting the identified proteins according to metabolic function, 15 proteins were transporter protein, 12 proteins were related to stress response and virulence, and 9 proteins were related to transcription factor activity. Moreover, nine proteins related to amino acid metabolism were over-expressed.

Keywords: *Rhizobium leguminosarum*, the glucose-methanol-choline oxidoreductase GmcA, symbiotic nitrogen fixation, antioxidant and symbiotic gene expression, quantitative proteomics

INTRODUCTION

Rhizobium leguminosarum bv. *viciae* is an aerobic, Gram-negative, nitrogen-fixing bacterium that can live under the conditions of microaerobe, aerobe and form symbiotic relationships with *Pisum sativum* (pea) and *Vicia cracca* (vetch) under the condition of nitrogen limitation (Karunakaran et al., 2009). Organisms of this genus play a critical role in soil fertility, inducing the formation

of symbiotic nodules on the roots of leguminous plants, where bacteroids reduce atmospheric nitrogen to ammonia available for plant uptake (Bhat et al., 2015). The symbiosis between rhizobia and legumes can be characterized by a mutual exchange of signal molecules between the two partners (Janczarek et al., 2015; López-Baena et al., 2016). After attachment of the bacteria to the plant root, the plant supports bacterial infection via host-derived infection threads (Haney and Long, 2010). Successful nodulation requires the activation of cell division in the cortex to form the nodule primordium (Blanco et al., 2009). In nodules, the nitrogenase enzyme, which is extremely sensitive to oxygen, has a low turnover number and a large requirement of chemical energy in the form of ATP and reducing potential (Clarke et al., 2011; Okazaki et al., 2015). In addition to reducing N_2 and protons, nitrogenase can also reduce several small, non-physiological substrates, including a wide array of carbon-containing compounds (Seefeldt et al., 2013). It was found that uptake hydrogenases allow rhizobia to recycle the hydrogen generated in the nitrogen fixation process within the legume nodule (Baginsky et al., 2002).

Oxidoreductases catalyze a large variety of specific reduction, oxidation, and oxyfunctionalization reactions, which are important in redox processes, transferring electrons from a reductant to an oxidant (Hollmann and Schmid, 2004; Jeelani et al., 2010). Oxidoreductases included laccases, GMC (glucose-methanol-choline) oxidoreductases, copper radical oxidases and catalases (Beckett et al., 2015). The family of GMC flavoprotein oxidoreductases, which includes glucose/alcohol oxidase and glucose/choline dehydrogenase from prokaryotic and eukaryotic organisms, was first outlined by Cavener (1992). Members of the GMC oxidoreductase family share a common structural backbone of an adenine-dinucleotide-phosphate-binding $\beta\alpha\beta$ -fold close to their amino terminus (Iida et al., 2007). The group of GMC flavoprotein oxidoreductases encompasses glucose oxidase from the mold *Aspergillus niger*, the glucose dehydrogenase from *Thermoplasma acidophilum* and *Drosophila melanogaster*, methanol oxidase from yeast *Hansenula polymorpha*, and choline dehydrogenase from *Escherichia coli* (Ahmad et al., 2010; Liu et al., 2013). In the leaf beetle subtribe *Chrysomelina sensu stricto*, GMC oxidoreductases enabled chemical defenses and were important for adaptive processes in plant-insect interactions (Rahfeld et al., 2014). In *E. coli*, choline dehydrogenase catalyzes the flavin-dependent, two-step oxidation of choline to glycine betaine, which acts as an osmoprotectant compatible solute that accumulates when the cells are exposed to drastic environmental changes in osmolarity (Yilmaz and Bülow, 2010). However, little is known about the functional diversity of the rhizobium GMC family.

Rhizobium leguminosarum bv. *viciae*, which has been widely used as a model to study nodule biochemistry, is able to nodulate and fix nitrogen in symbiosis with several legumes (Karunakaran et al., 2009). Here, we investigated the roles of a GMC oxidoreductase GmcA in free-living bacteria and during nitrogen-fixing symbiosis on pea by analyzing the phenotypes of a mutant strain. Proteome analysis provides clues to explain the differences between the *gmcA* mutant and wild-type nodules.

MATERIALS AND METHODS

Bacterial Growth and Media

The strains, plasmids and primers used in this study are listed in Table 1. *Rhizobium* strains were grown at 28°C in either Tryptone Yeast extract (TY) (Beringer and Hopwood, 1976) or Acid Minimal Salts medium (AMS) (Poole et al., 1994) with D-glucose (10 mM) as a carbon source and NH_4Cl (10 mM) as a N source (referred to as AMS Glc/ NH_4^+). For growth and qRT-PCR experiments, cells were grown in AMS Glc/ NH_4^+ . Antibiotics were used at the following concentrations ($\mu g/mL$): ampicillin (Amp), 50; gentamicin (Gm), 20; kanamycin (Km), 20; neomycin (Neo), 80; spectinomycin (Spe), 100; streptomycin (Str), 500; tetracycline (Tc), 5. Strains were grown at 28°C with shaking (200 rpm) for liquid media. To monitor culture growth, optical density at 600 nm (OD_{600}) was measured on three independent cultures.

Construction and Complementation of the *gmcA* Gene Mutant of *R. leguminosarum* 3841

Primers *gmcAF* and *gmcAR* were used to PCR amplify an internal region of the *gmcA* gene from *R. leguminosarum* bv. *viciae* 3841 genomic DNA (Johnston and Beringer, 1975). The 650-bp *gmcA* PCR product was cloned into the *Pst*I and *Xba*I sites of pK19mob, resulting in plasmid pK*gmcA*. The plasmid pK*gmcA* was conjugated with *R. leguminosarum* bv. *viciae* 3841 using pRK2013 as a helper plasmid, as previously described (Figurski and Helinski, 1979; Karunakaran et al., 2010). Insertions into the *gmcA* gene of strain RL3841 were selected by neomycin resistant AMS medium with 30mM pyruvate as a sole carbon source and confirmed by PCR using M*gmcA* and a pK19mob-specific primer (either pK19A or pK19B) (Karunakaran et al., 2010).

To complement the *gmcA* mutant, primers *cgmcAF* and *cgmcAR* were used to amplify the complete *gmcA* gene from strain RL3841. The PCR product was digested with *Kpn*I and *Xba*I and cloned into pBBR1MCS-5, resulting in plasmid pBBR*gmcA*. Plasmid pBBR*gmcA* was conjugated into the mutant strain RL*gmcA* using pRK2013 as a helper plasmid to provide the transfer genes, as previously described (Karunakaran et al., 2010).

Hydrogen Peroxide Resistance Activity

Logarithmic phase cultures of mutant strain RL*gmcA* and wild-type RL3841 were collected and washed twice in sterile phosphate-buffered saline (PBS) (1×; 136 mM NaCl, 2.6 mM KCl, 8.0 mM Na_2HPO_4 , and 1.5 mM KH_2PO_4). Cells with an optical density (OD_{600}) 1 were treated with H_2O_2 at different concentrations (0, 1, 5, and 10 mmol/L) for 1 h. Strains were thoroughly washed with distilled water to remove any remaining oxidant, and the diluted TY plate method was used to evaluate the bacterial survival rate. The experiment consisted of three independent experiments, each of which had three repeats, and statistical differences were analyzed with one-way ANOVA ($P < 0.05$).

TABLE 1 | Strains, plasmids, and primers.

Strains	Description	
RL3841	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> , Str ^r	
RLgmcA	RL3841 <i>gmcA</i> :pk19mob, Str ^r Neo ^r	
RLgmcA (pBBRgmcA)	RLgmcA carrying <i>gmcA</i> gene, Str ^r Neo ^r Gm ^r	
Plasmids	Description	
pK19mob	pK19mob pUC19 derivative <i>lacZ</i> mob; Km ^r	
pRK2013	Helper plasmid for mobilizing plasmids; Km ^r	
pKgmcA	<i>gmcA</i> AF/ <i>gmcA</i> R PCR product in pK19mob, Km ^r	
pBBRgmcA	<i>cgmcA</i> AF/ <i>cgmcA</i> R PCR product in pBBR1MCS-5, Gm ^r	
Primer	Description	Sequence ¹
<i>gmcA</i> AF	Sense primer for pRL100444 (<i>gmcA</i>) mutation	TTTAGATCTGGCGGGTTCCTTTCGCGTAA
<i>gmcA</i> R	Antisense prime for pRL100444 (<i>gmcA</i>) mutation	TTTCTGCAGTCAGCTCACCGGTGCGCTTT
MgmcA	Mapping PCR primer for <i>gmcA</i> gene	CGCCCCGACGGATTGTAGAAT
pK19A	pK19mob mapping primer	ATCAGATCTTGATCCCCTGC
pK19B	pK19mob mapping primer	GCACGAGGGAGCTTCCAGGG
<i>gyrB</i> 1-F	Sense primer for qRT-PCR of <i>GyrB</i> 1	GGCATCACCAAAAGGGAAAA
<i>gyrB</i> 1-R	Antisense primer for qRT-PCR of <i>GyrB</i> 1	GCGAGGAGAATTCGGATCA
<i>cgmcA</i> AF	Sense primer for <i>gmcA</i> complementation	TTTGGTACCAGCTCACTGTCGATCTCTCC
<i>cgmcA</i> R	Antisense prime for <i>gmcA</i> complementation	TTTCTAGACCTTTATCCGGTTGAGCTGG
<i>QgmcA</i> -for	Sense primer for qRT-PCR of <i>gmcA</i>	CGCCGCCTCGCTCGGCAAGA
<i>QgmcA</i> -rev	Antisense primer for qRT-PCR of <i>gmcA</i>	ATGCTCATGGAAGTGCAGAG
<i>gyrB</i> 1-F	<i>gyrB</i> 1 primers for qRT-PCR	GGCATCACCAAAAGGGAAAA
<i>gyrB</i> 1-R		GCGAGGAGAATTCGGATCA
<i>QkatG</i> -F	<i>katG</i> primers for qRT-PCR	GCAACTATTACGTCGGTCTG
<i>QkatG</i> -R		TCTCATCGATGACATTTTCC
<i>QkatE</i> -F	<i>katE</i> primers for qRT-PCR	CTCTCATCGATGACTTCCAT
<i>QkatE</i> -R		GGGACTCATATGTTTCGAAG
<i>QorhB</i> _F	Sense primer for qRT-PCR of <i>orhB</i>	CGGGCAGGCTGACATTGAGG
<i>QorhB</i> _R	Antisense primer for qRT-PCR of <i>orhB</i>	GCTGCTCAGAGAAAGATCAC
<i>QhmuS</i> -F	<i>hmuS</i> primers for qRT-PCR	AAGACCAGTCGCAGGAATTT
<i>QhmuS</i> -R		GAAGAACTCATGCGTATCGG
<i>QnifD</i> _F	Sense primer for qRT-PCR of <i>nifD</i>	GCAACTATTACGTCGGTCTG
<i>QnifD</i> _R	Antisense primer for qRT-PCR of <i>nifD</i>	TCTCATCGATGACATTTTCC
<i>QfdxB</i> _F	Sense primer for qRT-PCR of <i>fdxB</i>	ATGGCGAAGACGACTTTAAT
<i>QfdxB</i> _R	Antisense primer for qRT-PCR of <i>fdxB</i>	ATGAGTCTGGCAGTCTTGG

¹ Restriction sites in primer sequences are underlined.

Enzyme Activity Experiments

For analysis of glutathione reductase and glutathione peroxidase activities, logarithmic phase cultures of mutant strain RLgmcA and wild-type RL3841 with an optical density (OD₆₀₀) 1 were collected, and treated with 5 mM H₂O₂ for 1 h. H₂O₂-treated PBS cells were collected by centrifugation at 5,000 rpm for 5 min at 4°C. The cells were held in an ice-water bath and sonicated for 15 min. The sonicate was centrifuged at 12,000 rpm for 10 min at 4°C. Glutathione reductase and glutathione peroxidase activities were determined using a peroxidase assay kit (Beyotime, China). The experiment consisted of three independent experiments, each of which had three repeats, and statistical differences were analyzed with one-way ANOVA ($P < 0.05$).

Plant Growth and Microscope Study of Nodules

Pea seeds were surface sterilized in 95% ethanol for 30 s and then immersed in a solution of 2% sodium hypochlorite for 10 min. *R. leguminosarum* bv. *viciae* strains were inoculated

with 10⁷ CFU per seed at the time of sowing. Plants were incubated in a controlled-environment chamber with an 18-h photoperiod (day/night temperature, 22 and 20°C). For dry weight determination, plants were grown in a 2-L beaker filled with sterile vermiculite, watered with nitrogen-free nutrient solution and harvested at 7 weeks (Cheng et al., 2017). The shoot was removed from the root and dried at 70°C in a dry-heat incubator for 3 days before being weighed. Acetylene reduction was determined at flowering (4 weeks) in peas, as previously described (Allaway et al., 2000). The experiment consisted of two independent experiments, each of which had five repeats, and statistical differences were analyzed with one-way ANOVA ($P < 0.05$).

Nodules at 4 weeks post infection were fixed in 2.5% glutaraldehyde and postfixed in 1.5% osmium tetroxide. Root nodules were sectioned and were then stained with toluidine blue. Ultra-thin sections stained with uranyl acetate and lead citrate were observed using a Hitachi H-7100 transmission electron microscope (Yan et al., 2004). For light microscopy, thick sections were cut on a microtome and stained.

Rhizosphere Colonization

Rhizosphere colonization assays were performed as previously described (Cheng et al., 2017). Pea seedlings were grown for 7 days, as described above, for acetylene reduction, and inoculated with RLgmcA and RL3841 in the cfu ratios 1000:0, 0:1000, 1000:1000, and 10000:1000. After 7 days (14 days after sowing), shoots were cut-off and 20 mL of sterile phosphate-buffered saline (PBS) was added to the roots and vortexed for 15 min at speed 10 (Karunakaran et al., 2006). After vortexing, the samples were serially diluted and plate counted on TY medium plates containing either streptomycin (for wild-type RL3841 and mutant RLgmcA together) or streptomycin and neomycin (for RLgmcA), giving the total number of viable rhizosphere- and root-associated bacteria (Barr et al., 2008). Each treatment consisted of 10 replications, and statistical differences were analyzed with one-way ANOVA ($P < 0.05$).

RNA Isolation and Quantitative Reverse Transcription-PCR (RT-PCR)

Quantitative Real-Time RT-PCR was used to determine differences in the expression of genes. Cell samples were collected from free-living *R. leguminosarum* cultivated in AMS liquid medium, or free-living cells treated with 5 mM H_2O_2 for 1 h or root nodules, which were harvested from pea that had been inoculated with *R. leguminosarum* strains at 2, 4, and 6 weeks. The nodules of plants were harvested and grinded into a regular fine powder with liquid nitrogen. Total RNA of each sample was extracted using TRIzol Reagent (Invitrogen) and quantified by NanoDrop (Thermo Fisher Scientific) (Smith et al., 1985). cDNA was prepared using SuperScriptTM II reverse transcriptase and random hexamers. Quantitative real-time PCR was performed using the SYBR Premix ExTaq (Takara, Dalian, China) on the BIO-RAD CFX96 Real-Time PCR Detection System. Primers for *katG*, *katE*, *hmuS*, *ohrB*, *rhtA*, and *nifD* are detailed in **Table 1**. *GyrB1* was used as a reference housekeeping gene and the obtained data were analyzed as previously described (Prell et al., 2009). Statistical analysis of data sets was performed using REST (Pfaffl et al., 2002).

Protein Extraction and LC-MS/MS Analysis

The 4-week-nodule samples were grinded into cell powder in liquid nitrogen. The cell powder was transferred to a 5-mL centrifuge tube. Four volumes of lysis buffer (8 M urea, 1% protease inhibitor cocktail) was then added to the cell powder, and the slurry was sonicated three times on ice using a high intensity ultrasonic processor. Cellular debris was removed by centrifugation at 12,000 g for 10 min at 4°C, the supernatant was collected, and the protein content was determined using BCA protein assay kit (Pierce, Rockland, IL, United States). The resulting proteins were reduced by 5 mM dithiothreitol at 56°C for 30 min, and then alkylated in 11 mM iodoacetamide for 15 min at room temperature in the dark. Each protein sample was then diluted by 100 mM tetraethyl ammonium bromide (TEAB) to obtain a urea concentration of less than 2 and 1:100 trypsin-to-protein mass ratio for a second digestion of 4 h. Following

trypsin digestion, the peptides then were desalted by Strata X C18 SPE column and vacuum-dried. The peptides were reconstituted in 0.5 M TEAB and processed according to the manufacturer's instructions of the tandem mass tag (TMT) kit (Thermo Fisher Scientific, Bremen, United States). Concisely, one unit of TMT reagent was dissolved and reconstituted in acetonitrile. The peptide mixtures were then incubated at room temperature for 2 h and pooled, desalted and dried by vacuum centrifugation.

The tryptic peptides were dissolved in solvent A (0.1% formic acid in aqueous solution) and loaded directly onto a reversed phase analytical column (75 μ m i.d. \times 15 cm length). The loaded material was eluted from this column in a linear gradient of 6–22% solvent B (0.1% formic acid in 98% acetonitrile) over 26 min, 23–35% in 8 min, climbing to 80% in 3 min and holding at 80% for 3 min with a flow rate of 400 nL/min. The MS proteomics data were deposited to NSI source, followed by tandem mass spectrometry (MS/MS) by using a Q ExactiveTM Plus (Thermo) coupled online to the ultra-performance liquid chromatography (UPLC). The electrospray voltage was set to 2.0 kV. For the full scan mode, the m/z scan range was from 350 to 1,800. The intact peptides were detected in the Orbitrap at a resolution of 70,000. Peptides were selected to run MS/MS analysis using NCE setting as 28 and the fragments were measured using a resolution of 17,500 in the Orbitrap. The MS analysis alternated between MS and data-dependent tandem MS scans with 15.0 s dynamic exclusion. Automatic gain control (AGC) was set to accumulate 5×10^4 ions with the Fixed first mass of 100 m/z. Experiments were conducted in triplicate.

Data Analysis

The resulting MS/MS data were processed and prepared for a database search using the MaxQuant version 1.5.2.8 (Cox and Mann, 2008). The resulting tandem mass spectra were searched against the *R. leguminosarum* genome database concatenated with a reverse decoy database (Young et al., 2006). Trypsin/P was specified as the cleavage enzyme allowing up to four missing cleavages. The precursor mass tolerance was set to 20 ppm for the first search and 5 ppm for the main search, and the tolerance of the ions was set to 0.02 Da for fragment ion matches. Carbamidomethylation of cysteines was considered as a fixed modification, and oxidation of methionine was specified as variable modifications. A false discovery rate (FDR) of 1% was specified, and the minimal peptide score for modified peptides was set to 40. Protein expression was analyzed statistically using Student's *t*-tests ($p < 0.05$). Up-regulated and down-regulated proteins were defined as having fold changes (FC) > 1.2 and < 0.83 , respectively.

RESULTS

Bioinformatic Analysis of the *R. leguminosarum gmcA* Gene

Rhizobium leguminosarum gmcA gene (*pRL100444*) is predicted to encode a 550-amino acid polypeptide with an expected molecular mass of 60.5 kDa and a pI value of 8.19 (Young et al., 2006). The amino acid sequence of GmcA contained

TABLE 2 | Tolerance of *R. leguminosarum* strains to different concentrations of H₂O₂.

Strain	H ₂ O ₂ (mM)				
	0	0.5	1.0	5.0	10.0
RL3841	$(6.72 \pm 0.71) \times 10^{8a}$	$(4.27 \pm 0.27) \times 10^{8a}$	$(3.85 \pm 0.18) \times 10^{8a}$	$(2.73 \pm 0.14) \times 10^{7a}$	$(1.01 \pm 0.21) \times 10^{7a}$
RLgmcA	$(7.03 \pm 1.00) \times 10^{8a}$	$(3.80 \pm 0.17) \times 10^{8a}$	$(3.21 \pm 0.29) \times 10^{8a}$	$(1.59 \pm 0.19) \times 10^{7b}$	$(4.07 \pm 0.90) \times 10^{6b}$

All data are averages (\pm SEM) from three independent experiments. ^{a,b}Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, $P < 0.05$).

a consensus motif of a FAD/NAD(P)-binding domain in its N-terminal part and two GMC oxidoreductase signature patterns (Supplementary Figure S1), suggesting that GmcA should be included into the glucose-methanol-choline (GMC) flavin-dependent oxidoreductase family.

Antioxidation Analysis of a *R. leguminosarum* gmcA Mutant

To confirm the function of the *gmcA* gene in growth performance, antioxidation and symbiotic nitrogen fixation ability, a mutant RLgmcA strain of this gene was constructed by single crossover homologous recombination. In liquid AMS minimal medium with glucose as a carbon source and NH₄Cl as a nitrogen source, there is no significant difference in growth between the mutant RLgmcA and wild-type RL3841 (data not shown).

The importance of GmcA for protection against oxidative stress was investigated by carrying out survival assays of the mutant RLgmcA in the presence of oxide hydrogen peroxide (H₂O₂). The survival rates of RLgmcA were not significantly affected by H₂O₂ treatments at low concentrations of 0.5 and 1 mmol/L compared with the wild-type RL3841 strain, whereas the antioxidative capacity of mutant RLgmcA was significantly decreased by these treatments with H₂O₂ at higher concentrations of 5 and 10 mmol/L (Table 2). The role of *R. leguminosarum* GmcA in controlling protein glutathionylation status was investigated by quantifying glutathione reductase and glutathione peroxidase activities in 5 mM H₂O₂-induced oxidative stress conditions. The results showed that the glutathione reductase activity of mutant RLgmcA was not different from that of wild-type strain RL3841, but its glutathione peroxidase activity was significantly lower (Table 3). Thus, GmcA may play important roles in oxidative stress resistance and cellular detoxification in *R. leguminosarum*.

Pea Rhizosphere Colonization by *R. leguminosarum* Strains

Competition between the *gmcA* mutant RLgmcA and the wild type RL3841 for growth in the pea rhizosphere was measured by inoculating a low number of bacteria into the pea rhizosphere (10^3 to 10^4 bacteria per seedling) and determining total bacteria after 7 days. When the mutant RLgmcA and the wild type RL3841 were inoculated alone into short-term colonization of sterile pea rhizosphere, the percentage of bacteria recovered after 7 days was significantly lower for the mutant than for the wt strain (Figure 1). When inoculated in equal ratios, RLgmcA accounted

TABLE 3 | Oxidase activity of *R. leguminosarum* gmcA mutant.

Strains <i>R. leguminosarum</i>	Glutathione reductase (U/mg protein)	Glutathione peroxidase (U/mg protein)
RL3841	0.399 ± 0.041^a	0.406 ± 0.029^a
RLgmcA	0.408 ± 0.031^a	0.018 ± 0.006^b
RLgmcA(pBBRgmcA)	0.409 ± 0.030^a	0.385 ± 0.031^a

All data are averages (\pm SEM) from three independent experiments. ^{a,b}Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, $P < 0.05$).

for only 25% of bacteria recovered (t -test; $P \leq 0.01$). Even when strain RLgmcA was inoculated at a 10-fold excess over the wild type, it still accounted for only 41% of bacteria recovered (Figure 1). The decreased ability of the *gmcA* mutant to grow in a sterile rhizosphere of peas shows that GmcA is essential for colonization of the pea rhizosphere by *R. leguminosarum*.

The Symbiotic Phenotype of *R. leguminosarum* Strains

To observe the nodulation status and measure nitrogenase activity of the *gmcA* mutant strain, pea seedlings were inoculated with the mutant RLgmcA or wild-type RL3841. Four weeks later, the number, shape and structure, and acetylene reduction activity (ARA) values of the nodules were measured. No statistically significant difference was observed in the number of nodules per plant between plants inoculated strain RLgmcA and plants inoculated with wild-type RL3841 (Table 4 and Supplementary Figure S2). *R. leguminosarum* bv. *viciae* formed determinate nodules on pea, while the *gmcA* mutant elicited more elongated, rather than spherical, nodules compared to the wild type and showed a 30.36% decrease in ARA and a 40% drop in the dry weight of plants compared to the wild type (Table 4). When recombinant plasmid pBBRgmcA was introduced into mutant RLgmcA, plants inoculated with the resulting strain RLgmcA(pBBRgmcA) formed normal nodules and showed no significant difference in nitrogen-fixing ability and the dry weight of plants compared to the RL3841-inoculated plants (Table 4).

Four-week-old nodules were further examined by both light and electron microscopy. The nodules induced both by wild type RL3841 and by mutant RLgmcA turned blue when stained with toluidine blue. These observations were corroborated by light microscopic analysis. Both the nodules

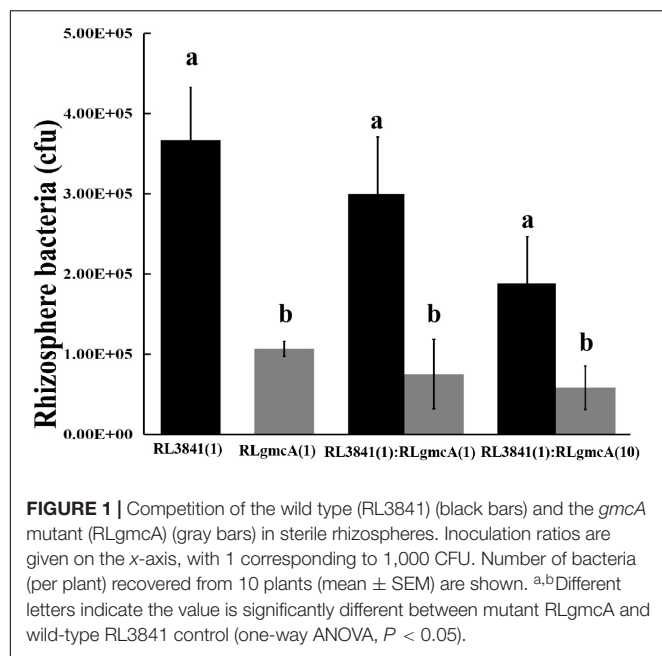


TABLE 4 | Symbiotic behavior of *R. leguminosarum gmcA* mutant.

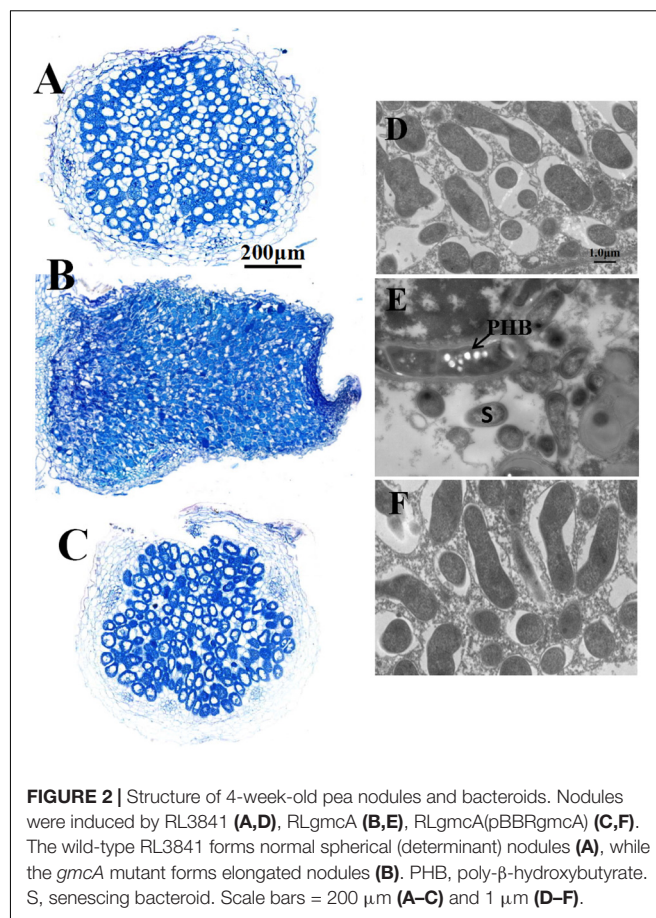
Strain	Nodules per plant	Acetylene reduction(μ moles acetylene per plant per h)	Dry weight per plant (g)
RL3841	137.3 \pm 13.2 ^a	2.24 \pm 0.14 ^a	1.86 \pm 0.20 ^a
LMB599	131.3 \pm 11.3 ^a	1.56 \pm 0.05 ^b	1.10 \pm 0.18 ^b
LMB675	135.5 \pm 11.3 ^a	2.12 \pm 0.16 ^a	1.80 \pm 0.16 ^a
WC	0	0	0.35 \pm 0.09 ^c

All data are averages (\pm SEM) from ten independent plants. ^{a,b,c}Different letters indicates the value is significantly different from that of the wild-type RL3841 control (one-way ANOVA, $P < 0.05$). WC, water control without inoculation.

were filled by Rhizobia-infected cells (Figures 2A,B). The ultrastructural structure of the infected cells was observed by transmission electron microscopy. In the mutant infected nodule cells, bacteroids underwent premature senescence. Bacteroids in pea plants inoculated by *R. leguminosarum* bv. *viciae* usually did not produce visible PHB granules, but in the mutant bacteroids, the poly- β -hydroxybutyrate (PHB) was also distinctly observed (Figure 2).

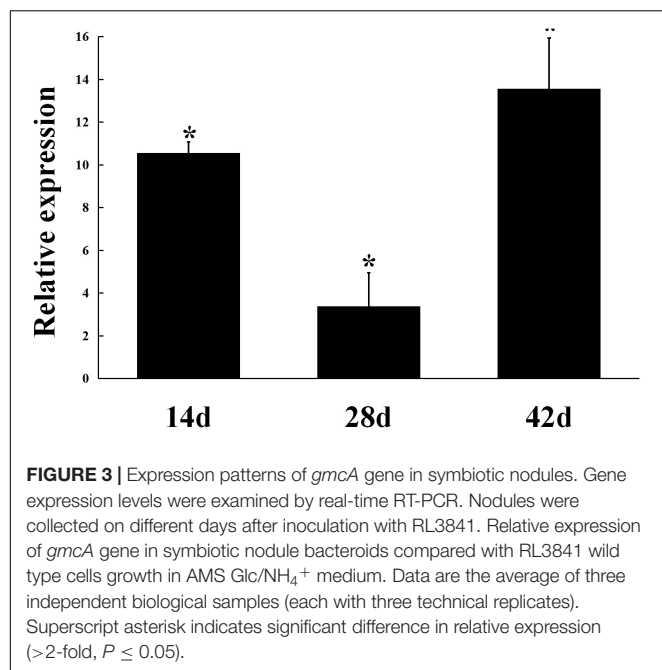
Expression Level of the *gmcA* Gene in Nodules Induced by *R. leguminosarum* 3841

The expression of *gmcA* was significantly up-regulated in the early stage (14 days), maturation stage (28 days) and late stage (42 days) of nodule development and senescence in comparison to that in free-living cells (Figure 3). During symbiosis, *gmcA* gene has the highest expression level in nodules at 42 days after inoculation. Thus, these results showed that *gmcA* gene expression was induced during *R. leguminosarum*-pea symbiosis and suggest that this gene plays an important role in bacteroid persistence in old nodules.



Analysis of the Relative Expression of Genes Involved in Redoxin Production and Nitrogen Fixation in the *gmcA* Mutant

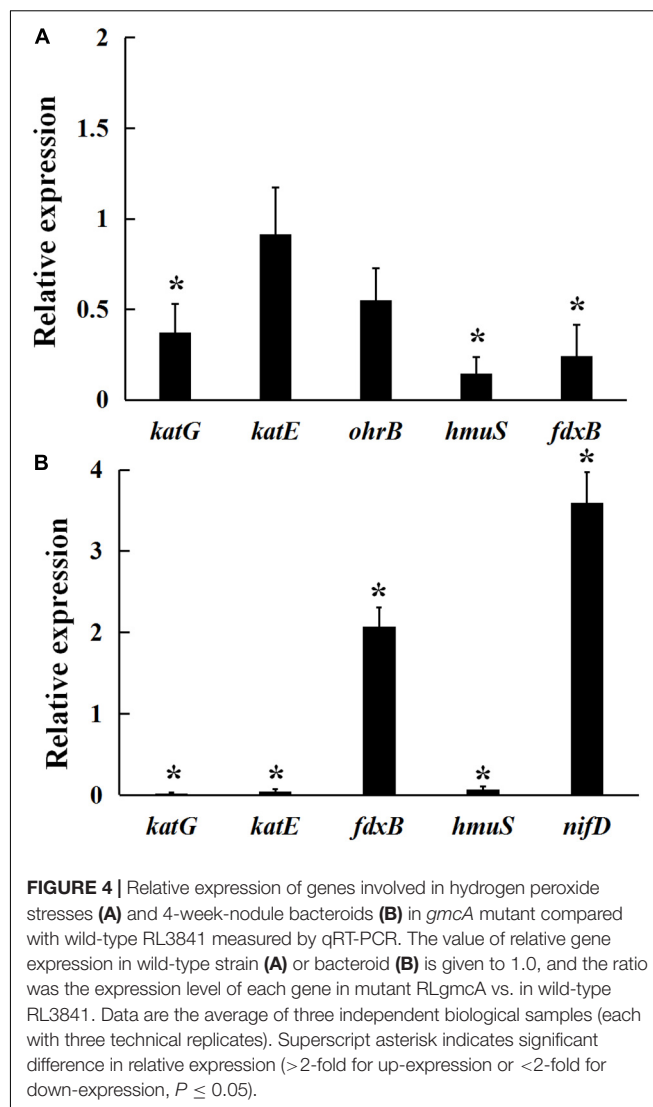
As shown in Figure 4A, under 5 mmol/L H_2O_2 -induced oxidative stress condition, a significant decrease in *katG*, *fdxB*, and *hmuS* gene expression was observed in the *gmcA* mutant, suggesting that GmcA plays an important role in cellular redox balance. Since a large reduction in the nitrogen-fixing capacity of nodules inoculated with mutant strain was observed, qRT-PCR was used to assess whether the N-fixation system, e.g., nitrogenase genes, was affected in the transcription of ribosomal RNAs in the GmcA-deficient mutant. The expression of *nifD* and *fdxB* was analyzed in pea root nodules using qRT-PCR (Figure 4B). Unexpectedly, the expression level of *nifD* and *fdxB* was found to be significantly increased in 4-week-old nodules inoculated with *gmcA* mutant strain compared with control nodules. Thus, GmcA may function in redox balance and antioxidant defense system in the pea root nodules. *hmuS* gene expression was significantly down-regulated in *gmcA* mutant, both under H_2O_2 -induced oxidative stress condition and in 4-week-old nodule, suggesting *gmcA* is involved in iron transport and regulation of iron homeostasis.



Protein Differential Expression Analysis

A quantitative proteomic approach using UPLC coupled with tandem mass spectrometry (LC/LC-MS/MS) was performed to compare differential *gmcA* mutant root bacteroids in response to wild type infection. Proteomics analysis identified peptides derived from a total of 2002 distinct protein groups in *gmcA* mutant bacteroids and 2000 in wild-type bacteroids, with molecular weights ranging from 7 to 317 kDa. A total of 60 differentially expressed proteins ($P < 0.05$) were identified. Among these proteins (Table 5), 33 proteins were up-regulated in *gmcA* mutant nodule bacteroids and 27 proteins were down-regulated. Cell surface protein (RL4381) was absent in the *gmcA* mutant bacteroids, while invasion associated protein (RL1020) and lipoate-protein ligase B (RL2555) were not found in the wild-type bacteroids. Thirty-two differential protein-encoding genes were localized in plasmids pRL7, pRL8, pRL9, pRL10, pRL11, and pRL12. Cellular localization of the differentially expressed proteins showed that thirty-nine proteins localized to the cytoplasm, thirteen proteins localized to periplasmic space, five proteins located in the outer membrane, two were extracellular proteins, and one protein existed in the inner membrane (Table 5).

By sorting the identified proteins according to metabolic function, most of the differences in expression were found among transporter activity (15 proteins), followed by 12 proteins related to stress response and virulence, 9 proteins related to transcription factor activity, 7 proteins related to amino acid metabolism, 6 proteins related to carbohydrate metabolism, and 4 proteins related to nucleotide metabolism. This change in metabolism was mirrored by corresponding changes in proteins involved in the regulation of transcription, among which, a *nif*-specific transcriptional activator NifA and a nitrogen regulatory protein PtsN were highly expressed in the mutant bacteroids.



The main groups of differentially expressed proteins identified were transport proteins, of which 6 were ABC-type nitrate/nitrite transporters. The result showed *gmcA* mutant was affected in transport, especially in nitrate transport. Further analysis of the differentially expressed proteins identified a subset involved in stress response and virulence. The number of affected oxidoreductases, cytochrome oxidase, dehydrogenase, hydrolase, dehydrogenase, surface, and invasion associated proteins also suggests that GmcA function in antioxidant capacity in the root nodules and that the loss of these proteins could result in antioxidant defect. Finally, the loss of GmcA resulted in the differential expression of seven proteins with unknown function in the nodule bacteroids.

DISCUSSION

The family of GMC oxidoreductases includes glucose/alcohol oxidase and glucose/choline dehydrogenase. Members of this

TABLE 5 | Differential expression proteins in 4-week nodule mutant bacteroids relative to wild-type bacteroids.

Gene ID	Gene Name	Cellular localization	Protein description	MW [kDa]	pI	Ratio	P-value
Stress response and virulence							
RL3853		Cytoplasmic	FAD-dependent oxidoreductase	47.38	5.71	6.15	0.0004
RL4381		Outer membrane	cell surface protein	66.06	4.46	1.58	NP1
pRL90097	<i>pdxA2</i>	Cytoplasmic	4-hydroxythreonine-4-phosphate dehydrogenase	34.66	6.39	1.23	0.0332
pRL100245		Cytoplasmic	LLM class flavin-dependent oxidoreductase	38.97	5.20	1.22	0.0082
pRL80022		Cytoplasmic	alpha/beta hydrolase	35.12	6.03	-0.40	0.0011
pRL80023	<i>cutM</i>	Cytoplasmic	carbon monoxide dehydrogenase subunit M protein	30.42	5.56	-0.48	0.0012
pRL80041	<i>hisD</i>	Cytoplasmic	Histidinol dehydrogenase	47.16	5.39	-0.60	0.0115
RL1020		Periplasmic	invasion associated protein	22.08	5.45	-0.85	NP2
pRL120603	<i>gabD3</i>	Cytoplasmic	NAD-dependent succinate-semialdehyde dehydrogenase	52.53	5.29	-0.74	0.0344
pRL90027	<i>adhA</i>	Cytoplasmic	alcohol dehydrogenase	37.15	5.93	-0.77	0.0008
pRL120056	<i>mcpR</i>	Cytoplasmic	methyl-accepting chemotaxis protein	68.73	5.01	-0.77	0.0426
pRL90018	<i>fixN2</i>	Inner membrane	Putative cytochrome oxidase transmembrane component FixN	60.90	8.98	-0.82	0.0074
Amino acid metabolism							
pRL100242		Cytoplasmic	amino acid synthesis family protein	21.17	6.29	1.45	0.0159
pRL110557	<i>glxB</i>	Cytoplasmic	glutamine amidotransferase	31.99	5.21	1.42	0.0190
RL0041	<i>hisE</i>	Cytoplasmic	Phosphoribosyl-ATP pyrophosphatase	11.51	5.19	1.30	0.0496
pRL100099		Cytoplasmic	Nif11 family protein	14.45	8.86	1.23	0.0152
RL2075	<i>gatC</i>	Cytoplasmic	Aspartyl/glutamyl-tRNA(Asn/Gln) amidotransferase subunit C	10.19	4.73	1.22	0.0082
pRL100192		Outer membrane	glutamate N-acetyltransferase	20.33	6.71	1.21	0.0024
pRL90221		Cytoplasmic	Putative glutamine amidotransferase protein	28.31	5.18	-0.72	0.0244
Carbohydrate metabolism							
pRL110453		Cytoplasmic	Concanavalin A-like lectin/glucanase domain	22.05	5.00	1.25	0.0237
RL0916	<i>dgoK</i>	Cytoplasmic	2-dehydro-3-deoxygalactonokinase	31.49	5.69	-0.73	0.0076
RL0874	<i>RL0874</i>	Cytoplasmic	aldo/keto reductase	38.21	5.45	-0.78	0.0000
pRL110598		Cytoplasmic	L-fuconate dehydratase	47.20	5.17	-0.82	0.0137
pRL120643	<i>groSp12</i>	Cytoplasmic	co-chaperone GroES	11.37	5.48	-0.83	0.0003
RL2555	<i>lipB</i>	Cytoplasmic	lipoate-protein ligase B	26.70	5.22	-0.99	NP2
Transporter activity							
RL4326		Periplasmic	Putative transmembrane protein	98.15	5.59	1.39	0.0122
RL3066		Periplasmic	Putative transmembrane protein	17.22	8.09	1.35	0.0090
RL2491		Cytoplasmic	Conserved hypothetical exported protein	9.75	5.38	1.28	0.0001
pRL100386		Periplasmic	VWA domain-containing protein	74.90	4.84	1.24	0.0122
RL3065		Periplasmic	Conserved hypothetical exported protein	14.77	5.88	1.23	0.0151
pRL70182		Periplasmic	Conserved hypothetical exported protein	36.84	5.32	1.21	0.0265
pRL80026	<i>livJ</i>	Periplasmic	ABC transporter substrate-binding protein	45.36	5.81	-0.33	0.0006
pRL80060		Periplasmic	ABC transporter substrate-binding protein	29.75	5.35	-0.53	0.0007
pRL80085		Cytoplasmic	Autoinducer 2 ABC transporter substrate-binding protein	35.70	6.02	-0.60	0.0448
RL2775	<i>ropA1</i>	Outer membrane	Porin	36.80	3.92	-0.71	0.0111
RL4402		Cytoplasmic	ABC transporter substrate-binding protein	35.94	4.96	-0.72	0.0115
RL1499	<i>ropA2</i>	Outer membrane	Porin	36.71	4.01	-0.77	0.0001
pRL100415		Periplasmic	ABC transporter substrate-binding protein	37.92	5.17	-0.79	0.0492
pRL120671		Periplasmic	nitrate ABC transporter substrate-binding protein	36.00	5.50	-0.83	0.0123
pRL100325	<i>fhuA1</i>	Outer membrane	outer membrane siderophore receptor	78.19	4.64	-0.83	0.0203
Nucleotide metabolism							
RL0952		Cytoplasmic	RNA-binding domain transcriptional regulator	83.38	6.67	1.34	0.0278
RL2475	<i>holB</i>	Cytoplasmic	Putative DNA polymerase III, delta subunit	36.35	5.90	1.26	0.0409
RL1785	<i>rplX</i>	Cytoplasmic	50S ribosomal protein L5	11.21	10.37	1.23	0.0038
RL2183		Cytoplasmic	nucleotidyltransferase	33.59	8.80	-0.82	0.017
Transcription factor activity							
pRL100146		Periplasmic	transcriptional regulator	23.94	9.57	1.33	0.0243
RL4412	<i>priA</i>	Cytoplasmic	primosome assembly protein PriA	80.82	6.32	1.30	0.0382

(Continued)

TABLE 5 | Continued

Gene ID	Gene Name	Cellular localization	Protein description	MW [kDa]	pI	Ratio	P-value
RL3457		Extracellular	SH3-like domain, bacterial-type; uncharacterized protein	21.84	4.62	1.25	0.0082
RL0425	<i>ptsN</i>	Cytoplasmic	PTS IIA-like nitrogen regulatory protein PtsN	16.65	5.70	1.23	0.0007
RL1379	<i>rosR</i>	Cytoplasmic	MucR family transcriptional regulator	15.62	6.96	1.22	0.0055
RL0133		Cytoplasmic	YbaB/EbfC family nucleoid-associated protein	11.42	5.18	1.22	0.0007
pRL100196	<i>nifA</i>	Cytoplasmic	nif-specific transcriptional activator	56.46	9.05	1.21	0.0011
pRL80079		Cytoplasmic	sugar-binding transcriptional regulator	35.34	5.71	−0.38	0.0072
pRL80046		Cytoplasmic	TetR/AcrR family transcriptional regulator	25.32	8.01	−0.46	0.0112
Unknown function proteins							
pRL100106		Periplasmic	Uncharacterized protein	28.65	6.19	1.59	0.0363
RL1874		Cytoplasmic	Uncharacterized protein	13.23	4.66	1.35	0.0185
RL3516		Extracellular	DUF2076 domain-containing protein	27.48	4.26	1.24	0.0017
RL4728		Periplasmic	DUF1013 domain-containing protein	26.14	5.91	1.23	0.0005
RL2820		Cytoplasmic	Uncharacterized protein	7.40	9.46	1.23	0.0494
pRL80010		Cytoplasmic	Uncharacterized protein	9.58	9.51	−0.21	0.0189
pRL80005		Cytoplasmic	Uncharacterized protein	59.58	5.52	−0.47	0.0028

Protein expression was analyzed statistically using Student's *t*-tests ($P < 0.05$). Np1, no protein in mutant; Np2, no protein detected in wild type.

family catalyze a wide variety of redox reactions with respect to substrates and co-substrates (Sützl et al., 2018). An important issue is that *gmcA* expression is elevated in nitrogen-fixing bacteroids of the pea root nodules, but the function of GmcA in root nodule bacteria nitrogen fixing system is poorly understood. In this study, we took advantage of a *gmcA* mutant strain of *R. leguminosarum* to examine what role GmcA may play in symbiotic nitrogen fixation. Our data demonstrated that GmcA is required for the nodule senescence and cellular detoxification that is affected, regarding its nitrogen fixation capacity and oxidative stress response.

Mutation of *R. leguminosarum gmcA* did not affect the growth of free-living bacteria but led to decreased antioxidative capacity under the conditions of 5 and 10 mM hydrogen peroxide H_2O_2 . The direct link between GmcA and H_2O_2 detoxification has been less reported, while in most wood-rotting fungi, the members of GMC oxidoreductase superfamily play a central role in the degradation process because they generate extracellular H_2O_2 , acting as the ultimate oxidizer (Ferreira et al., 2015). Our results suggested that cells with GmcA tolerate internally generated or exogenously applied H_2O_2 . Cellular oxidoreductases catalyze redox processes by transferring electrons from a reductant to oxidant and are important for protection against oxidative stress (Bisogno et al., 2010). The ferredoxin-like protein (FdxB) and iron transport protein HmuS are ubiquitous electron transfer proteins participating in the iron-sulfur cluster biosynthesis and a wide variety of redox reactions (Chao et al., 2005; Gu et al., 2008). In *Rhizobium*, the peroxidases and the catalases KatG (catalase HPI), KatE (catalase), and OhrB (organic hydroperoxide resistance) were known to participate in the antioxidant defense mechanism against H_2O_2 -induced stress (Vargas Mdel et al., 2003), and the two electron transfer proteins FdxB and HmuS are also involved in a wide variety of redox reactions (Chao et al., 2005; Gu et al., 2008). This cell cytotoxicity was relieved by inducing transcription of antioxidant genes (Jung and Kim, 2003). Expression levels of *katG*, *fdxB*, and *hmuS*

were significantly down-regulated in the *gmcA* mutant under H_2O_2 -induced oxidative stress. It has been reported that decreased ferredoxin-NADP(H) oxidoreductase (FNR) results in a more oxidized glutathione pool, while increasing FNR content results in a more reduced glutathione pool (Goss et al., 2012). Glutathione reductase activity in mutant RLgmcA was not different from that wild-type strain, but the absence of GmcA was associated with a 96.5% decrease in cellular glutathione peroxidase activity. Cellular peroxide deficit damages cellular macromolecules by reactive oxygen species (ROS), and glutathione peroxidases are one of the important ROS scavengers in the cell (Islam et al., 2015). The decrease of glutathione peroxidase activity is related to an uncontrolled increase of ROS (Giergiel et al., 2012).

Pea plants inoculated with the *gmcA* mutant exhibited a large decrease in the nitrogen-fixing activity of root nodules (reduced by more than 30%), although, the protein expression of NifA and PtsN was higher in the mutant bacteroids compared to that of wild type bacteroids. Two genes, *nifD* and *fdxB*, involved in metabolism related to nitrogen fixation and bacteroid maturation in pea root nodules (Capela et al., 2006) also had a higher level of expression in the mutant bacteroids. It has been reported that GMC oxidoreductases are involved in extracellular hydrogen peroxide and iron homeostasis (Rohr et al., 2013). Iron is required for symbiotic nitrogen fixation as a key component of multiple ferroproteins involved in this important biological process (Takanashi et al., 2013). *hmuS* was chosen based on previous studies, which showed that it was involved in iron transport (Chao et al., 2005). *hmuS* exhibited higher expression level in the mutant bacteroids, demonstrating the involvement of GMC in the regulation of iron homeostasis. Proteomic analysis of the mutant nodule bacteroids indicated that most of the differentially expressed proteins were involved in transporter activity, metabolism, and stress responses. These transporters may aid in regulation of ion and membrane potential homeostasis through their transport of nitrate, which is known to regulate

the symbiosis (Vincill et al., 2005). These results indicated that GmcA is involved in a variety of metabolic processes, as has been described in *A. niger* and *E. coli* (Etxebeste et al., 2012; Liu et al., 2013).

The electron microscope investigation revealed that *gmcA* mutant altered the ultrastructure of pea nodules. GmcA can likely play a role in nodule senescence, since senescent parameters such as increased activities of enzymes of amino acid metabolism, PHB production, and an increase in the number of disintegrated bacteroids occurred. In addition, glutathione peroxidase activity dramatically decreased, and amino acid metabolism reflecting arginase activity was increased. *R. leguminosarum* bv. *viciae* forms determinate nodules on pea and usually does not produce visible PHB granules during symbiosis. PHB granules occurred in undergoing senescence bacteroids, which indicated that the energy and carbon metabolism has shifted (Xie et al., 2011). The PHB and tricarboxylic acid (TCA) cycles both start with acetyl-CoA. Under aerobic conditions, the TCA cycle is responsible for the complete oxidation of acetyl-CoA and formation of intermediates required for ATP production, but under oxygen limitation condition, when there is an inhibition of the TCA cycle by NADH or NADPH, the bacteroids favor PHB synthesis. During PHB synthesis, there is apparently a concomitant reduction in protein synthesis, a process coupled to ATP formation and utilization (Tal et al., 1990). In the symbiosis of the GmcA-deficiency mutant RLgmcA, the low expression of the catalase-peroxidase gene (*katG*), alpha/beta hydrolase (pRL80022), carbon monoxide dehydrogenase (pRL80023), succinate-semialdehyde dehydrogenase (pRL120603), and alcohol dehydrogenase (pRL90027) inhibited NAD(P)H oxidase activity. To allow continued operation of the TCA cycle, NAD(P)H was channeled into other biosynthesis reactions, such as PHB synthesis, for acting as reducing equivalents (Xie et al., 2011).

The *gmcA* gene expression is significantly up-regulated during the whole nodulation process, and its highest expression level occurred at 42 days after inoculation. Moreover, the *R. leguminosarum gmcA* mutant was unable to compete efficiently in the rhizosphere with its wild-type parent, which shows that bacterial GmcA is important for adaptation to the microenvironment of the plant host. Overall, considering the poor nitrogen-fixing ability of its nodules, the mutant in *gmcA* gene had a profound influence on the whole nodulation process.

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DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017485.

AUTHOR CONTRIBUTIONS

GC conceived and designed the study. QZ, SL, and HW performed the experiments. GC, QZ, DH, and XL analyzed the results. GC and QZ wrote 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/fmicb.2020.00394/full#supplementary-material>

FIGURE S1 | Analysis of the protein domains of GmcA in *Rhizobium leguminosarum* 3841. BetA, choline dehydrogenase or related flavoprotein; GMC_oxred_C, GMC oxidoreductase; GMC_mycocaf_2, GMC family mycofactacin-associated oxidoreductase.

FIGURE S2 | Plant growth test of the symbiotic ability of *R. leguminosarum*. (A) Control plant root inoculated with the wild type RL3841, (B) Plant root inoculated with RLgmcA.

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Effects of *Epichloë gansuensis* Endophyte on the Root and Rhizosphere Soil Bacteria of *Achnatherum inebrians* Under Different Moisture Conditions

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This study was conducted to explore effects of the systemic fungal endophyte *Epichloë gansuensis* on root and rhizosphere soil bacterial diversity of *Achnatherum inebrians* host plants growing under different moisture conditions. Soil properties of different treatments were compared using standard techniques. A total of 4371379 16S rRNA gene sequences were obtained and assigned to 5025 operational taxonomic units (OTUs). These OTUs in roots and rhizosphere soil were divided into 13 and 17 phyla, respectively, and the Actinobacteria and Proteobacteria were the most abundant phyla both in roots and rhizosphere soil. Shannon diversity and Chao1 richness index of bacteria in rhizosphere soil was significantly higher than in roots. *E. gansuensis* decreased the Shannon diversity of the root-associated bacterial community, and increased Shannon diversity and Chao1 richness index of the rhizosphere soil bacterial community of *A. inebrians*. Meanwhile, Chao1 richness of the rhizosphere soil bacterial community of *A. inebrians* significantly increased with the increase of the soil moisture level. Structural equation modeling also emphasized that *E. gansuensis* decreased the diversity of the root-associated bacterial community and increased the diversity of the rhizosphere soil bacterial community through decreasing soil available N. Additionally, soil moisture increased the diversity of the rhizosphere soil bacterial community through increased soil pH, C/N, and NN, and decreased soil AP. The *E. gansuensis* endophyte and soil moisture effects on root and rhizosphere soil bacterial diversity were likely to be from responses to modifications of the rhizosphere soil properties.

Keywords: *Epichloë gansuensis*, soil moisture, *Achnatherum inebrians*, bacterial diversity, plant-microbe interaction

INTRODUCTION

Microbial associations are widely distributed in terrestrial ecosystems, and plant tissues are associated with a wide range of microbes, including fungi (Johnson et al., 2013), and bacteria (Bell-Dereske et al., 2017). Endophytic fungi belonging to the genus *Epichloë* have been found in many cool-season grasses (Schardl et al., 2004; Leuchtman et al., 2014). The associations between host

grasses and the genus *Epichloë* are generally considered to be mutualistic and the transmission of many species is completely vertical (Schardl et al., 2004; Christensen et al., 2008). Previous studies on these symbiotic relationships have focused on the genera *Lolium* and *Festuca* because they enhance the adaptability and productivity of host plants under abiotic and biotic stresses (Johnson et al., 2013; Soto-Barajas et al., 2016).

Another grass species that is host to an *Epichloë* endophyte and which has become the focus for intense research is *Achnatherum inebrians*. This is a widespread perennial bunchgrass in the Qinghai-Tibet Plateau, including Tibet, Qinghai, Xinjiang, and Gansu provinces (Nan and Li, 2000; Li et al., 2004). Nearly 100% of *A. inebrians* plants in these regions are host to an *Epichloë* endophyte (Nan and Li, 2000). The endophyte was originally classified as being a *Neotyphodium* species as the sole method of transmission is through the seed of host plants; stromata in which the sexual stage of some *Epichloë* species is formed have never been observed, almost certainly eliminating the possibility of horizontal transmission. The endophyte of *A. inebrians* was originally given the name of *Neotyphodium gansuense* (Li et al., 2004) but was later classified as *E. gansuensis* (Leuchtmann et al., 2014). A subsequent study revealed that *A. inebrians* plants originating from seed obtained from seven of eight populations from the grasslands of Xinjiang, Gansu and Inner Mongolia provinces of China were host to a distinctive *Epichloë* endophyte that was given the name *E. inebrians* (Chen et al., 2015). *A. inebrians* plants have long been associated with a narcotic-type effect on grazing livestock, giving rise to the species name *inebrians* and the common name of drunken horse grass (Zhang et al., 2011, 2014a; Liang et al., 2017). In 1996, high levels of ergonovine and lysergic acid amide were identified in leaves of *A. inebrians* plants infected with a systemic endophyte, then referred to as an *Acremonium* sp of the *albolaranosa* section Morgan-Jones and Gams (Miles et al., 1996). It is these endophyte-produced alkaloids that are present in *A. inebrians* plants host to *E. inebrians* (Chen et al., 2015). *A. inebrians* plants host to *E. gansuensis* were found in that study to contain the indole-diterpene alkaloid paxilline (Chen et al., 2015). The presence of an *Epichloë* endophyte in *A. inebrians* plants provides enhanced tolerance to abiotic stresses including drought stress (Xia et al., 2018), salt stress (Wang et al., 2018), heavy metals (Zhang et al., 2010), and low temperature (Chen et al., 2016), and as well as resistance to fungal pathogens (Xia et al., 2016) and insect pests (Zhang et al., 2012). The deterrence of grazing and the enhanced tolerance to abiotic and biotic stresses conferred by the presence of an *Epichloë* endophyte have led to the greatly increased distribution of *A. inebrians* throughout the grasslands of northwest China that have been degraded by overgrazing (Zhao et al., 2005; Yao et al., 2015).

Effects of *Epichloë* endophytes, hyphae of which are absent in roots, have been reported on belowground organisms, especially microorganisms, and under different ambient conditions (Rojas et al., 2016; Bell-Dereske et al., 2017; Zhong et al., 2018). Included in the microorganisms affected are arbuscular mycorrhizal fungi (AMF; Rojas et al., 2016) and phosphorus-solubilizing fungi (Arrieta et al., 2015). Additionally, previous studies also found that the presence of an *Epichloë* endophyte decreased the

abundance of gram-positive bacteria in soil of tall fescue (*Festuca arundinacea*; Buyer et al., 2011) and the root-associated bacterial diversity of American beachgrass (*Ammophila breviligulata*; Bell-Dereske et al., 2017), while increased the rhizosphere soil bacterial diversity associated with tall fescue (Roberts and Ferraro, 2015). The presence of an *Epichloë* endophyte in annual ryegrass (*Lolium multiflorum*) changed the composition of the soil bacterial community (Casas et al., 2011).

The composition and diversity of bacterial communities in soil and roots are considered as indicators reflecting plant biomass, mineral resources acquisition and biological processes, which are inevitably affected by host plant and environmental factors, including pH (Shen et al., 2013), nutrient availability (Meyer et al., 2013), soil moisture (Zhang et al., 2014b), and fertility (Yao et al., 2018). These factors alter rhizosphere soil microbial communities by changing soil physical and chemical properties, nutrient cycling and phytohormones production (Zhang et al., 2014b; Francioli et al., 2017; Zhahlnina et al., 2018). Previous research also indicated that plant genotype and vegetation growth stage transforms the plant bacterial diversity, and which can promote release of secondary metabolites from roots, influencing microbial diversity and community composition in rhizosphere soil (Guo et al., 2015; Vandegrift et al., 2015; Rojas et al., 2016; Soto-Barajas et al., 2016).

A previous study had found that the presence of *Epichloë gansuensis* increased the spore diversity of AMF in the *A. inebrians* plants rhizosphere soil under different growth conditions (Zhong et al., 2017), and decreased the root-associated fungal diversity under cultivation (Zhong et al., 2018). However, how *E. gansuensis* affects the bacterial diversity of rhizosphere soil and roots of *A. inebrians* is poorly understood. Our previous study had indicated that the presence of *E. gansuensis* could markedly improve water-use efficiency of *A. inebrians* plants under limited soil water content in greenhouse conditions (Xia et al., 2018). Furthermore, *E. gansuensis* also promoted the growth and development of *A. inebrians* roots under low soil moisture in the field (Xia, 2018). However, the effects of soil moisture on bacterial diversity in rhizosphere soil and roots of *A. inebrians* have not been reported. To address these questions, the objective of this present study was to investigate effects of *E. gansuensis* on bacterial diversity of rhizosphere soil and roots of *A. inebrians* plants under different moisture conditions. It was hypothesized that (1) *E. gansuensis* and soil moisture levels could influence the bacterial diversity in roots of *A. inebrians* plants and rhizosphere soil, (2) Changes in bacterial diversity in roots of *A. inebrians* plants and rhizosphere soil associated with the presence of *E. gansuensis* and changes in soil moisture may be related to the soil physical and chemical properties.

MATERIALS AND METHODS

Site Description and Experimental Design

This study was conducted in field plots at the Yuzhong campus (104°39'E, 35°89'N, and attitude 1653 m) of the College of Pastoral Agriculture Science and Technology of Lanzhou

University. The *A. inebrians* plants used in this study originated locally, from the location where the endophyte species present in this species of grass was assigned the name *N. gansuense* (Li et al., 2004). The endophyte infection status of 20 tillers from individual *A. inebrians* plants originating from that location was determined by aniline blue staining of leaf sheaths and observing under a microscope, and then in 2011, seeds were collected from the tillers of one *A. inebrians* plants with 100% endophyte-tiller infection (Li et al., 2016). Before planting in 2012, the collected seeds were divided into two parts, with one part treated with thiophanate methyl fungicide to eradicate *E. gansuensis*, while the other part was untreated. EF and EI *A. inebrians* seeds were planted separately at Yuzhong campus as described by Zhong et al. (2019). In 2013, seeds of *E. gansuensis*-free (EF) and *E. gansuensis*-infected (EI) *A. inebrians* plants were collected, the endophyte-infection status was confirmed in the laboratory, and then the seeds were stored at 4°C in a refrigerator. In 2014, EF and EI *A. inebrians* plants were established in the field, using seeds that had been collected from plants grown from seeds obtained from single EF and EI plants originating from the same population to lower variability of the plants used in experiments, as described by Xia et al. (2018). Before planting, 50 seeds were randomly selected from EI and EF seed stocks to determine their endophyte-infection status and confirm that seed stocks used in the study were 100% and 0% infected, respectively. There were nine plots (each plot: 4.0 m × 4.8 m), and each was divided equally into two parts by a cement wall. EF and EI *A. inebrians* plants were planted individually in 4 lines and 8 rows.

From May to October of 2014–2016, three water treatments were maintained on split plots including three replicate plots. The first treatment was maintained at normal water content (N) and only received natural precipitation, the annual precipitation from 2014 to 2016 was 321 mm, 282 mm, and 256 mm, respectively. The second treatment was drought stress (D), in which the plots were manually covered. The third treatment was the sufficient irrigation condition (W), which received water every 3 days by overhead automatic irrigation, and to maintain 45–60% of the relative saturated soil moisture.

Sample Description

Root and rhizosphere soil samples were collected at the end of water treatments in October 2016. For each sub-plot, roots and rhizosphere soil were obtained from 20 cm cores from five separate *A. inebrians* plants and following screening, the roots and rhizosphere soil were mixed to form mixed roots or rhizosphere soil samples. The 18 root and 18 rhizosphere soil samples were cooled and brought back to the laboratory. The root samples were gently rinsed several times with tap water then washed with sterile water, followed by drying on sterilized filter paper. Samples of these roots were stored at –80°C before DNA extraction. Before soil chemical analysis, soil samples were screened using a 2.0 mm sieve and stored at 4°C, while others were stored at –80°C.

Soil and Biological Properties

Soil pH was analyzed at a ratio of 1:2.5 in soil/water mixtures. A Shimadzu total organic carbon (TOC)-VCPH analyzer was

used to analyze TOC and total carbon (TC). According to the method of Nelson and Sommers (1982), 0.25 mm – sieved soil was used to measure the soil organic matter. Ammonium acetate and Flame Photometry were used to extract and analyze available potassium (AK; Helmke and Sparks, 1996). A molybdenum blue method was used to calculate the plant available phosphorus (AP; Robertson et al., 1999). A continuous flow analyzer (FIAstar 5000 Analyzer) was used to measure total nitrogen (TN), nitrate-N (NN), ammonium-N (AN), total P (TP), and available N in the soil (Zhao et al., 2014).

DNA Extraction, Amplification, and Sequencing

Total DNA was extracted from approximately 0.1 g and 0.5 g of root and rhizosphere soil samples, respectively, by using a plant DNA kit (Tiangen, Beijing) and a Soil DNA Kit (OMEGA, Shanghai) according to the manufacturer's instructions. Bacteria 16S rRNA genes were amplified by the primer pair of Eub518 (5'-ATT ACC GCG GCT GCT GG-3') and Eub338 (5'-ACT CCT ACG GGA GGC AGC AG-3'). Two different thermostable DNA polymerases were used in the 16S rDNA PCR amplifications for each sample in order to reduce PCR bias: (I) Phusion High-Fidelity DNA Polymerase (Thermo Scientific, Sweden): 98°C for 2 min followed by 30 cycles of 98°C for 30 s (denaturation), 56°C for 20 s (annealing), 72°C for 20 s (polymerization), and a final extension at 72°C for 10 min, and confirmed the size of amplified product was appropriate. Using 1% agarose gels to mixed and visualized each DNA samples after electrophoresis. Then PCR products were purified with a kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States), and submitted to Majorbio Pharm Technology (Shanghai, China) on the Illumina pyrosequencing¹ for sequences.

Bioinformatic Analyses

Pyrosequencing reads were assembled and filtered, and reads with ambiguous nucleotides, a quality score of less than 20, lacking complete barcode and primer were deleted and excluded from further analysis, and then the primer region was removed. The remaining sequences were assigned to operational taxonomic units (OTUs) using QIIME, requiring at least 97% threshold over at least 90% of the sequence length.² These sequences were performed on the Silva database³ to identify these OTUs which were obtained from Illumina pyrosequencing. After removing the non-bacteria OTUs, the abundance information of the OTUs was normalized using the sequence number standard, which corresponded to the sample with the minimum sequence, and the rarefaction curves were generated based on these OTUs. Subsequent analysis of alpha and beta diversity is based on this output of standardized data.

Alpha and Beta Diversity Analysis

Community richness was determined with the Chao1 index (Chao and Bunge, 2002) and community diversity was

¹<https://www.ncbi.nlm.nih.gov/sra/SRP117302>

²http://qiime.org/scripts/assign_taxonomy.html

³Release 128 <http://www.arb-silva.de>

determined by the Shannon index (H' ; Shannon, 1949), respectively, which was calculated using the formula.

$$\text{Chao1} = S_{\text{obs}} + \frac{F_1(F_1 - 1)}{2(F_2 + 1)}$$

Where the S_{obs} which represented the number of observed OTUs, and F_1 and F_2 are the number of singletons and doubletons, respectively.

$$H' = - \sum_{i=1}^s (P_i \log_2 P_i)$$

Where s is the number of OTUs and P_i is the proportion of the bacteria community represented by the OTUs.

For diversity analysis, dissimilarity of *A. inebrians* plant root and rhizosphere soil bacterial communities were calculated using principal-coordinate analysis (PCoA) by pairwise analysis, which was performed using R software (version 2.14.0) by pairwise Bray–Curtis dissimilarity. Based on Bray–Curtis distances, analysis of similarity (ANOSIM) and permutational multivariate two-way analysis of variance (PERMANOVA) were performed to calculate the statistically significant differences of root and rhizosphere soil bacterial communities under different treatments. Redundancy analysis (RDA) among *A. inebrians* plant root and rhizosphere soil bacterial community abundance at the phylum level and rhizosphere soil properties, were conducted by CANOCO for Windows 4.5.

Statistical Analyses

These differences of rhizosphere soil properties, and root and rhizosphere soil bacterial community diversity under different endophyte and soil moisture levels were tested using two-way analysis of variance (Two-way ANOVA) by SPSS 22.0 (SPSS Inc., Chicago, IL, United States). Significant differences among different soil moisture levels were tested by one-way analysis of variance (One-way ANOVA). Fishers least significant differences (LSD) test was used to determine whether differences between means were statistically significant. In all tests, P -value < 0.05 was considered statistically significant.

Structural Equation Modeling

Structural equation modeling (SEM) was used to identify potential causal relationships between explanatory variables and bacterial diversity. According to the results of linear regression, we calculated the degree of intimacy of direct and indirect relationships between variables, and checked the binary relationship between variables to ensure the appropriateness of the linear model. Based on the potential relationship between known factors and driving factors of bacterial diversity, SEM models were constructed. χ^2 test was used to evaluate the fitting of each model. In addition, AMOS 24.0 (Amos Development Co., Greene, MD, United States) was used for performing SEM analysis and others statistical analyses were calculated by SPSS 22.0 (SPSS, Inc., Chicago, IL, United States).

RESULTS

Root and Rhizosphere Soil Bacterial Community Composition

Using the pair of primers, a total of 1876756 and 2494623 sequences were obtained from rhizosphere soil and root samples, respectively (Figure 1 and Supplementary Table S1). There were 4994 OTUs detected in rhizosphere soil and 2627 OTUs detected in roots, and 2596 OTUs were present in both the roots and the rhizosphere soil. These OTUs in roots and rhizosphere soil were divided into 13 phyla and 17 phyla, respectively (Figure 1 and Supplementary Table S1). The bacterial communities in the roots of the three treatments harbored relatively fewer phyla compared to those in rhizosphere soil bacterial communities (Figure 1 and Supplementary Table S1). In addition, the overall patterns of relative abundance of the main groups at the phylum level in roots and rhizosphere soil is different among different endophyte plus soil moisture treatments (Figure 1 and Supplementary Table S1).

Actinobacteria (583 OTUs, 25.21% sequences) was the most abundant phylum in roots, while Proteobacteria (268 OTUs, 27.54% sequences) was the dominant phylum in rhizosphere soil bacterial communities under different treatments (Figures 1E,F and Supplementary Table S1). The following four most abundant phyla in roots were Proteobacteria (124 OTUs, 19.84% sequences), Cyanobacteria (67 OTUs, 18.86% sequences), Firmicutes (329 OTUs, 14.44% sequences), and Bacteroidetes (235 OTUs, 4.33% sequences; Figures 1B,D and Supplementary Table S1). In contrast, in rhizosphere soil the four next most abundant phyla were Actinobacteria (814 OTUs, 21.68% sequences), Firmicutes (483 OTUs, 15.68% sequences), Chloroflexi (314 OTUs, 6.07% sequences), and Fusobacteria (192 OTUs, 5.12% sequences; Figures 1B,D and Supplementary Table S1).

Rarefaction curves were generated for all root and rhizosphere soil treatments by using a 97% identity cutoff, which was used to depict the bacterial richness among different root (Supplementary Figure S1A) and rhizosphere soil samples (Supplementary Figure S1B). As the results show, the species of the bacterial community in roots (Supplementary Figure S1A) were less diverse than in rhizosphere soil (Supplementary Figure S1B). Principal coordinates analysis indicated that the root-associated and rhizosphere soil bacterial community composition between EI and EF *A. inebrians* differed among the D, N, and W treatments (Figures 2A,B and Table 1). *E. gansuensis*, soil moisture and their interactions had no significant ($P > 0.05$) effects on the diversity of the bacterial community in *A. inebrians* rhizosphere soil (Table 1), while, *E. gansuensis*, soil moisture and their interactions had significant ($P < 0.05$) effects on root-associated diversity of the bacterial community of *A. inebrians* (Table 1).

Root and Rhizosphere Soil Bacterial Community Diversity

The results indicated that the Shannon diversity and Chao1 richness index of bacterial diversity in rhizosphere soil ($F = 1.885$;

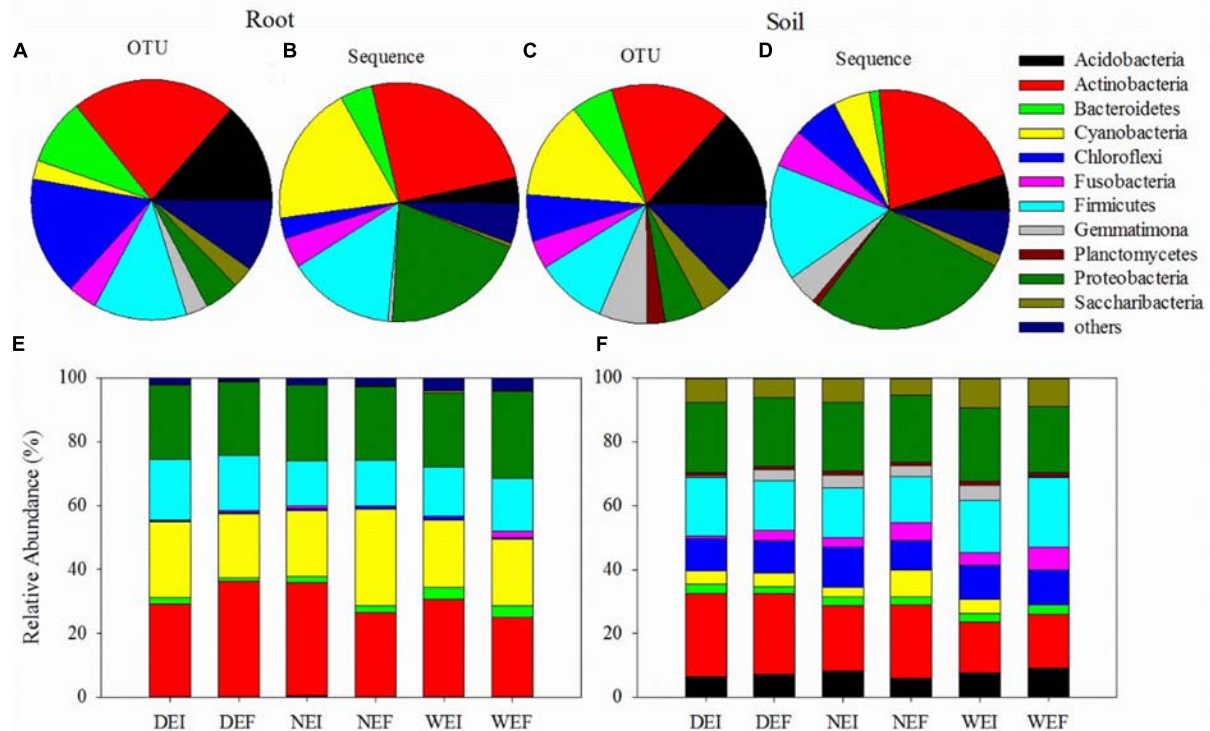


FIGURE 1 | The bacterial community structures (at the phyla level) of samples from roots (**A,B**) and rhizosphere soil (**C,D**) using plant and soil DNA kits, as well as the composition of different phyla of bacteria from the (**E**) roots and (**F**) rhizosphere soil of *A. inebrians* on account of the classification of partial 16S rRNA sequences ($n = 3$, D: drought, N: normal, W: well-watered, EI: endophyte-infected, and EF: endophyte-free).

$P < 0.001$) was significantly higher than in roots ($F = 0.009$; $P < 0.001$; **Figure 3**). Furthermore, the presence of *E. gansuensis* significantly ($P = 0.003$) decreased the Shannon diversity of the bacterial community in roots of *A. inebrians* (**Figure 3A**). The interactions between *E. gansuensis* and soil moisture had no significant effects on the Shannon diversity and Chao1 richness of bacterial diversity in roots of *A. inebrians* (**Figures 3A,B**).

The presence of *E. gansuensis* significantly increased the Shannon diversity ($P = 0.027$) and Chao1 richness ($P = 0.02$) of the rhizosphere soil bacterial community of *A. inebrians* (**Figures 3C,D**). Meanwhile, compared to the normal soil moisture, drought markedly decreased the Chao1 richness of the rhizosphere soil bacteria community of *A. inebrians*, and the well-watered treatment significantly increased the Chao1 richness of the rhizosphere soil bacteria communities (**Figure 3D**). The interactions between *E. gansuensis* and soil moisture had no significant effects on the Shannon diversity and Chao1 richness of rhizosphere soil bacterial community of *A. inebrians* (**Figures 3C,D**).

Relationship Between Bacteria and Soil Properties

The soil moisture had significant ($P < 0.01$) effects on soil properties, especially AN, NN, TN, AP, and available N (**Table 2**).

E. gansuensis had significant ($P < 0.01$) effects on soil properties, especially NN, AP, and available N (**Table 2**). The interactions between *E. gansuensis* and soil moisture had significant ($P < 0.01$) effects on *A. inebrians* rhizosphere soil AN and NN (**Table 2**). Spearman correlations results revealed that the Chao1 index of the *A. inebrians* rhizosphere soil bacterial community was positively and significantly ($P < 0.05$) associated with rhizosphere soil NN, available N, and N/P (**Table 3**), and negatively correlated with rhizosphere soil AP (**Table 3**). The Shannon index of the *A. inebrians* rhizosphere soil bacterial community had no significant ($P > 0.05$) correlation with rhizosphere soil properties (**Table 3**). Additionally, according to the RDA between the rhizosphere soil bacterial community and soil properties, the first and second axis of RDA explained 34.3% and 17.8% of the variance, respectively, as the length of each arrow represents the contribution of parameters to structural variation (**Figure 4B**). In addition, Proteobacteria was positively associated with rhizosphere soil AK, pH, AN, NN, and available N, while negatively associated with rhizosphere soil SOC and TN (**Figure 4B**). Meanwhile, Actinobacteria was positively associated with rhizosphere soil AK, AP, TP, TN, and negatively associated with rhizosphere soil pH, AN, NN, available N, SOC, and C/N (**Figure 4B**).

Spearman correlations results revealed that the Chao1 and Shannon index of *A. inebrians* root bacterial communities had no

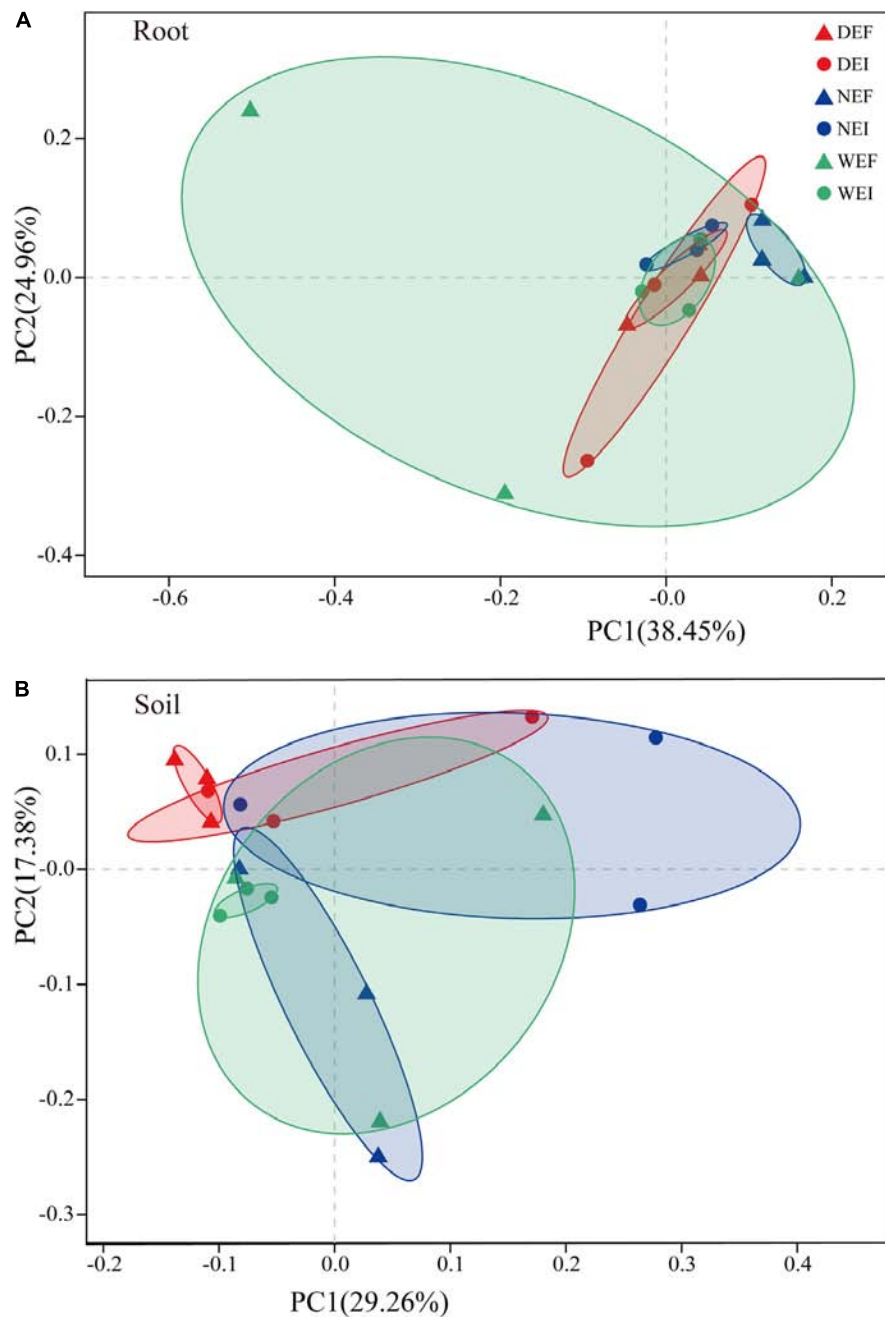


FIGURE 2 | The structure of plant-related bacterial communities. Principal Coordinates analysis (PCoA) of pairwise Bray–Curtis dissimilarity between **(A)** root and **(B)** rhizosphere soil comparison within each of the three datasets tested with randomly sampled microbial community ($n = 3$, D: drought, N: normal, W: well-watered, EI: endophyte-infected, and EF: endophyte-free).

significant ($P > 0.05$) correlation with soil properties (**Table 3**). Additionally, according to the RDA between the root bacterial community and soil properties, the first and second axis of the plotted RDA results explained 19.3% and 10.3% of the variance, respectively, (**Figure 4A**). Furthermore, Actinobacteria was positively correlated with soil AN, AP, pH, and TP, while negatively correlated with soil NN, AK, and available N (**Figure 4A**). Meanwhile, Proteobacteria was positively correlated

with soil AK, NN, and available N while negatively correlated with soil TP, pH, AP, and AN (**Figure 4A**).

The best SEM ($\chi^2 = 18.674$, $df = 23$, $P = 0.720$, $NFI = 0.858$, and $RMSEA = 0.336$) explained 33.5% of the variations in the root-associated bacterial community diversity and 81.5% of variations in the rhizosphere soil bacterial community diversity (**Figure 5**). *E. gansuensis* decreased the diversity of the root-associated bacterial community and increased the diversity of

TABLE 1 | The statistical test of similarity (ANOSIM) and permutational multivariate two-way analysis of variance (PERMANOVA) to analyze differences of *Achnatherum inebrians* root and rhizosphere soil bacterial community composition calculated by Illumina sequencing.

Type	Treatment	df	PERMANOVA		ANOSIM	
			Bray–Curtis		Bray–Curtis	
			<i>F</i>	<i>P</i>	<i>R</i>	<i>P</i>
Soil	E	1	0.7429	0.6102	−0.0823	0.4929
	W	2	0.4314	0.7612	−0.0247	0.5542
	W*E	2	0.7324	0.6264		
Root	E	1	5.0327	0.0058	0.5185	0.0055
	W	2	5.7199	0.0045	0.3251	0.0115
	W*E	2	5.5094	0.0052		

Bold values indicate significant differences.

the rhizosphere soil bacterial community through decreasing soil available N content (Figure 5). Soil moisture increased the diversity of the rhizosphere soil bacterial community through significantly increasing soil NN, and non-significantly increasing pH and C/N, and significantly decreasing soil AP (Figure 5).

DISCUSSION

Our study that examined the influence of the presence of a mutualistic seed-borne, systemic fungal endophyte and also the effects of different available soil moisture on bacterial communities revealed that *E. gansuensis* influenced the diversity and richness of the bacterial community in the roots and rhizosphere soil of *A. inebrians* plants, and soil moisture only affected the diversity and richness of the bacterial community of *A. inebrians* plants rhizosphere soil. As with some other studies, different soil moistures can have negative, neutral or positive effects on the diversity of both the rhizosphere and the root-associated bacteria (Zhang et al., 2014b; Xu et al., 2018). In general, the diversity of both the rhizosphere and the root-associated bacteria tended to be lowest under low moisture (Xu et al., 2018). Our study also indicated that the presence of *E. gansuensis* in *A. inebrians* plants decreased the diversity of the root-associated bacterial community, but enhanced the diversity and richness of the rhizosphere soil bacterial community.

Diversity of Root and Rhizosphere Soil Bacterial Communities

The previous studies on the diversity in the bacterial community in roots and the rhizosphere soil, using different plants and different growing conditions, found that as for our findings, the rhizosphere soil had a higher diversity than actually in the roots. Donn et al. (2015) found that the composition of bacterial communities in roots of wheat plants and rhizosphere soils showed obvious differences, and the diversity of these bacterial communities decreased from soil to roots. Edwards et al. (2015) also found a similar pattern in rice, which supported the conclusion that bacterial microbial diversity in soil was

higher than that in roots. In contrast to these findings was the one that looked at the rhizosphere and root-associated bacteria in *Am. breviligulata*, a grass that thrives in sand dunes. The bacterial diversity and richness in the root system of this grass was significantly higher than in the soil, and it may be that root exudates offer much-needed resources for root bacteria than are present in the sandy soil in dune ecosystems (Bell-Dereske et al., 2017). Bulgarelli et al. (2012) revealed that Actinobacteria and Bacteroidetes are the dominant phyla in *Arabidopsis* roots and rhizosphere soils. And then, Schlaeppli et al. (2014) found that the dominant bacterial populations in roots and soil microbiota of *Ar. thaliana* are Actinobacteria, Bacteroidetes and Proteobacteria. Our current experimental results showed that Actinobacteria and Proteobacteria were the most abundant phyla of bacterial communities in roots and rhizosphere soil, which indicated that genera of Actinobacteria and Proteobacteria may be key bacteria in the root and rhizosphere.

Effects of *Epichloë* Endophyte on the Belowground Bacteria Community

Our continuing studies are being conducted to investigate the effects of aboveground *Epichloë* endophytes on belowground microbial communities associated with *A. inebrians* host plants. As part of these studies Zhong et al. (2018) showed that the presence of an *Epichloë* endophyte reduced the diversity of root-associated fungal communities associated with *A. inebrians*. Studies on other plant species/*Epichloë* endophytes associations have also investigated these effects and these are valuable comparative studies to compare and contrast with our studies. The study by Bell-Dereske et al. (2017) indicated that the presence of an *Epichloë* endophyte in *Am. breviligulata* decreased the diversity of the root-associated bacteria community under elevated soil moisture. Similarly, our present study indicated that *E. gansuensis* decreased the Shannon diversity of the root-associated bacterial community of *A. inebrians*. The presence of an *Epichloë* endophyte altered the composition of the soil bacterial community associated with *Lolium multiflorum*, while having no apparent effect on the soil fungal community (Casas et al., 2011). Roberts and Ferraro (2015) showed that the *Epichloë* endophyte of tall fescue increased the rhizosphere soil bacteria diversity. Our study similarly found that *E. gansuensis* enhanced the diversity and richness of rhizosphere soil bacteria community.

Some previous studies had shown that the effects of *Epichloë* endophytes on belowground bacteria may be caused by secondary metabolites (Vandegrift et al., 2015; Rojas et al., 2016; Soto-Barajas et al., 2016), such as root exudates (Guo et al., 2015), alkaloids (Franzleubbers and Hill, 2005), and root volatile organic compounds (Rostás et al., 2015). A study had demonstrated that an *Epichloë* endophyte altered the composition of root exudates, such as the total phenolic content and TOC (Guo et al., 2015); in addition, studies also found that root exudates could construct the belowground bacterial community (Badri and Vivanco, 2009; Bakker et al., 2013). Although studies have been conducted on the content of alkaloids in the aboveground tissue of *A. inebrians* (Zhang et al., 2011, 2014a), the presence of these alkaloids in

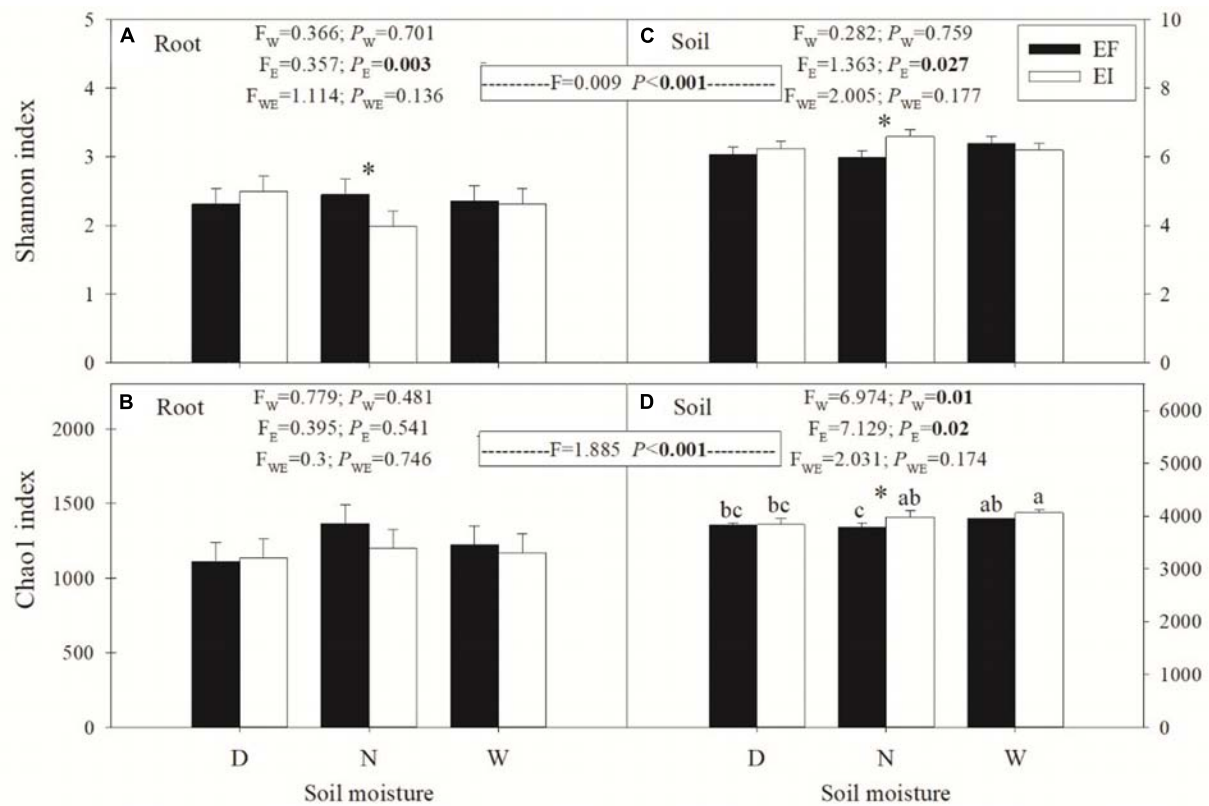


FIGURE 3 | Bacterial community diversity in roots (A,C) and soil (B,D) under different water and endophyte treatments ($n = 3$, D: drought, N: normal, W: well-watered, EI: endophyte-infected, and EF: endophyte-free). Values are mean \pm standard error (SE), with bars indicating SE. The asterisk (*) means significant difference at $P < 0.05$ (independent t -test) between EI and EF plants at corresponding water content. The A, B means significant difference at $P < 0.05$ among corresponding water content and endophyte status.

TABLE 2 | The chemical properties of root-associated and rhizosphere soil bacterial community composition of *A. inebrians* under different soil moisture and endophyte treatments at phylum levels ($n = 3$, D: drought, N: normal, W: well-watered, EI: endophyte-infected, and EF: endophyte-free).

Treatment	AN (mg/Kg)	NN (mg/Kg)	TN (%)	TP (%)	AP (mg/Kg)	AK (mg/Kg)	pH	SOC (%)	Available N (mg/Kg)
DEI	3.384 \pm 0.145	4.987 \pm 0.188	0.060 \pm 0.001	0.074 \pm 0.002	7.734 \pm 0.179	20.463 \pm 1.210	7.993 \pm 0.062	0.621 \pm 0.023	8.373 \pm 0.315
DEF	4.398 \pm 0.064	6.144 \pm 0.156	0.059 \pm 0.000	0.076 \pm 0.000	6.553 \pm 0.084	16.342 \pm 1.169	8.061 \pm 0.086	0.601 \pm 0.037	10.542 \pm 0.216
NEI	5.250 \pm 0.128	4.291 \pm 0.350	0.058 \pm 0.001	0.075 \pm 0.003	6.512 \pm 0.083	16.016 \pm 1.779	8.014 \pm 0.068	0.639 \pm 0.006	9.539 \pm 0.410
NEF	4.726 \pm 0.097	7.662 \pm 0.083	0.057 \pm 0.001	0.074 \pm 0.001	5.786 \pm 0.315	17.292 \pm 2.089	8.022 \pm 0.064	0.632 \pm 0.034	12.391 \pm 0.017
WEI	4.061 \pm 0.110	6.18 \pm 0.308	0.061 \pm 0.001	0.077 \pm 0.001	5.381 \pm 0.159	20.161 \pm 0.565	8.041 \pm 0.075	0.614 \pm 0.064	10.232 \pm 0.336
WEF	4.393 \pm 0.153	7.889 \pm 0.184	0.061 \pm 0.000	0.074 \pm 0.003	5.118 \pm 0.152	17.609 \pm 1.461	8.070 \pm 0.052	0.650 \pm 0.022	12.264 \pm 0.312
E	0.067	0.000	0.160	0.125	0.000	0.142	0.543	0.885	0.000
W	0.000	0.000	0.005	0.165	0.000	0.285	0.861	0.859	0.000
E*W	0.000	0.001	0.143	0.430	0.061	0.183	0.848	0.650	0.337

Values are mean \pm standard error ($n = 3$). Soil factors indicated include AN, Ammonium Nitrogen; NN, Nitrate Nitrogen; TN, Total Nitrogen; TP, Total Phosphorus; AP, Available P; AK, Available potassium; pH, SOC, Soil Organic Carbon; and Available N. Bold values indicate significant differences.

the roots and rhizosphere soil of *A. inebrians* has not been reported. Previous studies also showed that *Epichloë* endophytes produced changes in soil properties, including soil total nitrogen content (Buyer et al., 2011), inorganic nitrogen (Franzluebbers and Hill, 2005), TOC content (Guo et al., 2015), biomass C (Buyer et al., 2011), and pH (Shen et al., 2013). Our present study also found that *E. gansuensis* decreased soil available

N content and this was associated with enhanced diversity of the *A. inebrians* rhizosphere soil bacterial community and decreased diversity of the root-associated bacterial community. Compared with previous findings, our second hypothesis that *E. gansuensis* affect bacterial diversity of *A. inebrians* by changing soil physical and chemical properties was supported by the present study.

TABLE 3 | Correlations between chemical properties in root or rhizosphere soil of *A. inebrians* under different soil moisture and endophyte treatments with alpha diversity was analyzed by Pearson's correlation coefficient.

Soil properties	Root		Soil	
	Shannon	Chao1	Shannon	Chao1
AN	0.112	0.295	0.01	-0.094
NN	-0.23	-0.14	0.387	0.742**
TN	-0.042	0.116	-0.162	-0.201
TP	-0.064	0.095	-0.038	-0.561*
AP	0.093	-0.133	-0.292	-0.680**
AK	-0.074	-0.445	0.19	-0.044
pH	0.275	0.35	-0.085	0.164
SOC	0.172	0.366	0.13	0.304
C/N	0.185	0.312	0.171	0.347
Available N	-0.162	-0.007	0.352	0.630**
N/P	-0.167	0.065	0.339	0.758**

The asterisk indicates significant differences at the 0.01 and 0.05 level (** $P < 0.01$; * $P < 0.05$). Soil factors indicated include AN, Ammonium Nitrogen; NN, Nitrate Nitrogen; TN, Total Nitrogen; TP, Total P; AP, Available P; AK, Available potassium; pH, SOC, Soil Organic Carbon; C/N, Total Organic Carbon: Total Nitrogen; Available N, and N/P, Available N: Available P. Bold values indicate significant differences.

Effects of Soil Moisture on Diversity of the Belowground Bacterial Communities

Previous studies on the effects of precipitation and drought stress on microbial community changes have also revealed that

underground microorganisms are affected by soil moisture. According to Xu et al. (2018) proposed that drought stress reduced the diversity of bacterial communities in sorghum-related rhizosphere soil. Naylor et al. (2017) and Santos-Medellín et al. (2017) also highlighted that drought stress changed the composition of bacterial communities associated with rice and some grass/crop species. In our present study, compared to the normal soil moisture, drought markedly decreased the richness of the rhizosphere soil bacterial communities of *A. inebrians*, and the well-watered treatment significantly increased the richness of the rhizosphere soil bacterial community, and this supports our first hypothesis that soil moisture could influence the bacterial diversity in roots and rhizosphere soil of *A. inebrians*.

Soil moisture, as a significant contributor to belowground bacterial community changes, has been reported to have profound effects on soil microbial activity, thus affecting carbon input as well as decomposition of soil organic matter, and this will contribute to plant growth (Zhang et al., 2014b; Naylor et al., 2017; Olatunji et al., 2018). Furthermore, previous findings indicated that soil properties are a major driver of differences in the distribution and composition of bacterial communities (Zhang et al., 2014b; Olatunji et al., 2018). These soil properties include physical structure, microbial activity, organic compounds, nutrient transformation and the presence of root exudates (Zhang et al., 2014b; Francioli et al., 2016; Zhelnina et al., 2018). Our current study also found that soil moisture changed the diversity of rhizosphere soil bacteria of

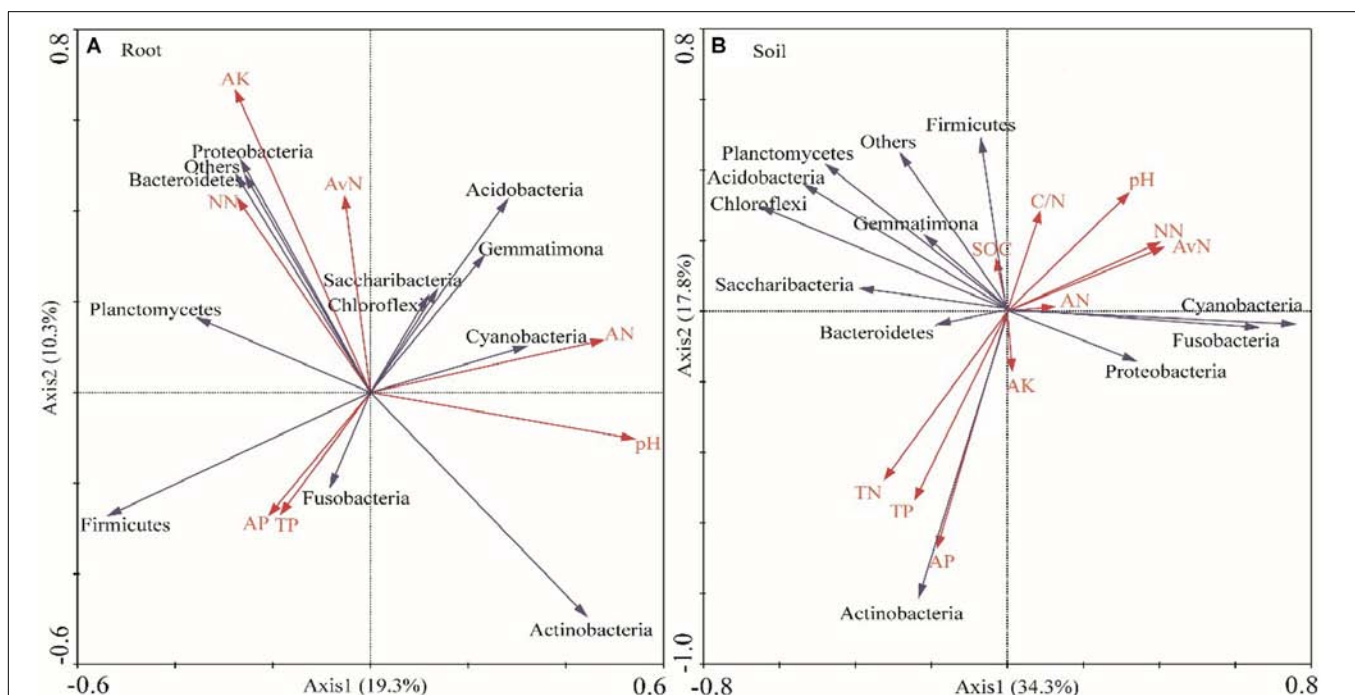
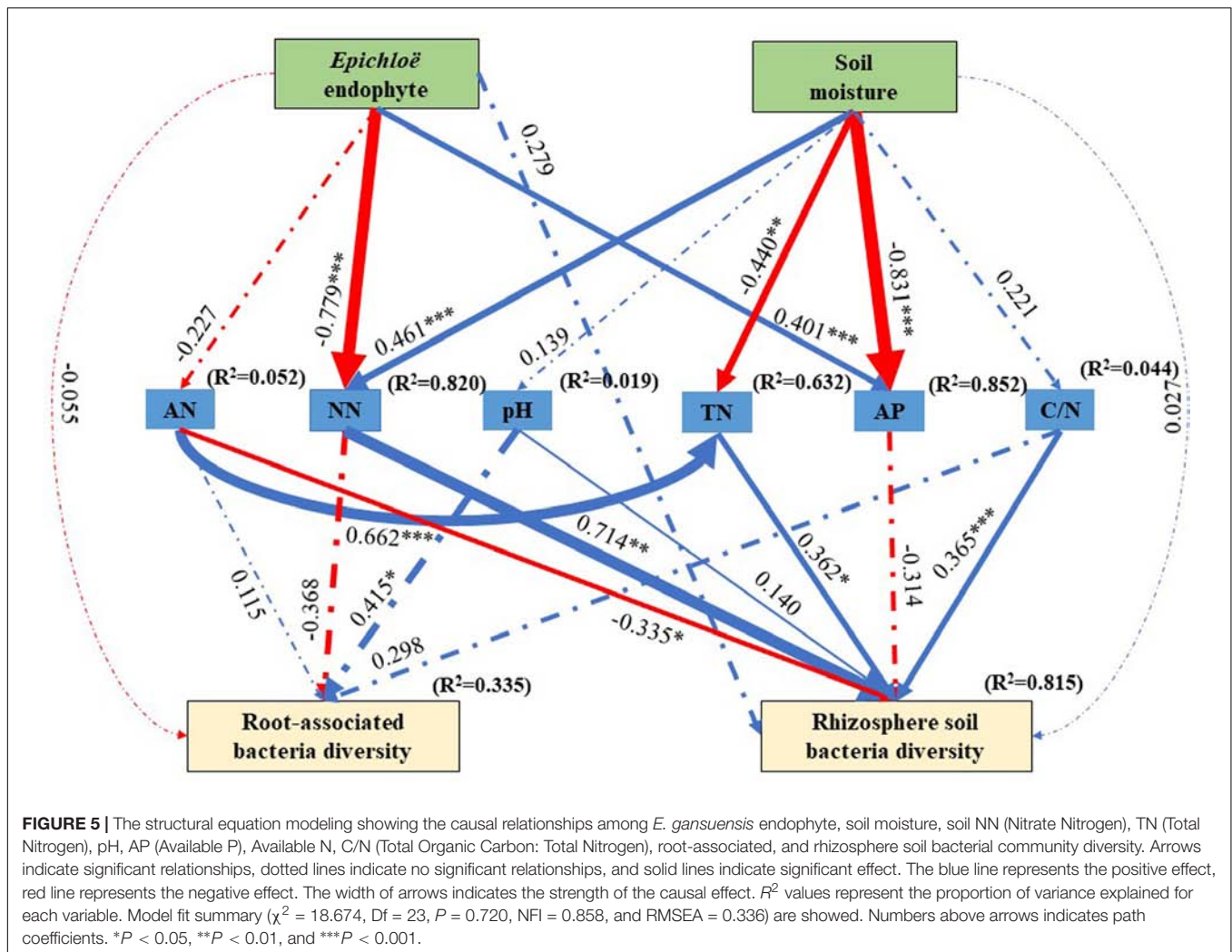


FIGURE 4 | Redundancy analysis (RDA) of relative abundance of root (A) and rhizosphere soil (B) bacterial communities and soil properties under different water and endophyte treatments ($n = 3$; D: drought, N: normal, W: well-watered, EI: endophyte-infected, and EF: endophyte-free). Soil factors indicated include AN (Ammonium Nitrogen), NN (Nitrate Nitrogen), TN (Total Nitrogen), TP (Total Phosphorus), AP (Available P), AK (Available potassium), pH, SOC (Soil Organic Carbon), and Available N.



A. inebrians by increasing soil pH, C/N, and NN content and decreasing soil AP content. This is consistent with previous research results (Shen et al., 2013; Zhang et al., 2014b; Van der Bom et al., 2018). Meanwhile, our results also supported the second hypothesis that different soil moisture treatments led to the changes of soil properties, which in turn can bring changes to the diversity and richness of the soil bacterial community of *A. inebrians*.

Relationships Between Bacteria Community and Environmental Factors

Previous studies have shown that the diversity and composition of bacterial communities associated with plant roots and rhizosphere soil are affected by a series of biotic and abiotic factors, such as fertility, pH and soil moisture (Shen et al., 2013; Zhang et al., 2014b; Yao et al., 2018). These factors normally lead to changes in the physical and chemical properties of rhizosphere soil and soil nutrient levels, which are closely related to the diversity of rhizosphere soil and root bacteria (Bulgarelli et al., 2012; Fan et al., 2017; Francioli et al.,

2017). Meyer et al. (2013) demonstrated that the availability of inorganic nitrogen regulates the relative diversity of bacteria and archaea of soil microbial communities among different types of land use intensity in grassland ecosystem, and bacteria are involved in the whole process of inorganic nitrogen cycling. Van der Bom et al. (2018) also highlighted that the effects of N inputs on the soil bacterial community structure in the field was greater than that of P or K inputs. Nie et al. (2018) revealed and high levels of N addition decreased soil bacterial diversity and altered the composition of the forest soil bacterial community. The present study indicated that with decreasing soil available N, the diversity of the root-associated bacterial community was decreased and the diversity of the rhizosphere soil bacterial community was increased. Our study also found that soil N, AP, and pH content was closely correlated with rhizosphere soil bacterial diversity of *A. inebrians*. Rousk et al. (2010) also found that the richness and diversity of bacterial communities were positively correlated with pH, and the diversity of the bacterial community almost doubled when pH was increased from 4 to 8. Dimitriu and Grayston (2010) noted that the relative abundance

of Acidobacteria increased with lower pH. Meanwhile, Shen et al. (2013) also found that bacterial communities differed sharply at different altitudes, and bacterial community composition is closely related to soil pH, which also emphasized that pH was a good predictor of the diversity distribution of soil bacterial communities at different altitudes. Our present study also demonstrated that soil pH was closely correlated with the diversity and composition of the *A. inebrians* bacterial community, which fully supported our second hypothesis that the presence of *E. gansuensis* and soil moisture treatments can bring changes in soil physical and chemical properties, and a close relationship was observed between underground bacteria and soil properties in our research.

CONCLUSION

This study revealed that rhizosphere soil of *A. inebrians* plants harbored a richer and more diverse bacterial community than the roots. In addition, the presence of *E. gansuensis* in *A. inebrians* plants significantly decreased the Shannon diversity of the root-associated bacterial community, and increased the Shannon diversity of the rhizosphere soil bacterial community. In addition, soil moisture increased the Shannon diversity of the rhizosphere soil bacterial community. Meanwhile, Chao1 richness of the rhizosphere soil bacterial community of *A. inebrians* significantly increased with the increase in the soil moisture level. Moreover, the present study also indicated that the diversity and richness of *A. inebrians* root-associated and rhizosphere soil bacterial communities were intimately associated with soil properties of available N, C/N, NN, AP, and pH. Therefore, two hypotheses were proved by our present study. Further experiments should systematically study the mechanism of soil moisture and the aboveground *E. gansuensis* endophyte on the richness and diversity of root and rhizosphere bacterial communities.

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DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Sequence Read Archive (SRA) <https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA590316>.

AUTHOR CONTRIBUTIONS

YJ and XZ designed this experiment. YJ and RZ performed the soil moisture treatment, managed the experimental field. YJ and RZ measured the soil data. YJ and RZ analyzed the data. YJ and XZ wrote this manuscript. MC revised this manuscript and polished the English. All authors contributed to revise 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/fmicb.2020.00747/full#supplementary-material>

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A Tale of Two Grass Species: Temperature Affects the Symbiosis of a Mutualistic *Epichloë* Endophyte in Both Tall Fescue and Perennial Ryegrass

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Many cool-season grasses form permanent, mutualistic symbioses with asexual *Epichloë* endophytes. These fungal symbionts often perform a protective role within the association as many strains produce secondary metabolites that deter certain mammalian and invertebrate herbivores. Although initially a serious issue for agriculture, due to mammalian toxins that manifested in major animal health issues, selected strains that provide abiotic stress protection to plants with minimal ill effects to livestock are now commercialized and routinely used to enhance pasture performance in many farming systems. These fungal endophytes and their grass hosts have coevolved over millions of years, and it is now generally accepted that most taxonomic groupings of *Epichloë* are confined to forming compatible associations (i.e., symptomless associations) with related grass genera within a tribe. The most desired compounds associated with *Epichloë festucae* var. *lolii*, an endophyte species associated with perennial ryegrass, are peramine and epoxy-janthitrems. No other major secondary metabolites with invertebrate bioactivity have been identified within this association. However, other agriculturally beneficial compounds, such as lolines, have been discovered in related endophyte species that form associations with fescue grasses. A rationale therefore existed to develop novel grass-endophyte associations between loline-producing endophytes originally isolated from tall fescue with elite cultivars of perennial ryegrass to achieve a wider spectrum of insect bioactivity. A suitable loline-producing endophyte strain of *Epichloë* sp. FaTG-3 was selected and inoculated into perennial ryegrass. We hypothesized that endophyte transmission frequency, endophyte mycelial biomass and endophyte-derived alkaloid production would differ between the original tall fescue host and the artificial association. Consistent with our hypothesis, our data strongly suggest that plant species significantly affected the plant-endophyte association. This effect became more apparent for transmission frequency and endophyte biomass as the plants matured. Overall, the viable endophyte infection frequency was greater in

the tall fescue host than in perennial ryegrass, at all sampling dates. Additionally, temperature was found to be a significant factor affecting endophyte transmission frequency, endophyte mycelial biomass and alkaloid production. Implications for the development of novel grass-endophyte associations are discussed.

Keywords: alkaloid, cool-season grass, lolines, mycelial biomass, peramine, vertical transmission

INTRODUCTION

Members of the sub-family Pooideae (family Poaceae) form permanent, symbiotic associations with fungal endophytes of the genus *Epichloë* (family Clavicipitaceae) and their asexual morphs, previously known as *Neotyphodium* (Leuchtmann et al., 2014). The grass host provides shelter and nutrients to the endophyte, while the plant benefits through increased tolerance to abiotic and biotic stresses (Malinowski and Belesky, 2000; Popay and Bonos, 2005). Asexual *Epichloë* species have lost the power of contagion, being exclusively vertically transmitted via host seed after colonization of inflorescences, flower and seed tissues (Zhang et al., 2017). In New Zealand (NZ) agriculture, the most economically important associations are those between selected “animal friendly” *Epichloë festucae* var. *lolii* strains and elite cultivars of perennial ryegrass (*Lolium perenne*) (Easton, 2007; Johnson et al., 2013), the dominant pasture species cultivated in NZ (Valentine and Kemp, 2007).

These fungal-grass associations were detrimental to NZ agriculture around the late 1970s and early 1980s as some natural associations produced a number of mammalian toxins (indole-diterpenes, e.g., lolitrem B, and ergot alkaloids, e.g., ergovaline) that manifested in major animal health issues including ryegrass staggers, a neurological disorder (di Menna et al., 2012). Since this era, a great deal of fundamental research was undertaken with respect to the endophyte’s biology, chemistry and genetic diversity (Johnson et al., 2013). This knowledge led a NZ government-owned institute, AgResearch Limited, to develop an endophyte bioprospecting pipeline that identifies, characterizes and selects agriculturally beneficial strains (those that produce bio-protective properties to the host while conferring notably low or no detriment to grazing livestock) that can be incorporated into elite grass cultivars and marketed for increased pasture persistence and productivity (Johnson et al., 2013; Card et al., 2014; Bonth et al., 2015; Johnson and Caradus, 2019).

One of the most challenging steps in developing *Epichloë* endophytes for commerce is the ability to transfer suitable fungal strains from their original wild grass host to elite grass cultivars (Easton, 2007; Johnson and Caradus, 2019). The strain designated as AR1 was one of the first endophytes to be commercialized in 2001 (Johnson et al., 2013; Johnson and Caradus, 2019) and by 2008 over 70% of the proprietary seed sold in NZ was infected with this agriculturally beneficial fungal strain (Caradus et al., 2013). AR1 primarily produces the alkaloid peramine, which is responsible for insect deterrence, particularly toward Argentine stem weevil (ASW) while expressing no animal toxicity (Rowan and Gaynor, 1986; Rowan et al., 1986; Fletcher, 1999). The next leap in endophyte discovery arrived with the advent of epoxy-janthitrems, a unique indole diterpene compound with a wider

range of insect deterrence than peramine and in 2006, strain AR37, a producer of this class of alkaloid was released onto the NZ market (Caradus et al., 2013; Johnson et al., 2013; Hennessy et al., 2016; Johnson and Caradus, 2019). No other major secondary metabolites with invertebrate bioactivity have been identified within this fungal taxon. However, other agriculturally beneficial alkaloids, such as lolines, have been discovered in related species that pre-dominantly form associations with tall fescue (*Festuca arundinacea*) and meadow fescue (*F. pratensis*) (Scharidl et al., 2007). A rationale therefore existed to develop novel grass-endophyte associations between loline-producing endophytes originally isolated from tall fescue with elite cultivars of perennial ryegrass to achieve a wider spectrum of insect bioactivity than possible with ryegrass endophyte species (Easton, 2007; Easton et al., 2009).

A high frequency of viable endophyte infection in seed is desired by the seed industry for commercial endophyte products going to market, although this can be difficult to achieve with certain novel grass-endophyte associations (Rolston and Agee, 2007). Failure in vertical endophyte transmission has been documented for many endophyte-grass associations, including novel and wild-type associations, with no single factor responsible (Gundel et al., 2008). As well as genetic factors (Ju, 2011; Gagic et al., 2018), some environmental factors can contribute to incompatibility issues between these endophytic fungi and their grass hosts that may culminate by inhibiting the endophyte’s transmission pathway, with temperature suggested as one of the most important (Ju et al., 2006).

This study investigated the effects of different temperature regimes on the vertical transmission of an *Epichloë* endophyte strain within tall fescue (its original host species) and perennial ryegrass (a novel host species) and further analyzed endophyte infected plants with respect to their mycelial biomass and production of insect deterrent alkaloids. We hypothesized that endophyte transmission frequency, endophyte mycelial biomass and endophyte-derived alkaloid production would differ between the original tall fescue and the novel, or artificial, association developed with perennial ryegrass.

MATERIALS AND METHODS

Two grass lines were used in this study, namely T9886, a tall fescue line, cv. Grasslands Flecha (a summer dormant Mediterranean-type cultivar) and KLp903, a tetraploid perennial ryegrass line, derived from crosses of the cultivars Banquet, Banquet II and Bealey. Both seed lines were infected with the same strain of fungal endophyte, *Epichloë* sp. FaTG-3, strain AR501, previously designated TF16 (Christensen et al., 1993).

AR501 produces peramine and loline alkaloids but none of the ergot or indole diterpene alkaloids linked to animal toxicosis when associated with its original native grass host or within novel (or artificial) associations with tall fescue or perennial ryegrass. The lack of animal toxins is due to the absence of key genes in both alkaloid pathways. The seeds of the tall fescue line were harvested in 2012 and subsequently stored at near optimal storage conditions for endophyte, 0°C with 30% relative humidity (Rolston et al., 1986; Rolston et al., 1991) in the Margot Forde Germplasm Center, New Zealand's national gene-bank of grassland plants. Seed from the perennial ryegrass line was harvested in 2011 and stored by PGG Wrightson Seeds Ltd. under the same conditions as for the tall fescue line. The viable endophyte infection frequencies of each grass line were determined before the experiment commenced by assessing ~96 seedlings per line using an established tissue-immunoblot technique (Simpson et al., 2012).

Seeds from each seed line were sown in separate 24-cell plastic trays (196 cm³ per cell) containing seedling mix (120 L Southland peat, 80 L pumice) with the following fertilizer additions per cubic meter: 4 kg Osmocote® exact mini (16% N, 3.5% P, 9.1% K), 8 kg dolomite lime and 2 kg Hydroflo® (granular wetting agent manufactured by Everris Australia Ltd.). Two seeds were sown in each cell at a depth of 1 cm. The experiment was set up in October 2013 using a randomized block design with four blocks and 14 trays in each block (56 trays in total, 28 trays of tall fescue and 28 trays of perennial ryegrass). All the trays were initially placed in a heated glasshouse (~20°C) and watered as required until the seedlings had emerged. Two weeks after sowing, seedlings were thinned to one seedling per cell by hand, and the trays randomly assigned to treatment groups consisting of four controlled temperature regimes (A–D, see **Figure 1**).

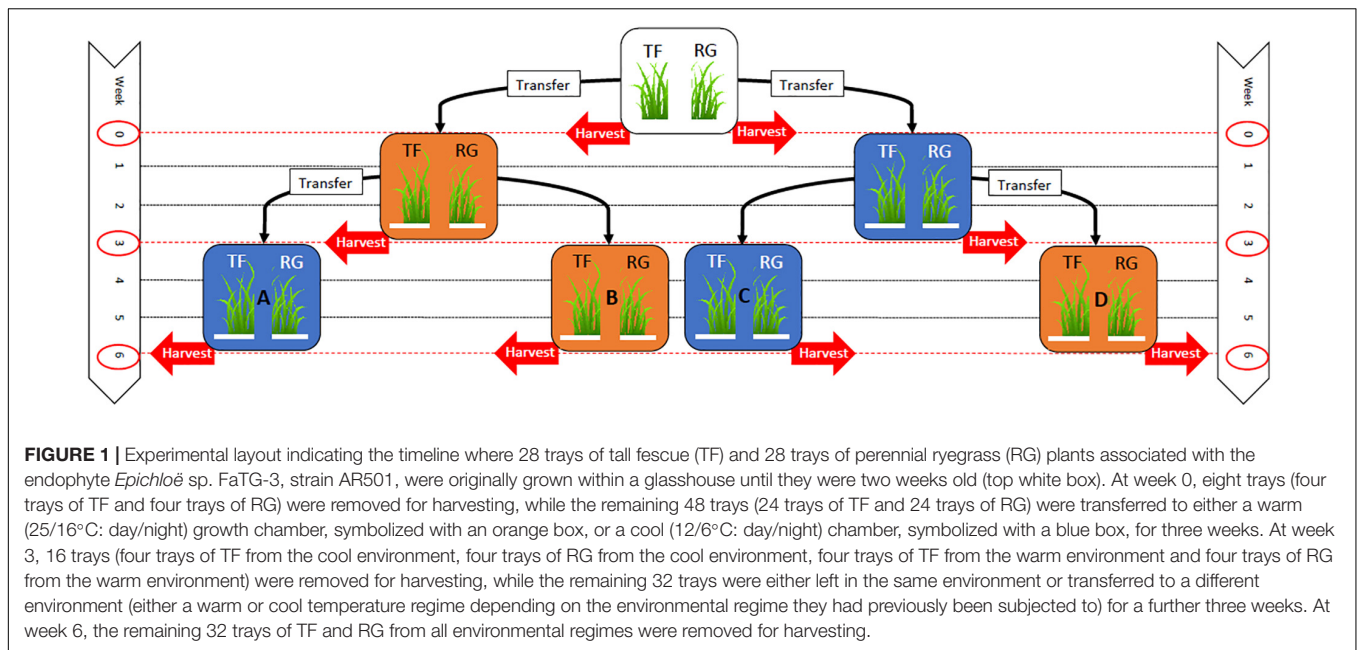
At week 0, at the commencement of the experiment, two trays from each block (four trays containing tall fescue plants and four trays containing perennial ryegrass plants), eight trays in total, were randomly selected and six plants per tray were randomly selected and harvested. This involved removing seedlings from their cell trays and separating the foliar tissues (pseudostem and leaves) from root tissues with the aid of a scalpel. The foliar tissues were bulked and freeze dried using a bench top freeze dryer (MicroModulyo, Thermo Savant™, United States) and ground using a coffee grinder (Breville Group Ltd., China) before being stored at –20°C. The root samples were discarded. The biomass of endophyte mycelia per plant was then determined using an enzyme-linked immunosorbent assay (ELISA) developed by AgResearch (Faville et al., 2015). The remaining 48 seedling trays were split into two groups, of an equal number of trays and grass species, and transferred from the glasshouse to two walk-in growth chambers (PGV36, Conviron®, Canada) with a photoperiod length of 16 h light/8 h dark. One chamber with a temperature regime set at 25/16°C: day/night (termed from now on as warm) and the other chamber set at 12/6°C: day/night (termed from now on as cool). The light intensity was 244 and 214 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for the cool and warm growth chambers, respectively.

At week three, eight trays from each of the two temperature regimes (warm and cool), 16 trays in total, were randomly

selected and six plants per tray were harvested as described previously. Additionally, the viable endophyte infection frequency of these plants was assessed as described earlier. At this time, eight of the sixteen remaining trays from the warm temperature regime were placed into the cool temperature regime, and eight of the sixteen remaining trays from the cool temperature regime were placed into the warm temperature regime (**Figure 1**). Plants were left to grow for a further three weeks before six plants per tray were again randomly harvested and assessed for viable endophyte (week 6 harvest). Plants (including tillers previously assessed by TPIB) were freeze dried, ground and endophyte mycelial biomass plus the concentration of agriculturally beneficial grass-endophyte derived alkaloids, peramine and lolines, determined by ELISA. Samples were analyzed for peramine and loline alkaloids using ELISAs developed by AgResearch (Briggs et al., 2017). The plate coating conjugate, and the polyclonal sheep anti-peramine antibody were originally described by Garthwaite et al. (1994) but the immunoassay was reformatted and all other reagents, buffers and the protocol were replaced (Briggs, pers. comm.).

At all harvest dates (week 0, 3, and 6) trays of tall fescue and perennial ryegrass were removed from the growth chambers and six plants per tray randomly selected and harvested. At week 0, a total of 48 plants were selected from eight trays (4 trays of tall fescue and 4 trays of perennial ryegrass) and assessed for mycelial biomass. Plants at this stage were too small to be assessed for the presence of viable endophyte by the tissue print immunoblot technique. At week 3, a total of 96 plants were selected from 16 trays (8 trays of tall fescue and 8 trays of perennial ryegrass) and assessed for mycelial biomass and their endophyte infection status. At week 6, a total of 192 plants were selected from 32 trays (16 trays of tall fescue and 16 trays of perennial ryegrass) and assessed for mycelial biomass, their endophyte infection status and alkaloid concentration as described previously. In total, 336 plants were harvested from the three harvest dates.

Statistical analyses for the endophyte frequency data was performed using R (R Core Team, 2019), whilst the mycelial biomass data was analyzed using GenStat for Windows (18th Edition, VSN International, Hemel Hempstead, United Kingdom). A binomial generalized linear mixed model (GLMM) was used to model endophyte infection frequencies using lme4 (Bates et al., 2015), where block was treated as a random effect. Pairwise comparisons for the GLMM used Tukey's *P*-value adjustment for multiple comparisons using the emmeans R package (Lenth et al., 2019) with statistical significance defined as *P* < 0.05. Analysis of variance (ANOVA) and Fisher's protected test of least significant difference (LSD; *P* < 0.05) were performed to compare treatment effects for the mycelial biomass and alkaloid concentration data. Mycelial biomass and alkaloid (peramine and lolines) concentrations were natural log transformed prior to analysis to stabilize the variance. Loline (*N*-acetyl loline and *N*-formyl loline) and peramine concentrations in both perennial ryegrass and tall fescue hosts were each regressed against endophyte mycelial biomass to investigate potential relationships. Analysis of covariance, which fitted parallel lines through the scatter plots (one point per treatment) for the two grass



hosts, was used to correlate alkaloid and endophyte biomass within hosts, after adjusting for differences in overall mean values between hosts.

RESULTS

Endophyte Infection Frequency

The initial viable endophyte infection frequency of AR501 was 99 and 87% for the tall fescue and perennial ryegrass lines, respectively. At week 0 (the first harvest date), the endophyte infection frequency was not determined as the plants were too young, and therefore too fine, for assessment using the tissue print-immunoblot technique. At week 3 (the second harvest date), there was no variation in the tall fescue data. Therefore, the analysis only compared the data from the ryegrass datasets (cool vs. warm) and observed means rather than fitted means displayed in **Table 1**. The viable endophyte infection frequency of AR501 was significantly lower (32%) in the perennial ryegrass line exposed to the warm temperature regime compared to the ryegrass line exposed to the cool temperature regime (84%) (**Tables 1, 2**). Additionally, there was a slight trend indicating a higher endophyte infection frequency of AR501 in tall fescue than perennial ryegrass (**Table 1**).

At week 6 (the third harvest date), there were no overall significant interactions between plant species and temperature, although there was a slight trend indicating that plants transferred from the warm to the cold temperature regime showed a lower viable endophyte infection frequency, regardless of host plant species (**Tables 1, 2**). There was also a significant ($P < 0.001$) effect of plant species, i.e., there was a higher viable endophyte infection frequency in tall fescue than perennial ryegrass (**Table 2**).

Biomass of Endophyte Mycelia

At week 0 (the first harvest date), the mycelial biomass of AR501 did not differ significantly between the tall fescue (2.22 mg/g) and perennial ryegrass (1.24 mg/g) host populations grown at 20°C (**Tables 1, 2**). At week 3 (the second harvest date), there was no overall significant interaction between treatments (**Tables 1, 2**), although there was a significant ($P = 0.004$) effect of host plant species, i.e., there was a greater amount of endophyte mycelia detected in tall fescue than perennial ryegrass (**Tables 1, 2**) and a significant ($P = 0.005$) effect of temperature on the biomass of mycelia within endophyte-infected plants. At week 6 (the third harvest date), there was a highly significant interaction between treatments ($P = 0.001$) indicating that plant species and temperature influenced the biomass of endophyte mycelia (**Tables 1, 2**). Within the perennial ryegrass treatments, the lowest amount of endophyte mycelia (0.81 mg/g) was detected in plants transferred from the warm to the cool temperature regime (**Table 1**). For tall fescue, the lowest amount of mycelia was also detected in the plants transferred from the warm to the cool temperature regime (1.93 mg/g) and for plants kept at the cool temperature regime (1.92) for the entire experiment (**Table 1**). The greatest biomass of endophyte mycelia was detected in plants transferred from the cool to the warm temperature regime, regardless of host plant species, with a mean of 5.18 mg/g detected in perennial ryegrass plants and 10.82 mg/g detected in tall fescue (**Table 1**).

Concentration of Insect Deterrent Alkaloids

At week 6 (the third harvest date), there were no overall significant interactions among treatments (**Tables 3, 4**), although there were highly significant ($P < 0.001$) effects with regards to temperature for both peramine and loline concentrations.

TABLE 1 | Fitted mean % of viable endophyte infection (95% CI) and mean mycelial biomass (\pm SD) of *Epichloë* sp. FaTG-3 strain AR501 within perennial ryegrass and tall fescue host backgrounds after plants were incubated for fixed periods at selected temperature regimes.

Temperature regime (day/night)	Fitted mean % of viable endophyte infection (95% CI)		Mean mycelial biomass (mg/g \pm SD)	
	Perennial ryegrass	Tall fescue	Perennial ryegrass	Tall fescue
Initial seed infection frequency				
~20°C constant	87*	99*	n/a	n/a
Week 0				
~20°C constant	nt	nt	1.24 (\pm 0.56) a	2.22 (\pm 2.66) a
Week 3				
12/6°C (cool)	84 (52, 99) a	100*	1.07 (\pm 0.11) a	2.27 (\pm 0.54) a
25/16°C (warm)	32 (4, 73) b	100*	0.57 (\pm 0.24) a	1.45 (\pm 1.29) a
Week 6				
12/6°C (cool)	75 (54, 88) a	96 (76, 99) a	2.72 (\pm 0.46) b	1.92 (\pm 0.41) ab
25/16°C (warm)	75 (54, 88) a	92 (72, 98) a	2.73 (\pm 1.07) b	7.29 (\pm 1.80) d
25/16°C transferred to 12/6°C (warm to cool)	58 (38, 76) a	87 (68, 96) a	0.81 (\pm 0.42) a	1.93 (\pm 0.64) ab
12/6°C transferred to 25/16°C (cool to warm)	75 (54, 88) a	92 (72, 98) a	5.18 (\pm 1.02) c	10.82 (\pm 1.70) e

Means within a sampling period (e.g., week 6) followed by the same letter are not significantly ($P > 0.05$) different as determined by Tukey's HSD test for the week 6 endophyte infection data or Fisher's protected LSD test for mycelial concentration data.

*No variation in the data, therefore observed means are displayed rather than fitted means, n/a is not applicable, nt is not determined as the tillers were too young, and therefore too fine, to be assessed by the tissue print-immunoblot technique.

TABLE 2 | P -values for the effects of plant species (perennial ryegrass and tall fescue), temperature (cool and warm for weeks 0-3, and cool, warm, transfer from cool to warm and transfer from warm to cool for week 6) on *Epichloë* sp. FaTG-3 strain AR501 endophyte infection frequencies (%) and mycelial biomass (mg/g).

Source of variation	P -value		
	Week 0	Week 3	Week 6
Viable endophyte infection frequency			
Plant species	–	–	< 0.001
Temperature	–	0.002	0.385
Plant species \times Temperature	–	–	0.950
Biomass of endophyte mycelia			
Plant species	0.495	0.004	< 0.001
Temperature	–	0.005	< 0.001
Plant species \times Temperature	–	0.973	0.001

P -values for endophyte infection frequency data were generated via Type II Wald Chi-square tests, whilst P -values for endophyte mycelia data were generated by Fisher's protected LSD test ($P < 0.05$).

Values in bold are statistically significant ($P < 0.05$), – no analysis possible.

The lowest concentrations of peramine were recorded for plants transferred from the warm to cool temperature regime and the highest concentrations recorded for plants transferred from the cool to warm temperature regime, irrespective of plant species (Table 3). For lolines, the greatest concentrations were again recorded for plants transferred from the cool to warm temperature regime (Table 3). Additionally, there was a highly significant ($P < 0.001$) effect of plant species on the production of loline alkaloids indicating that more lolines were produced by tall fescue plants infected with AR501 than perennial ryegrass (Tables 3, 4). There was a near significant ($P = 0.052$) effect with respect to plant species on the production of peramine (Tables 3, 4).

Correlation Between Mycelial Biomass and Alkaloid Concentration

Peramine and loline alkaloid concentrations increased when endophyte biomass increased (Figure 2), with the tall fescue endophyte association producing greater amounts of both alkaloids [1.04 and 1.70 mg/g (log10) of peramine and loline alkaloids, respectively] when endophyte biomass reached its maximum levels. The common slope of the parallel regression lines was significantly ($P = 0.012$ and $P < 0.001$) positive for both loline and peramine alkaloids, respectively.

DISCUSSION

We hypothesised that the endophyte transmission frequency, endophyte mycelial biomass and endophyte-derived alkaloid production would differ between associations formed between endophyte strain AR501 and the original host species, tall fescue, and the novel association developed with perennial ryegrass. Consistent with our hypothesis our data strongly suggests that plant species significantly affects the plant-endophyte association. This effect became more apparent for transmission frequency and endophyte biomass as the grass plants matured. Overall, the viable endophyte infection frequency was greater in the tall fescue host than in perennial ryegrass, at all sampling dates. However, as the endophyte infection frequency was higher in the original seed line of tall fescue compared to perennial ryegrass, and as asexual *Epichloë* cannot naturally infect endophyte-free plants due to their lack of horizontal transmission (Zhang et al., 2017), it was not surprising that this trend continued at all subsequent sampling dates. What was surprising was the effect of temperature at the second sampling date, which negatively impacted the endophyte infection status of perennial ryegrass

TABLE 3 | Mean concentration of peramine and total loline alkaloids at the week 6 harvest in perennial ryegrass and tall fescue plants, infected with the endophyte *Epichloë* sp. FaTG-3, strain AR501.

Temperature regime (day/night)	Mean concentration of peramine ($\mu\text{g/g} \pm \text{SD}$)		Mean concentration of total loline alkaloids ($\mu\text{g/g} \pm \text{SD}$)	
	Perennial ryegrass	Tall fescue	Perennial ryegrass	Tall fescue
12/6°C (cool)	5.62 (± 2.99) cde	4.22 (± 1.56) bcd	1.50 (± 0.00) a	14.78 (± 8.08) b
25/16°C (warm)	4.26 (± 3.24) bc	11.02 (± 5.40) ef	7.30 (± 7.68) a	89.53 (± 74.73) c
25/16°C and transferred to 12/6°C (warm to cool)	1.53 (± 1.47) a	2.92 (± 1.78) b	1.50 (± 0.00) a	27.00 (± 19.26) b
12/6°C and transferred to 25/16°C (cool to warm)	12.03 (± 6.25) ef	17.28 (± 3.89) f	26.05 (± 18.36) bc	171.30 (± 79.30) d

Means associated with the same alkaloid followed by the same letter are not significantly different as determined by ANOVA and Fisher's protected LSD test ($P > 0.05$).

TABLE 4 | *P*-values for the effects of Plant Species (perennial ryegrass and tall fescue), Temperature (cool, warm, transfer from cool to warm and transfer from warm to cool) and their interaction with respect to the concentration of peramine and loline alkaloids ($\mu\text{g/g}$) associated with the endophyte, *Epichloë* sp. FaTG-3, strain AR501.

Source of variation	<i>P</i> -value	
	Peramine	Lolines
Plant species	0.052	<0.001
Temperature	<0.001	<0.001
Plant species \times Temperature	0.317	0.966

P-values were generated by Fisher's protected LSD test ($P < 0.05$).

Values in bold are statistically significant ($P < 0.05$).

but not tall fescue. However, the subsequent assessment of the endophyte infection frequencies at the final sampling date, showed that the perennial ryegrass plants that were exposed to the warm temperature regime for the first three weeks recorded a higher infection frequency. A possible explanation for this anomaly is that these plants had a mixed infection status (plants possessed both endophyte infected and endophyte-free tillers) and this ratio changed between the two sampling dates. An alternative, and possibly a more plausible explanation, is that as a low mycelial biomass was also detected in plants exposed to the warm temperature regime at the second sampling date, this could have resulted in false-negative blots developing on the nitrocellulose membrane used to determine the viable endophyte infection status of grass tillers (Gwinn et al., 1991). Ju et al. (2006), also used an immunoblot technique when studying tall fescue cv. Jesup infected with the MaxQ endophyte and noted a low endophyte infection frequency in winter compared to summer and this correlated to a low endophyte biomass within individual plants.

In addition to plant host species, temperature was also found to be a significant factor affecting the endophyte transmission frequency, endophyte mycelial biomass and endophyte-derived alkaloid production of strain AR501. At week 6, the biomass of AR501 mycelia was highest for both plant species when plants were grown in the cool temperature regime for three weeks and then transferred to the warm temperature regime. The biomass of endophyte mycelia within tall fescue was, however, almost twice as that detected in the perennial ryegrass association. When

this situation was reversed (i.e., plants were transferred from the warm to the cool temperature regime), both grass hosts also had significantly less mycelium than if they were subjected to a constant warm temperature regime. Although the concentration of peramine and loline alkaloids were generally higher in the tall fescue host, trends for both plant species remained similar and constant. Peramine significantly increased when plants of both grass species were transferred from the cool to the warm temperature regime, compared to plants grown at a constant cool temperature. This resulted in a 2-fold increase for perennial ryegrass and a 4-fold increase for tall fescue. This trend was more dramatic for loline alkaloids with a 17-fold increase for perennial ryegrass and a 12-fold increase for tall fescue. The opposite trend was detected when plants of both species were transferred from the warm to the cool regime.

Other researchers have also recorded strong impacts of temperature on endophyte mycelial biomass and endophyte-derived alkaloids (Reinholz and Paul, 2000; Ryan et al., 2015; Fuchs et al., 2017). In our study the concentration of both peramine and loline alkaloids was highly correlated to the biomass of endophyte mycelia and this has been documented previously in many grass-endophyte associations and as mycelial biomass increases so do the concentration of these alkaloids (Bush et al., 1993; Keogh et al., 1996; Easton et al., 2002; Rasmussen et al., 2007; Ryan et al., 2015). Endophyte-derived alkaloids are independently regulated and are controlled by both plant and endophyte genotype (Roylance et al., 1994). These secondary metabolites are a product of the grass-endophyte association and although there are reports of *in vitro* production of some compounds from axenically cultured endophyte strains (Blankenship et al., 2001), the concentrations are minimal to those produced *in planta* (TePaske et al., 1993; Adhikari et al., 2016). Fuchs et al. (2017) showed that climatic conditions in spring and summer enhanced endophyte growth and alkaloid production (peramine, ergovaline and lolitrem B) in perennial ryegrass infected with *E. festucae* var. *lolii*. Additionally, Justus et al. (1997) showed that the highest concentrations of loline alkaloids were found in early spring and summer in meadow fescue infected with the endophyte *E. uncinata*. All these factors are ecologically linked, showing that alkaloids that protect against herbivory are produced when the threat of herbivory is at its highest. Endophytes are now known to play a major role in the structure of grassland communities and

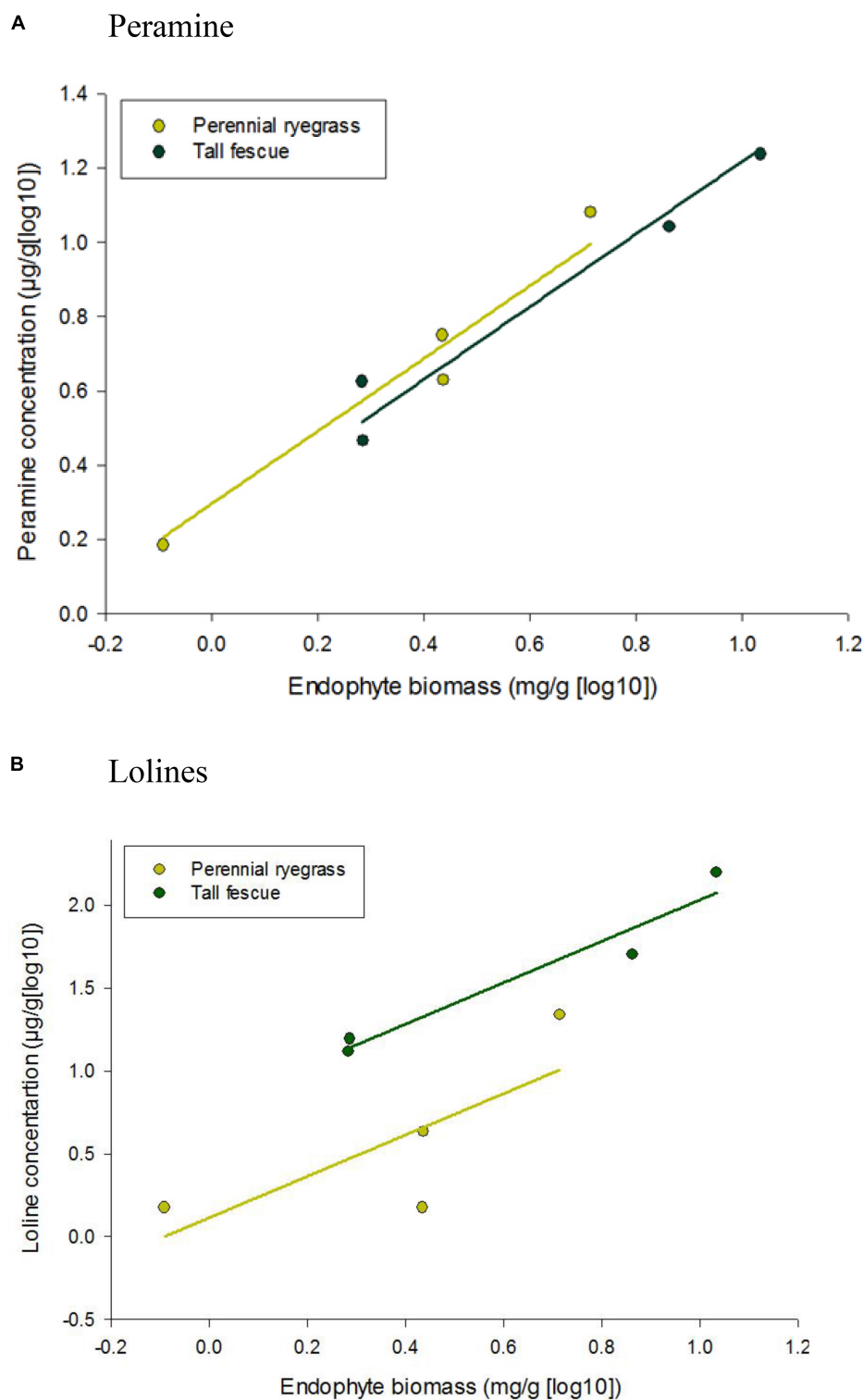


FIGURE 2 | Correlation between alkaloid concentration, **(A)** peramine and **(B)** lolines, and endophyte biomass of *Epichloë* sp. FaTG-3 strain AR501 in perennial ryegrass and tall fescue plant hosts. Equations for fitted curves were **(A)** perennial ryegrass, $y = 0.979x + 0.2955$ and tall fescue, $y = 0.979x + 0.2391$ and **(B)** perennial ryegrass, $y = 1.25x + 0.1143$ and tall fescue, $y = 1.25x + 0.7832$. The parallel lines were fitted using analysis of covariance. Note that the scale on the y axis differs for each alkaloid.

tropic interactions are likely to have significant consequences on the entire ecosystem (Saikkonen et al., 2016). The growth of *Epichloë* hyphae is synchronized with that of the plant with the endophyte behaving more like a plant tissue than a separate organism (Christensen et al., 2008). As both the fungus and plant have co-evolved over a period of 40 million years (Schardl et al., 2008), it is not unexpected that the association has developed ways to adapt to environmental cues in order to protect the plant from herbivory via the production of alkaloids. As these compounds are energy-rich, the association has also adapted ways to efficiently manage the production of these alkaloids.

High concentrations of endophyte-derived alkaloids are also found in seed, sometimes at higher levels than in vegetative plants, and probably evolved to reduce the probability of predation by granivores such as insects, small rodents and birds (Madej and Clay, 1991; Knoch et al., 1993; Finch et al., 2016; Pennell et al., 2017). Even seeds containing endophyte that has died can retain high alkaloid concentrations, which can protect seed from predation (Stewart, 1985; Ball et al., 1993). With the onset of seed germination, these compounds are subsequently translocated into seedlings to provide protection to the developing plant, although there is a period of vulnerability toward certain herbivores, such as ASW, up until the seedlings are six weeks old as these alkaloids are diluted within the increasing plant biomass (Ruppert et al., 2017). At six weeks it is then speculated that the endophyte becomes fully metabolically active and production of endophyte-derived alkaloid production can then commence. In a mature plant, alkaloid levels are generally highest in young leaves, stems and panicles (Saikkonen et al., 2013). Concentrations and the ratio of the different loline alkaloids are also dependent upon endophyte and host genotype (Ball and Tapper, 1999). Unfortunately assessing the different types of loline produced by AR501 within tall fescue and perennial ryegrass was outside the scope of this study. This would be advantageous information to gain from future research as the three loline alkaloids, *N*-formyl loline (NFL), *N*-acetyl loline (NAL), and *N*-acetyl norloline (NANL), have differing bioactive properties. Some tall fescue associations lack NFL and NAL and some lack lolines completely (Ball and Tapper, 1999). Concentrations of NFL, NAL, and NANL were generally higher in meadow fescue than tall fescue when infected with the same endophyte genotype. The study by Ball and Tapper (1999) also showed that mycelial biomass and production of the alkaloids peramine and lolines were heavily influenced by host plant species with greater amounts of mycelia and secondary metabolites produced in tall fescue. Additionally, recent research has uncovered greater diversity in the pyrrolizidine alkaloids (i.e., peramine) than previously thought (Berry et al., 2019) and future investigations need to account for the potential derivations produced by these fungal endophytes.

Epichloë strain AR501, an FaTG-3-type endophyte, was originally isolated from a Mediterranean-type tall fescue plant growing in southern Spain and, as with other strains of this grouping, is an asexual hybrid formed between *Epichloë baconii* and *E. typhina* (Moon et al., 1999). Endophyte strains

from this taxonomic grouping are known for their production of peramine and loline alkaloids but produce none of the metabolites linked to animal toxicity (i.e., ergovaline). As only one single AR501-infected plant of the original wild accession was present within the AgResearch parent plant collection, an alternative host was required for experimental purposes. Cultivar Grasslands Flecha, a summer dormant, Mediterranean cultivar of tall fescue, with a similar genetic background to the original host, was chosen as the host grass for AR501 alongside perennial ryegrass (line KLP903) for experimentation. Flecha was selected from germplasm native to the Mediterranean region and can exhibit less summer activity and more winter growth than cultivars developed from germplasm indigenous to central and northern Europe (Norton et al., 2006; He et al., 2017). Therefore, we have assumed that the endophyte biomass, chemical profile and colonization behavior of AR501 would be similar within Flecha as with the original wild *Festuca* host.

Christensen et al. (1993) were the first to investigate and document the compatibility (or lack of) within novel or artificially developed *Epichloë*-grass associations. After creating novel associations using endophyte strains from six recognizable taxonomic groupings and inoculating them into the apical meristem of three grass species, symptoms of incompatibility were observed. These included stunted tillers and necrosis of the apical meristem. Koga et al. (1993) documented less severe symptoms within incompatible novel associations created with perennial ryegrass and *E. coenophiala*. These symptoms, however, only affected the endophyte rather than the plant, and manifested as distorted, collapsed and dead hyphae. *Epichloë* fungal endophytes and their grass hosts have coevolved, and it is now generally accepted that endophyte taxonomic groupings, including those from tall fescue, are not host-specific but are confined to forming compatible associations (i.e., symptomless associations) with related grass genera within a tribe (Schardl and Phillips, 1997; Schardl et al., 1997). Based on chloroplast genome analysis, both tall fescue and perennial ryegrass are classified within chloroplast group 2 of the subtribe Lollinae, with rationale for aligning *Festuca* subg. *schedonorus*, the broad-leaved fescue species with the inclusion of tall fescue, within the genus *Lolium* (Darbyshire, 1993). The severe symptoms (described by Koga et al., 1993; Christensen, 1995) from incompatible endophyte-grass associations were not observed with the novel associations used for experimental purposes described in our study. We speculate that the close phylogenetical proximity of the two grass species is an explanation for this. Whether low endophyte transmission frequencies and low mycelial biomass within plants is a symptom of grass-endophyte incompatibility is an area of contention as low frequencies of endophyte can be found throughout many natural grass stands (Leyronas and Raynal, 2001).

Tall fescue is a long-lived, perennial, bunchgrass indigenous to Europe. Tall fescue can form mutualistic associations with asexual *Epichloë* endophyte species, all of which are interspecific hybrids derived from one or two parasexual hybridization events. These events likely occurred when an

endophyte-infected plant was colonized by *Epichloë* ascospores of a different species, subsequently leading to anastomosis followed by karyogamy (Tsai et al., 1994; Schardl and Craven, 2003). Tall fescue typically associates with three taxonomic groupings of *Epichloë*, as defined by isozyme, alkaloid and morphological characteristics, namely *Festuca arundinacea* taxonomic group 1 (FaTG-1) = *E. coenophiala*, FaTG-2 and FaTG-3, with the latter two groupings yet to receive Linnaean names (Christensen et al., 1993; Christensen, 1995; Leuchtman et al., 2014). These associations are believed to have coevolved over thousands of years with *E. coenophiala* possibly predating its tall fescue host, being identified within the ancestral tetraploid grass *Festuca arundinacea* spp. *fenas* (Schardl and Craven, 2003; Vazquez de Aldana et al., 2003). Tall fescue is an outbreeding, allohexaploid species and is more accurately described as a species complex (Hand et al., 2010; Ekanayake et al., 2012) with three distinct morphotypes described; a Mediterranean morphotype indigenous to North Africa, a Continental morphotype indigenous to Northern Europe and a rhizomatous morphotype indigenous to the Iberian Peninsula (Dierking et al., 2012). *E. coenophiala* associates with the Continental morphotype while FaTG-2 and FaTG-3 associate closely with the Mediterranean morphotype. Little information is available on the endophyte associations formed with the rhizomatous grass morphotype. However, this morphotype shares the same progenitors as the Continental type, namely *F. pratensis* and *F. arundinacea* var. *glaucescens* (Hand et al., 2010) and therefore may naturally associate more closely with *E. coenophiala* than the other taxonomic groups.

Perennial ryegrass is native to southern Europe, the Middle East, North Africa and eastwards to central Asia. The low growing, tufted, hairless grass is now regarded as an important forage species in many countries around the world including Australia, New Zealand, North America and South Africa. This grass species naturally associates with two taxonomic groupings of mutualistic asexual *Epichloë* endophyte, namely *Lolium perenne* taxonomic grouping 1 (LpTG-1 = *Epichloë festucae* var. *lolii*) and LpTG-2 (= *Epichloë hybrida*). Perennial ryegrass does not respond well to hot temperatures during its establishment stage (Kemp et al., 1999) and we speculate that the low biomass of AR501 mycelia detected at the second harvest date (week 3) within this grass host, as compared to tall fescue, was due to the plant exhibiting stress under the warmer temperature regime. By the third harvest date (week 6) the perennial ryegrass-endophyte association was less susceptible to this environmental stress as indicative of a similar biomass of hyphae being detected in plants exposed to a constant warm or a constant cool temperature regime. In contrast, at week 6, the biomass of endophyte mycelia within the tall fescue host was lowest at the constant cool temperature regime and when plants were transferred after three weeks from the warm to the cool temperature regime. This was highly significant, with mycelial biomass 80% less in plants exposed to both these temperature regimes compared to plants transferred after three weeks from a cool to a warm temperature regime.

Christensen et al. (2008) established that *Epichloë* species colonize their grass hosts by a unique mechanism termed intercalary hyphal extension and not by the general model of hyphal tip growth. However, there is still substantial variation within the *in-planta* colonization patterns linked to different groups of these *Epichloë* species. For example, *Epichloë occulta*, associated with annual ryegrasses, are found as a dense mycelial mass located at the base of the leaf sheath while the hyphae of most *Epichloë* species are found throughout the leaf sheath, aligned to the leaf axis and are seldom branched (Christensen et al., 2002). Leaf blade colonization can differ between *Epichloë* species, host, genotype, novel and natural associations and with plant age (Christensen et al., 2002). Tall fescue associations differ in their distribution of *Epichloë* hyphae with continental-type associations exhibiting poor colonization of their leaf blades compared to Mediterranean associations where hyphae can be readily observed in blades and sheaths (Christensen and Voisey, 2006; Takach et al., 2012). It is assumed that the dense ligular zone found between the leaf sheath and blade is a physical barrier to the advancement of *Epichloë* hyphae. As grass leaves age, hyphae increase in diameter but not in frequency (Christensen and Voisey, 2006) and this could therefore have implications for the accumulation of mycelial biomass. Further research with AR501 would aim to determine if the distribution of hyphae differed between the perennial ryegrass and tall fescue hosts and if this had any significant effect on mycelial biomass and/or production of secondary metabolites.

Host and endophyte genotype, plant age, nutrition and environmental conditions (such as air and soil temperature, day length, solar radiation, and precipitation) are critically important factors in the production of endophyte-derived secondary metabolites (Bush et al., 1993; Reinholz and Paul, 2000; Krauss et al., 2007; Fuchs et al., 2017). Concentrations of certain alkaloids, such as lolines, can also be stimulated *in-planta* after wounding, e.g., after insect attack (Schardl et al., 2007), showing that the grass-endophyte association is able to respond not only to biotic cues but also abiotic ones. Herbivory is one of the most important threats for plants, impacting net primary productivity in natural ecosystems and causing important economic losses in agriculture (Bastias et al., 2017). It is therefore logical that these grass plants would have devised complex mechanisms to enhance their fitness by associating with alkaloid producing Clavicipitaceous endophytic fungi.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

PF carried out this research as a part of her Doctor of Philosophy (Research) and performed the experiments. JH, MR, TG, and SC were co-supervisors. PF, JH, MR, TG, and SC contributed conception and design of the study. PM, PE, and SC performed the statistical analysis. PF and SC wrote the first draft of the

manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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Impact of Soybean Nodulation Phenotypes and Nitrogen Fertilizer Levels on the Rhizosphere Bacterial Community

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The effects of nodulation properties of legumes on the rhizosphere bacterial community are still not clear. To determine the effects of nodulation phenotypes on bacterial communities in the rhizosphere of soybean plants, we performed high-throughput sequencing of the 16S rRNA gene to estimate the rhizosphere bacterial community of three soybean lines with different nodulation phenotypes grown in soil supplied with different levels of N fertilizer. The results revealed that both the soybean nodulation phenotypes and the N levels affected the rhizosphere bacteria community, but the nodulation phenotypes contributed more than the N-supply. The diversity of bacteria was decreased in the rhizosphere of super-nodulating phenotype. The response of rhizosphere bacterial communities to the soil available nitrogen (AN) concentrations was different than the response with the three nodulation phenotypes of soybean which was more stable in the wild-type (Nod⁺) soybean samples than that in the mutant samples (Nod⁻ and Nod⁺⁺). *Bradyrhizobium* in the rhizosphere was positively correlated with nodule number and negatively correlated to AN in the soil, while *Burkholderia* and *Dyella* were positively correlated with nodule biomass and nitrogenase activity. These results demonstrated that the nodulation phenotype of soybean affects the rhizosphere microbiome.

Keywords: rhizosphere, bacterial community, diversity, soybean, nodulation phenotypes, nitrogen levels, super-nodulating

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is an important leguminous crop worldwide, and it can form a nitrogen-fixing symbiosis with some soil bacteria: so-called rhizobia. The SNF by soybean plants contributes 16.4 Tg of combined nitrogen (N) annually, accounting for 77% of the total N fixed by the legume crops (Herridge et al., 2008), which can meet 50–60% of the nitrogen

Abbreviations: AN, available nitrogen; AON, autoregulation of nodulation; ARA, acetylene reduction assay; DCA, detrended correspondence analysis; GmNARK, *Glycine max* nodule autoregulation receptor kinase; N, nitrogen; OTUs, operational taxonomic units; PGPB, plant growth-promoting bacteria; RDA, redundancy analysis; SNF, symbiotic nitrogen fixation; WHC, water holding capacity.

demand of soybean plants during their life cycle (Salviaggiotti et al., 2008). After harvesting, the crop residue of soybeans can also increase the nitrogen level of the soil (Kumar and Babalad, 2018). Therefore, improving the nodulation and nitrogen fixation capability of soybean is an important way to reduce the amount of nitrogen fertilizer usage and in turn to reduce the carbon emissions in fertilizer production.

The SNF could be promoted by the appropriate supply of nitrogen in its early growth stage (Marschner, 1995), but was inhibited by excess nitrogen supplementation (Zahran, 1999) through reducing the nodule numbers and nitrogenase activity. Thus, we should take into account of the nitrogen level in the soil for studying the nodulation of soybean. In wild-type (*nod*⁺) soybean, the number of nodules per plant is regulated by sophisticated machinery, so-called AON (Reid et al., 2011), and GmNARK has been identified as the key gene controlling the AON (Searle et al., 2003). The deficiency of AON could make the soybean plants as super-nodulating mutants (Akao and Kouchi, 1992), while non-nodulating soybean mutants were also obtained (Mathews et al., 1987).

The rhizosphere is the transit region between the surface of plant roots and the bulk soil in which the physicochemical features are strongly affected by the growth, respiration, and nutrient exchange of roots. Therefore, the microbial abundance, diversity, and activity in the rhizosphere are different from those in the vicinity of bulk soil and endosphere of the root (Xiao et al., 2017a, 2019). Numerous studies have demonstrated that plant root exudates mediate the interactions between plant roots and the microbial communities in the rhizosphere; for example, under N-limiting conditions, legumes secrete more flavones and flavonols to attract and initiate a symbiosis with rhizobia (Huang et al., 2014). Considering their SNF trait, the interactions among the microbes associated with the roots of leguminous plants are more complex than those in the rhizosphere of other plants. The differences in the microbiome in distinct rhizocompartments (nodule endophytes, root endophytes, rhizosphere, and root zone) of soybean and alfalfa have been revealed by high-throughput sequencing (Xiao et al., 2017a). Meanwhile, the effects of soil type on rhizomicrobiome of *Phaseolus vulgaris* estimated with a double pot system revealed that the bacterial community in rhizosphere is regulated by long-distance plant signaling (Xiao et al., 2019). These previous studies demonstrated that the root nodules are restricted microhabitats for both the rhizobia and the other bacteria.

Studies on the microbiota of soybean plants with different nodulation phenotypes have revealed that the stem- and leaf-associated bacterial communities were affected by the nodulation phenotypes and nitrogen fertilization levels (Ikeda et al., 2010, 2011). In addition, a ribosomal intergenic transcribed spacer analysis (RISA) described that the microbial communities (bacteria and fungi) associated with stems and roots varied with the different nodulation phenotypes of the soybean plants (Ikeda et al., 2008). Since the rhizosphere microbes can improve plant health and growth via different mechanisms (Huang et al., 2014), it has great value to learn the interactions between the rhizosphere bacteria and

the soybean genotypes. Several studies have been performed on the diversity and community shifting in relation to the cultivars and growth stages of soybean, as well as to the soil types (Xu et al., 2009; Xiao et al., 2017b). However, in the previous studies, the effects of nodulation genotypes on the soybean rhizosphere microbial communities were not clearly described.

Considering the deficiency of information about the effects of nodulation genotypes on the rhizosphere microbes, we performed the present study to evaluate the impacts of soybean nodulation phenotypes and N fertilizer levels on the rhizosphere bacterial community, using the Illumina MiSeq platform.

MATERIALS AND METHODS

Plant Materials, Soil, and Pot Culture Experimental Designs

The soybean lines used in this study were the spring cultivar Heihe 38 (wild-type nodulating cultivar; *Nod*⁺), the non-nodulating summer cultivar En 1282 (derived from non-nodulating mutant Enrei; *Nod*⁻) (Francisco and Akao, 1993), and the super-nodulating spring cultivar Dongfu 4 (a hybrid descendant derived from male parent super-nodulating mutant ZX 4 × female parent Heihe 38; *Nod*⁺⁺). Some growth features of the three cultivars are presented in **Supplementary Table S1**.

The soil used in this study was sampled from 0 to 20 cm in a degraded wetland (typical field for local corn-soybean rotation) near the city of Harbin, China (GPS location: N45, E126), where corn was cultivated previously without the addition of N fertilizer. The soil was a sandy loam with the following characteristics: pH 7.65 ± 0.09, available N content 28.67 ± 1.07 mg/kg, P₂O₅ 47.3 ± 0.9 mg/kg, K₂O 116.9 ± 1.9 mg/kg, Electrical Conductivity (EC) 0.050 ± 0.002 ds/m, salinity 0.002 ± 0%, and total organic carbon 3.69 ± 0.22 g/kg. The WHC of the soil was 32.6%, as determined by the gravity method. The soil physicochemical characteristics were determined using standard methods (Carter et al., 2007) in triplicate. To obtain the samples, soybean seeds were planted on May 15, 2018, in plastic pots (diameter, 11 cm; height, 40 cm) (patent No. ZL 2015 2 0193626.9), filled with 3.5 kg soil per pot with a moisture of 60% WHC. The plants were grown in a greenhouse with natural day/night cycling, and water was supplied from the bottom to maintain the moisture when necessary.

The experimental designation was four levels of N as urea (N0, 0 N fertilizer; N1, 50 mg N/kg soil; N2, 100 mg N/kg soil; and N3, 150 mg N/kg soil), combined with P as calcium superphosphate (450 mg/kg soil) and K as potassium sulfate (150 mg/kg soil), all in analytical grade. Six seeds were planted in each pot, but only one seedling remained after sprouting by thinning out the excess seedlings. The mixed fertilizers were dressed around the seedling root, following by watering. Six repeats were set for each treatment, covering a total of 72 pots (plants). The pots corresponding to different treatments were randomly arranged in the greenhouse.

Sampling of Plants, Nodules, and Soils

Soil and plant samples were obtained at the full-bloom stage, for which the pots were split without damage to the soybean roots. After removing the roots, soils in pots of each treatment were mixed and air-dried for available N determination in triplicate with the standard method (Carter et al., 2007). For the collection of rhizosphere soil, three plants from each treatment were gently shaken to eliminate the excess root-attached soil particles and the soil adhering to the roots was brushed off with soft toothbrush. The rhizosphere soil samples were then stored at -80°C until use.

Nodulation Characterization

Nitrogenase activity of the plants at full bloom stage was measured for the entire roots with nodules by the ARA according to Hardy et al. (1973) and Xia et al. (2017). Briefly, the intact roots of three plants from each treatment were cut at the cotyledonary node, washed, blotted for drying without detaching the nodules, and then placed separately in a 500-mL amber glass wide-mouth bottle fitted with a rubber stopper. 50 mL of air was replaced in each bottle with an equal volume of acetylene gas (at a concentration of 99.9%). After 2 h of incubation at room temperature, gas sample (5 mL) was transferred with a syringe to a 5-mL head space bottle (pre-evacuated). A GC 7900 gas chromatograph (Shanghai Techcomp Scientific Instrument Co., Ltd., China) was used for detecting the ethylene. Acetylene reduction activity (ARA) was expressed in the μmole of ethylene formed per plant per hour. The data were given as statistical analysis with the SPSS Statistics V 20.0 (SPSS: IBM Corp., United States) software.

Biomass of Plants

Plant height was measured for each treatment at the sampling time. After ARA, the nodule number and fresh nodule weight were counted for each plant. Then, all the plant samples were dried at 60°C for 48 h to determine the dry weights of the aboveground part and the root. Chlorophyll content of the last but one ternate compound leaf was measured just before the plant was sampled by the portable chlorophyll detector (CCM-200, OPTI-Sciences, United States). All the data were obtained in triplicate for the subsequent statistic analysis.

DNA Extraction and PCR Amplification

For microbial diversity analysis, metagenomic DNA was extracted from 0.25 g of the rhizosphere soil sample using the PowerSoil DNA Isolation kit (MO BIO, Carlsbad, CA, United States) according to the manufacturer's protocol. The V4–V5 region of the bacterial 16S rRNA gene (about 400 bp) was amplified by PCR (95°C for 2 min, followed by 25 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min) with primers 515F (5'-barcode-GTG CCA GCM GCC GCG G-3') and 907R (5'-CCG TCA ATT CMT TTR AGT TT-3'). The barcode was an eight-base sequence, unique to each sample. PCR reactions were performed in triplicate in 20- μL mixture containing 4 μL of $5 \times$ FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. Amplicons

were extracted from 2% (w/v) agarose gels after electrophoresis and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to the manufacturer's instructions and then quantified using the QuantiFluorTM-ST (Promega, United States).

Library Construction and Sequencing

Purified PCR products were quantified by Qubit[®]3.0 (Life Invitrogen), and every 24 amplicons with different barcodes were mixed equally. The pooled DNA products were used to construct an Illumina Pair-End library following the Illumina genomic DNA library preparation procedure. Then, the amplicon library was paired-end sequenced (2×250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd.), according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database under the accession number of SRP151632.

Processing of Sequencing Data

Raw FASTQ files were demultiplexed and quality-filtered using QIIME (version 1.17) with the following criteria: (i) The 250-bp reads were truncated at any site receiving an average quality score of <20 over a 10-bp sliding window, discarding the truncated reads that were shorter than 50 bp. (ii) Exact barcode matching, 2-nucleotide mismatches in the primer matching, and reads containing ambiguous characters were removed. (iii) Only sequences with overlaps longer than 10 bp were assembled according to their overlap sequence. Reads that could not be assembled were discarded.

Operational taxonomic units (OTUs) were defined with a 97% similarity cutoff using UPARSE (version 7.1¹), and chimeric sequences were identified and removed using UCHIME. We combined the three replicates into one sample by summing up the values of OTUs in the three replicates. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier² against the SILVA (SSU119) 16S rRNA database using a confidence threshold of 70% (Amato et al., 2013).

Alpha- and Beta-Diversity Analyses

Based on the results of OTU cluster analysis, the Alpha-diversity of the 12 samples was estimated by calculating the indices Chao, ACE, Shannon, and Simpson, while the sequencing coverage was also calculated for each treatment (Bai et al., 2015). Sequences in each OTU ranged from large to small according to the OTU richness, and the Rank-abundance curves were drawn with the relative abundances of each OTU ranked against the OTU ranks.

Principal coordinate analysis (PCoA) was performed with QIIME program to examine dissimilarities in the community composition of the samples by plotting the 12 samples in (12–1)-dimensional space, and the samples were grouped based on unweighted and weighted UniFrac distance metrics. The OTUs defined in this study were further compared with the defined bacteria to determine their species affiliation by blasting in the

¹<http://drive5.com/uparse/>

²<http://rdp.cme.msu.edu/>

NCBI database, and the sequence similarity of 97% was used as the threshold of species.

Redundancy Analysis (RDA)

Detrended correspondence analysis (DCA) showed that the largest axis length was 1.33 and 1.42 at the genus level. Consequently, RDA was selected, and the significance of nodulation characteristics, AN, and plant biomass factors were tested with Monte Carlo permutations (permu = 999). The analyses of RDA were conducted in *R* for statistical computing (R Development Core Team, 2013), using the vegan package (Oksanen et al., 2013).

RESULTS

Nodulation and Growth Characterization of Soybean Cultivars

As expected, no nodule was observed on the roots of En 1282 (Nod⁻) plants in any treatments, while 129 and 900 nodules per plant on average were counted on the roots of Heihe 38 (Nod⁺) and Dongfu 4 (Nod⁺⁺) plants in N0 treatments (Table 1), which fitted the symbiotic characters for each soybean cultivar. The results in Table 1 showed that the nodule numbers, nodule mass, and ARA of Dongfu 4 plants were significantly higher than those of the Heihe 38 plants at all four N levels. The nodule numbers of Dongfu 4 were decreased as the N levels increased from the N0 to N3 (no significant difference between N2 and N3) treatments, while no significant change was observed in the nodule numbers of the Heihe 38 plants at the four N levels. The nodule mass of the Heihe 38 plants was significantly decreased in the N2/N3 treatments compared with the N0/N1 treatments, but this value of Dongfu 4 was significantly increased in the N1 treatment relative to the N0 treatment; then, the nodule mass of Dongfu 4 gradually decreased in the N2 and N3 treatments. For the ARA, the Dongfu 4 plants presented significantly greater values in the N1 and N2 treatments than those in the N0 and N3 treatments, while no significant difference was observed in the Heihe 38 plants at all the four N levels.

In this study, the chlorophyll contents of leaves presented in the order of Dongfu 4 > Heihe 38 > En 1282, while it was constant in Dongfu 4 and Heihe 38 at all the four N levels, but significantly increased in En 1282 at the highest N level. For plant height, both the nodulation cultivars presented a positive response to the N1 level and a supplement of more N fertilizer did not cause more growth, while the Nod⁻ cultivar En 1282 presented the positive response only for the highest N level (N3). For the dry weight of aerial parts, the three cultivars presented the order of En 1282 > Heihe 38 > Dongfu 4. While the dry weight was constant for the two nodulating cultivars despite the N levels, it was significantly increased for En 1282 from N0 through N2 levels and decreased at N3 level. For root biomass, the situation was similar to that of aerial parts (Table 1).

Bacterial Community

In the high-throughput DNA sequencing, 1,115,186 valid sequences with average lengths of 396 bp were obtained from the

TABLE 1 | Effects of Nitrogen levels on the nodulation characteristics of different soybean cultivars.

Sample code	Treatment		Nodule no. (plant ⁻¹)	Nodule mass (g plant ⁻¹)	Ethylene (μmol/plant/h)	Available nitrogen (mg/kg)	Chlorophyll content (SPAD)	Height (cm)	Dry weight of upground (g/plant)	Dry weight of root (g/plant)
	Cultivar	N-supp. mg/kg								
H-N0	Heihe 38	0	129.67 ± 27.43a	6.50 ± 1.01b	92.37 ± 23.42a	53.02 ± 2.22a	18.81 ± 2.38cd	27.82 ± 2.87bc	9.24 ± 1.18ab	5.13 ± 0.70abc
H-N1		50	255.33 ± 21.55a	6.93 ± 0.76b	125.59 ± 24.34a	56.49 ± 2.03abc	16.67 ± 0.84bc	34.42 ± 4.64d	12.25 ± 3.84b	5.91 ± 1.15c
H-N2		100	207.00 ± 14.18a	4.03 ± 0.61a	131.13 ± 32.81ab	62.57 ± 3.32d	21.38 ± 2.76de	30.17 ± 4.40cd	14.67 ± 3.68b	6.42 ± 0.78c
H-N3		150	123.67 ± 46.48a	3.00 ± 0.90a	75.53 ± 44.37a	70.52 ± 2.49e	22.51 ± 5.16de	31.18 ± 5.54cd	12.29 ± 1.49b	5.28 ± 1.96bc
D-N0	Dongfu 4	0	900.67 ± 219.00d	13.53 ± 1.76c	210.21 ± 61.08c	52.21 ± 5.57a	25.31 ± 2.62ef	22.33 ± 3.85a	6.16 ± 0.67a	2.51 ± 0.15a
D-N1		50	684.00 ± 154.28c	19.32 ± 1.55e	351.85 ± 34.32d	60.57 ± 2.67cd	27.39 ± 1.55f	28.65 ± 3.95c	10.72 ± 2.1ab	2.94 ± 0.24ab
D-N2		100	490.00 ± 43.59b	17.00 ± 1.61d	298.84 ± 39.32d	76.03 ± 3.75f	27.47 ± 4.18f	27.08 ± 3.00abc	8.70 ± 1.40ab	3.11 ± 0.39ab
D-N3		150	465.67 ± 55.82b	13.17 ± 2.10c	196.70 ± 30.76bc	58.40 ± 2.92bcd	26.67 ± 4.48f	31.55 ± 2.35cd	11.29 ± 0.98ab	3.11 ± 0.65ab
E-N0	En 1282	0				60.00 ± 0.33bcd	5.57 ± 1.15a	23.02 ± 3.11ab	12.37 ± 0.56b	6.02 ± 0.57c
E-N1		50				55.27 ± 1.36ab	7.44 ± 0.70a	26.60 ± 57.07abc	27.77 ± 3.51c	10.32 ± 2.02e
E-N2		100				60.88 ± 1.06cd	7.23 ± 1.32a	28.78 ± 4.50c	47.75 ± 7.16d	7.68 ± 2.23cd
E-N3		150				61.31 ± 1.26cd	13.92 ± 4.18b	34.30 ± 3.79d	32.40 ± 4.35c	9.74 ± 3.00de

Data are mean ± standard deviation (n = 3). Different lowercase letters in the same column show significant differences between treatments (*P* < 0.05). "H, D, E" represent the soybean cultivars "Heihe 38, Dongfu 4 and En1282" and "N0, N1, N2, N3" represent the four levels of N as urea, "0 N fertilizer, 50 mg N/kg soil; 100 mg N/kg soil; 150 mg N/kg soil".

TABLE 2 | Comparison of the observed OTUs and Estimators of bacterial communities in the rhizosphere of three nodulation phenotypes soybean at different N levels.

Sample ID*	Reads	OTU	Ace	Chao	Coverage	Shannon	Simpson
D-N0	86014	2037	2596 ± 93	2593 ± 117	0.99	3.32 ± 0.02	0.23 ± 0.00
D-N1	83282	2404	2804 ± 70	2827 ± 93	0.99	4.27 ± 0.02	0.11 ± 0.00
D-N2	78574	2142	2708 ± 92	2695 ± 115	0.99	3.47 ± 0.02	0.22 ± 0.00
D-N3	92995	2197	2658 ± 78	2662 ± 101	0.99	3.42 ± 0.02	0.21 ± 0.00
E-N0	91206	2767	3044 ± 52	3026 ± 63	0.99	5.02 ± 0.02	0.09 ± 0.00
E-N1	72048	2693	3037 ± 62	3071 ± 86	0.99	6.00 ± 0.02	0.01 ± 0.00
E-N2	72318	2422	2851 ± 74	2880 ± 100	0.99	5.03 ± 0.02	0.05 ± 0.00
E-N3	80078	2358	2805 ± 77	2833 ± 103	0.99	5.07 ± 0.01	0.04 ± 0.00
H-N0	89451	2786	3086 ± 56	3113 ± 77	0.99	5.55 ± 0.02	0.03 ± 0.00
H-N1	74059	2514	2938 ± 72	2943 ± 93	0.99	4.97 ± 0.02	0.05 ± 0.00
H-N2	68345	2195	2717 ± 86	2710 ± 108	0.99	4.21 ± 0.02	0.10 ± 0.00
H-N3	68591	1983	2683 ± 111	2696 ± 144	0.99	3.21 ± 0.03	0.22 ± 0.00

*See footnote of **Table 1** for the corresponding treatments.

12 samples, after filtering the raw reads (**Table 2**). The optimized sequences were divided into 3,310 OTUs at 97% sequence identity after cluster analysis. The coverage for all the samples was >99%, implying that almost all of the OTUs were detected. The Alpha-diversity in the 12 samples (**Table 2**) showed that the samples of En 1282 at different nitrogen levels had the highest diversity and that of Dongfu 4 had the lowest diversity. Within the single cultivars, samples of Dongfu 4 and En 1282 showed higher community diversity at the N1 level than at the other three N levels, while the samples of Heihe 38 exhibited a decreased diversity as the N level increased.

The rarefaction curves (available as **Supplementary Figure S1**) obtained using the randomly selected sequences trended to be flat when the number of sequences was >40,000, indicating that most OTUs have been recovered and that the sizes of the sequencing data were reasonable for our analysis. The rank-abundance curve (**Supplementary Figure S2**) obtained in this study demonstrated that the OTUs with relative abundance values as low as 0.001 were found in each sample and that most of the OTUs were recovered in this study. The community composition at the genus level is summarized in **Supplementary Table S2**.

Influence of Cultivar and N Supply on Bacterial Diversity

The PCoA based on the Bray-Curtis distance metrics of OTU distribution generated two principal coordinates (PCs), which collectively explained nearly 77% of the variation among the samples (**Figure 1**). In the direction of PC1, the communities in the rhizosphere of Dongfu 4 were separated from those in the rhizosphere of En 1282, while the communities in Heihe 38 rhizosphere were distributed at both sides of the Y-axis. These results indicated that the nodulation phenotypes of the soybeans might affect the community composition of rhizosphere bacteria, as they accounted for the largest source of variation (55.76%). In the direction of PC2, H0 was distinct from H1, H2, and H3; D1 was separated from D0, D2, and D3; and E0 and E1 were different from E2 and E3. These results revealed that the N level might

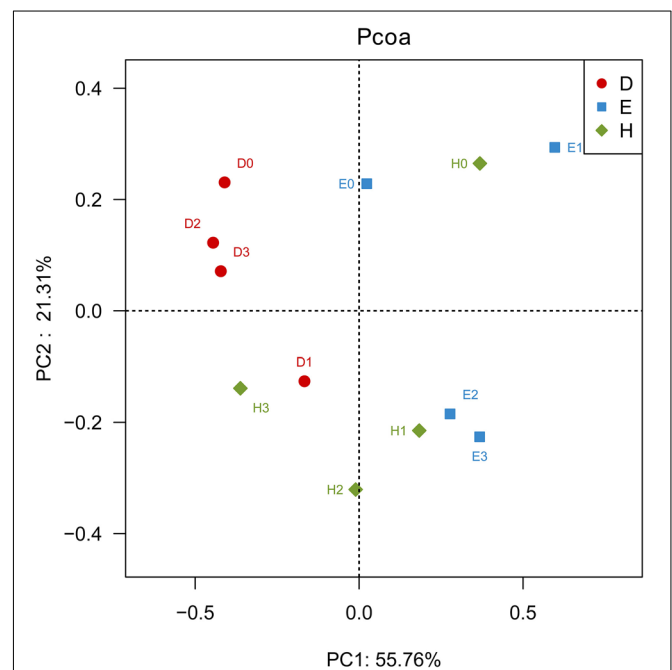
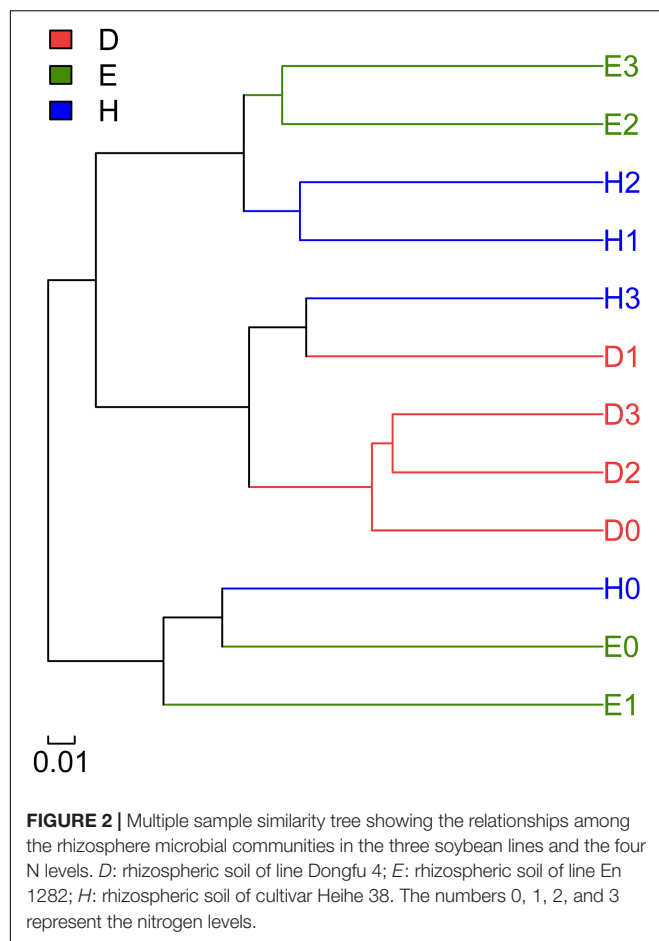


FIGURE 1 | Principal coordinate analysis (PCoA) based on the Bray-Curtis distance metrics of bacterial communities in the rhizosphere of soybeans with different nodulation phenotypes at different nitrogen levels ($n = 36$). *D* represents the rhizospheric soils of the line Dongfu 4; *E* represents the rhizospheric soils of the line En 1282; *H* represents the rhizospheric soils of the cultivar Heihe 38. Arabic numerals 0, 1, 2, and 3 represent the Nitrogen levels.

be the second factor used to explain the 21.31% variation of the community composition of rhizosphere bacteria.

Community Similarity and Differences Between the Different Samples

A hierarchical clustering tree was constructed to describe and compare the similarities of multiple samples (**Figure 2**). Based on the similarities between the community compositions, the

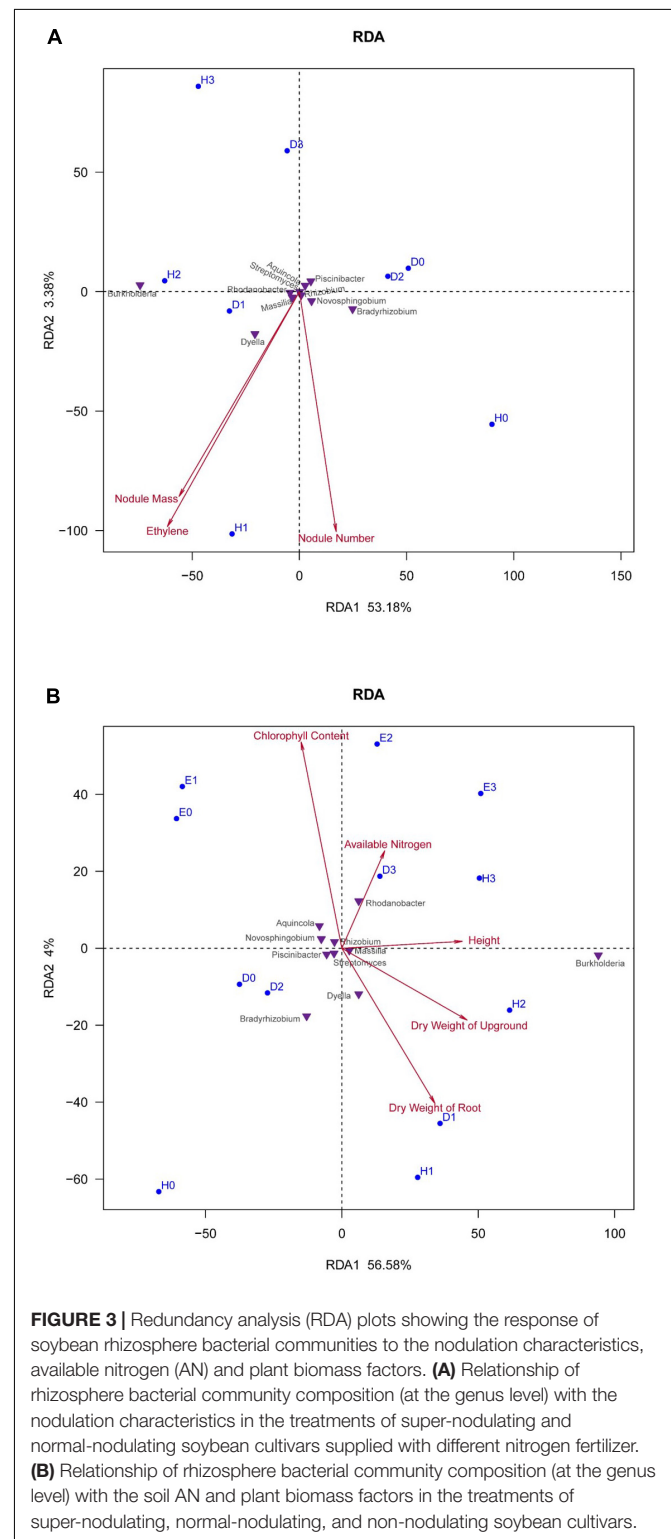


12 soil samples were divided into three groups. The first group included the bacterial communities of the Heihe 38 rhizosphere at the N3 level and the bacterial communities of the Dongfu rhizosphere at all the four N levels, implying that the bacterial community compositions of the Dongfu rhizosphere were not considerably affected by the N levels. The second group covered the communities of Heihe 38 rhizosphere at N0 level and the samples of En1282 at N0 and N1 levels. The third group was composed of bacterial communities of Heihe 38 rhizosphere at N1 and N2 levels together with the samples of En1282 at N2 and N3 levels.

Response of Bacterial Community to the Nodulation Characteristics, Available N, and Plant Biomass

The available N in soil and plant biomass observed in different treatments are shown in **Table 1**. In general, the available N (AN) in soil (ranging between 52 and 76 mg/kg) was increased across all the treatments after culturing soybean with or without N supply. As to the cultivar, the AN in Heihe 38 cultured soil was similar at N0 and N1 levels and increased at N2 and N3 levels; in Dongfu 4 cultured soil the AN was significantly increased in all the N supplied treatments; and in En 1282 cultured soil, the AN contents are almost the same, despite the doses of N supplement.

The response of soybean rhizosphere bacterial community to the nodulation characteristics, AN in soil, and plant biomass factors were expressed by RDA plots in **Figure 3**. It could be observed that the nodulation characteristics (nodule biomass,



nodule number, and nitrogenase activity) contributed a total of 56.56% (RDA1 + RDA2) for the variance in the bacterial communities (**Figure 3A**); while the soil AN and plant biomass contributed 60.58% (RDA1 + RDA2) of the bacterial community variation (**Figure 3B**). The sum of contributions in **Figures 3A,B** greater than 100% (117.14%) might be explained by the interactions between the nodulation characters and the plant biomass factors. In general, the correlations between the nodulation characters of the soybean cultivars and the bacterial communities were demonstrated by their distances of foot points to arrows in the plot (**Figure 3A**). The nodule mass and nitrogenase activity (ethylene production) presented a very similar correlation to the bacterial communities. The H-N1 (wild-type Heihe 38–50 mg N) community showed the highest and positive correlation with all the three nodulation characters, followed by that of H-N2, D-N1, and H-N0 samples; while the rhizosphere bacterial communities in D-N2, D-N0, D-N3, and H-N3 samples were negatively correlated to the nodulation characters (**Figure 3A**). As for the bacterial genera, *Dyella* and *Bradyrhizobium* in the rhizosphere were positively correlated with the nodule number, while *Burkholderia* and *Dyella* were positively correlated to the nodule mass and nitrogenase activity (**Figure 3A**).

In **Figure 3B**, it seemed that the rhizosphere bacterial communities in all the treatments were highly correlated by the chlorophyll content and plant biomass (shoot and root weight). The response of bacterial communities to the soil AN was enhanced with the increase of N levels for the wild-type soybean (Heihe 38) samples. The rhizosphere bacterial communities in super-nodulating soybean (Dongfu 4) samples D-N0, D-N1, and D-N2 had a similar correlation to the AN in soil, and D-N3 has a high correlation to the AN. The rhizosphere bacterial communities of non-nodulating soybean samples E-N0 and E-N1 have a higher correlation to soil AN than those of E-N2 and E-N3. The rhizosphere communities of bacteria of non-nodulating AON mutant En 1282 had a higher correlation with the chlorophyll content than those of the nodulating cultivars. Moreover, at the genus level, *Bradyrhizobium* and *Dyella* were negatively correlated with the AN, while *Rhodanobacter* had the highest positive correlation with the AN.

DISCUSSION

As one of the most important legume crops, many studies related to the SNF of soybeans have been performed, assessing the amounts of nitrogen required for promoting or inhibiting the nodulation (Marschner, 1995; Zahran, 1999), the diversity and effectiveness of its symbionts (Chen et al., 2002; Yan et al., 2017), and the genes involved in nodulation/nitrogen fixation in both soybean plants (Searle et al., 2003; Ikeda et al., 2008; Lim et al., 2010) and rhizobia (Shamsel, 2013). However, to the best of our knowledge, the effects of nodulation characters on soybean rhizosphere microbes have rarely been studied. As a part of root system for N₂-fixation, the presence and number of nodules on the roots might affect soybean rhizosphere bacteria.

In the present study, we used soybean cultivars with normal, absent, and enhanced nodulation abilities to reveal the effects of nodulation phenotypes and N fertilization on symbiosis formation and rhizosphere bacteria composition. To eliminate the impact of soil type on the microbiomes in the rhizosphere (Liu et al., 2019), only one soil was used in this study. The results indicated that the non-nodulating AON mutant En 1282, super-nodulating AON mutant Dongfu 4, and wild-type nodulating cultivar Heihe 38 formed a set of models for investigating the effects of nodulation on the rhizosphere bacterial community. Indeed, the nodulation characters regulated the rhizosphere bacterial communities, since the Chao value and Shannon index at the treatments N0 (no N supply) demonstrated a tendency of $\text{Nod}^+ > \text{Nod}^- > \text{Nod}^{++}$ (**Table 2**). In other words, in the soil with a background N level of 28.67 mg/kg, the wild-type cultivar Heihe 38 harbored the most diverse rhizosphere bacteria, while the AON mutants (Nod^- and Nod^{++}) decreased the diversity of rhizosphere bacteria. Previously, differences in rhizosphere microbiomes were observed among various cultivars (Wang et al., 2014), but all were nodulation wild types. In Ikeda et al. (2005), different compositions in root-associated microbiomes were revealed by a ribosomal intergenic spacer analysis (RISA), but no diversity index was reported for the microbiomes. So, our study was the first one to connect the diversity of rhizosphere microbiome with the nodulation genotypes. Since the diversity tendency was changed according to the nodulation phenotypes when the N level increased, we estimated that both the nodulation phenotype and the N levels regulated the diversity of rhizosphere bacteria, but the nodulation characteristics contributed more (56.56%) (**Figure 3A**) than the N levels under the conditions in this study (**Figures 1, 2**).

The effects of nodulation phenotype and N fertilizer levels on the rhizosphere bacteria were further revealed by the grouping of all the Dongfu 4 rhizosphere samples (D0 through D3) in the same cluster (**Figures 1, 2**). It seems that the bacterial community in the rhizosphere of the super-nodulating mutant (Dongfu 4) was not so sensitive to the soil nitrogen level, which might be related to the effects of nodulation traits on the rhizosphere bacteria, since it always presented a great number of nodules and high nitrogenase activity against different levels of N fertilizer.

It is well known that excessive N fertilization decreases the diversity of microbes in rhizosphere and bulk soils (Sun et al., 2019; Wang et al., 2019). In the present study, the decrease of the diversity of rhizosphere bacteria at the high dose of N fertilization (N2 and N3) compared with that in the N1 treatment for all the three cultivars (**Table 2**) demonstrated that only the excessive N fertilization decrease the diversity of rhizosphere bacteria, which might be through the selection pressure of high concentrated AN and alteration of soil pH on the microbes (Li et al., 2016). Also, some of the plant physiological features, such as the chlorophyll content and leaf area index, are regulated by the N level (Basal and Szabó, 2018), which could in turn affect the rhizosphere microbes via changing root exudates or signaling of the plant (Pfenning et al., 2009).

Previously, it has been reported that the nodulation and nitrogenase activity of soybean plants were stimulated by low-level N fertilization, and inhibited by high level (>50 mg-N/L)

N fertilization (Xia et al., 2017). In general, similar results were observed in our present study (Table 1). However, the nitrogenase activity was more sensitive to the concentration of N fertilizer in Nod^{++} than in the Nod^{+} phenotypes, which were also found among other soybean cultivars (Abdel Wahab and Abd-Alla, 1995). Although the mechanism for the difference in sensitivity of nitrogenase to N-supply among the cultivars is not clear, King and Purcell (2005) described nodule ureides, nodule aspartate, and several amino acids (Asp, Gln, etc.) in leaves as the possible molecules for feedback inhibition of nitrogen fixation of soybean.

The similar bacterial community compositions in rhizospheres of the treatments D1 and H3 (Figures 1, 2) might imply that similar root exudates were produced by soybean plants in these two treatments, since the rhizosphere microbiome was strongly regulated by root exudates (Haichar et al., 2008; Subbarao et al., 2009; Huang et al., 2014; White et al., 2015; Szoboszlay et al., 2016) and compounds sloughed off root tips (Bulgarelli et al., 2013), which could be related to N fertilization, colonization of endophytic microbe, and N_2 fixation of legume plant (Xie et al., 2019). To confirm this estimation, a comparative study on the root exudates is needed.

The greater similarities among the rhizosphere bacterial community compositions among the treatments of E0, E1, and H0, as well as among E2, E3, H1, and H2 (Figures 1, 2), implied that the Biological nitrogen fixation (BNF) by Heihe 38 nodules and N supply for En 1282 have similar effects on rhizosphere microbiomes. The mechanism of these effects needs further study, such as comparative analysis of root exudates of the soybean plants in the corresponding treatments. All these results suggested again that both the nodulation characteristics of the soybean plants and the nitrogen supply regulated the rhizosphere bacterial community, and their contribution varied depending on the cultivars of soybean plants.

In the RDA analysis, the *Bradyrhizobium* abundance in the rhizosphere was positively correlated with the nodule number, as *Bradyrhizobium* was the main microsymbionts of soybean in the region where the tested soil was taken (Yan et al., 2014, 2017). The positive correlation of *Burkholderia* with nodule mass and ethylene production demonstrated it to be beneficial for BNF, which was consistent with other reports that some PGPR *Burkholderia* strains could activate the energy production pathways of plants under both aerobic and microaerobic conditions, and in turn promote the BNF (Chanyarat et al., 2016). The positive correlation of *Dyella* with the nodule number/mass and ethylene production suggested it to be a possible PGPR, since *Dyella* isolated from the nodules of *Lespedeza* sp. could enhance the plant growth (Pitchai et al., 2010), probably by their production of indole acetic acid or 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Correa-Galeote et al., 2018).

As an important biochemical parameter of plants, chlorophyll content has been used to estimate plant productivity and health status (Casa et al., 2015; Chen et al., 2017). However, the orders of Dongfu 4 > Heihe 38 > En 1282 for chlorophyll content and En 1282 > Heihe 38 > Dongfu 4 for the biomass of shoots and roots across all N treatments (Table 1) are somewhat surprising. We postulate that this counterintuitive situation might be related

to the nodulation and BNF activities of the cultivars. Previously, it has been reported that the colonization and BNF of the endophytic bacteria could decrease the biomass accumulation of poplar and cedar plants, especially in the early growth stage (Anand and Chanway, 2013; Knoth et al., 2014). It was estimated that about 5.6–8.0 g of carbon were lost for fixing 1 g of N in the nodulation legumes (Phillips, 2003). So, we speculate that the greater BNF activity would require greater photosynthesis (more chlorophyll content) since BNF is an energy-consuming process. Therefore, the higher nodule number/biomass and higher ARA values in Dongfu 4 treatments than those in Heihe 38 treatments might explain why the chlorophyll contents in Dongfu 4 were the highest, but why its biomass was the lowest (Table 1). It is possible that in Dongfu 4 and Heihe 38, a remarkable proportion of carbohydrates produced by photosynthesis was used for N_2 fixation.

Another point in this study is the fact that the amounts of AN in the soil samples at harvest are similar across all treatments, despite the significant differences in N supply at the beginning, which is similar to the results in previous report (Eickhout et al., 2006). In the N0 treatments, the increase of AN in the soil might from the BNF by rhizobia for the nodulating cultivars (Heihe 38 and Dongfu 4) and from BNF by other diazotrophic bacteria for the non-nodulating cultivar En 1282. Indeed, the abundances of diazotrophs (*Azoarcus*, *Azospira*, *Azospirillum*, *Azotobacter*, and *Azovibrio*) (Supplementary Table S2) were greater in the rhizosphere of En 1282 and Heihe 38 than that of Dongfu 4. In the other treatments, the supplied N might be removed by plant absorption, NO_3^- leaching, denitrification, and NH_3 volatilizing as reported in fields of other crops (Raun and Johnson, 1999; Eickhout et al., 2006). Also, high N supply could decrease the utilization efficiency of N fertilizer by crops (Raun and Johnson, 1999; Eickhout et al., 2006) and enhance the denitrification as high as 10 times (up to 0.3 to 1.0 kg N ha⁻¹ day⁻¹) in the pasture soil (Colbourn, 1992). Considering the fact that the biomass of soybean was not significantly increased by the addition of N fertilizer for two nodulation cultivars and, in addition, an increase of height was obtained at N1 level and the height was not further increased at N2 and N3 levels, demonstrating that the symbiotic BNF might completely fit the N nutrient requirement of soybean growth, excessive supply of N fertilizer was not necessary, as reported in other studies (Ju et al., 2009).

Conclusively, (1) both the nodulation characters and the N level affected the bacterial community in soybean rhizosphere, but the soybean nodulation phenotypes contributed more than the N-supply; (2) the diversity of bacteria was decreased in the rhizosphere of super-nodulating phenotype; (3) the responses of rhizosphere bacterial communities to the soil AN concentrations varied according to the nodulation phenotypes of soybean, which was more stable in the wild-type (Nod^{+}) soybean samples than that in the mutant samples (Nod^{-} and Nod^{++}); (4) *Bradyrhizobium* in the rhizosphere was positively correlated with nodule number and negatively correlated to AN in the soil, while *Burkholderia* and *Dyella* were positively correlated with nodule biomass and nitrogenase activity. These results demonstrated that the nodulation phenotype of soybean affects the rhizosphere microbiome.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP151632).

AUTHOR CONTRIBUTIONS

HW was the first and corresponding author of this article, who was responsible for the manuscript writing and organizing research. CG was mainly responsible for bioinformatics analyzing. XL and CY were responsible for sample collection and processing. WL was responsible for linguistic modification. SW was responsible for field management.

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Breeding for Beneficial Microbial Communities Using Epigenomics

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INTRODUCTION

Traditionally, breeding programs have tapped into two main sources of diversity for the identification of alleles capable of conferring beneficial traits to the crop of interest: (1) The genetic diversity of landraces and wild relatives, and (2) the creation of novel alleles using random or directed mutation approaches.

The generation of mutant populations has been the base for many successful breeding programs, including barley, soybean, tomato, and wheat (Jankowicz-Cieslak et al., 2017). Genetic variability is used to develop novel agronomically beneficial traits but also to determine the putative function of non-characterized genes (reverse epigenetics), and to identify genomic locations responsible for traits of interest (forward epigenetics) in crops (Rodríguez López and Wilkinson, 2015). Genetic variation has been conventionally achieved through insertional mutagenesis (transfer DNA, transposons, and entrapment tagging) (Ram et al., 2019), chemical (i.e., ethyl methanesulfonate, EMS) or ionizing radiation (i.e., gamma ray) treatment (Jankowicz-Cieslak et al., 2017), and more recently through targeted gene editing approaches (TALEN, ZNF, and CRISPR/Cas9) (Wolter et al., 2019).

This approach has been pivotal in transforming food production systems but with an ever-changing environmental landscape and increasing global population, improvement rates fall short of providing food security (Mehrabi et al., 2018). Concerted research efforts have been made to address this pitfall. Advancements in recent years include marker-assisted selection for genes of interest (Karanjalkar and Begane, 2016), the development of speed breeding methodologies to shorten generation time (Mehrabi et al., 2018), targeted breeding using directed approaches (as opposed to trial-by-error breeding strategies), reverse-breeding strategies to introduce genetic diversity (ancestral traits) back into commercial crops (Palmgren et al., 2015), and random chemical mutagenesis (Jankowicz-Cieslak et al., 2017). These progressive breeding programs have made strides in increasing crop quality and quantity, but it is increasingly recognized that they do not target all possible sources of phenotypic variability (Rodríguez López and Wilkinson, 2015).

EPIGENETIC MECHANISMS AS A SOURCE VARIABILITY FOR CROP IMPROVEMENT

Epigenetic mechanisms regulate gene expression in response to plant development and environmental stimuli, ultimately affecting the plant's phenotype (Kumar, 2018). The field of applied epigenetics is a rapidly evolving area of research, stimulating new opportunities for the improvement of crop productivity. It is now widely accepted that epigenetic mechanisms have been the source of useful variability during crop varietal selection (Rodríguez López and Wilkinson, 2015; Crisp et al., 2016; Fortes and Gallusci, 2017; Gallusci et al., 2017). An early example of epigenetic breeding demonstrated the potential to improve crop performance and energy use efficiency (an important yield determinant) in a commercially valuable crop, rapeseed (*Brassica napus*), through recurrent epigenetic selection of isogenic lines (Hauben et al., 2009).

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The major epigenetic mechanisms mediating these effects include histone modifications, DNA methylation and small RNA molecules, which act in an interactive, and redundant fashion to affect gene expression (Rodríguez López and Wilkinson, 2015). DNA methylation involves the addition of a methyl group to the 5th carbon of cytosines (forming 5-methylcytosine) by a set of enzymes called DNA methyltransferases. Gene promoter methylation has been associated to transcriptional repression (Kass et al., 1997). Importantly, this classic promoter methylation–gene expression model does not seem to be universal (Anastasiadi et al., 2018). A more complex model has been suggested where the methylation of the promoter and the gene body exerts separate influences on gene expression (Wang et al., 2015). In general, a negative association has been found between gene body methylation (GbM) and gene expression (Anastasiadi et al., 2018; Magris et al., 2019). Nevertheless, GbM has also been linked to higher gene expression in certain gene subclasses (Dubin et al., 2015; Anastasiadi et al., 2018).

Exploiting the relationship between gene DNA methylation and expression through deliberate perturbation of DNA methylation via exogenous interventions, has been proposed as a fast method to generate variability for crop improvement (Rodríguez López and Wilkinson, 2015; Gallusci et al., 2017). This can be achieved by using methods that are analogous to those used in mutation breeding, application of chemical inhibitors of DNA methyltransferases, which causes stochastic genome-wide DNA demethylation (Geyer et al., 2011; Amoah et al., 2012; Browne et al., 2020) and so, generates new variants carrying epi-alleles (defined here, as any of a group of otherwise identical genes that differ in the extent of their methylation). The use of targeted epigenome editing techniques capable of altering DNA methylation or histone modifications in the genes of interest may also be employed (Vojta et al., 2016). The induction of such epialleles lead to changes in gene expression and phenotype. This strategy, similar to mutation breeding, can be used to generate novel, and valuable epigenetic variation for crop improvement (Amoah et al., 2012). Novel epialleles can be inherited, even over multiple rounds of sexual reproduction, (Amoah et al., 2012; Tricker et al., 2013a,b). More importantly, they can become fixed in hybrids, resulting in heritable molecular and physiological phenotypes (Wibowo et al., 2018) without the need for genetic modification.

THE HOLOBIONT AS A POTENTIAL BREEDING TARGET

Thus far, breeding approaches consider the crop as a single species. However, in nature, plants do not exist as an entity, but cohabit with diverse microbes (collectively termed the plant microbiota). The assemblage of the host and the microbiota is referred to as the holobiont, while the term hologenome is used to indicate the entire set of genomes within the holobiont (Figure 1).

Microbial communities provide multiple benefits to their hosts, including better access to nutrients, enhanced growth, and improved tolerance to biotic and abiotic insult (Powell et al., 2015; Harman and Uphoff, 2019). The realization of

the importance of microbiotas for crop health, has led to the development of prebiotic and probiotic cocktails intended to enhance the holobiont (Rodríguez et al., 2019). However, their effectiveness has been proven to be highly inconsistent (Ownley et al., 2003). This inconsistency has been attributed to different causes, including the host plant or pathogen genotype (Yang et al., 2018), agricultural practices (Schippers et al., 1990), and loss of activity due to mutation of the biocontrol strain (Duffy and Défago, 2000). These findings highlight the on-going need to better understand the host-microbiota interactions for crop improvement, and suggest non-specific additive cocktails are sub-optimal for general application (Rodríguez et al., 2019).

Interestingly, a handful of recent studies have shown that crop domestication and breeding have inadvertently altered the microbial communities of the target crops (Leff et al., 2017; Chaluvadi and Bennetzen, 2018), suggesting that microbiota composition is a trait that can be bred (Wissuwa et al., 2009). Unfortunately, very little research effort has been invested in understanding host-microbe interactions from a community perspective (Beilsmith et al., 2019). This makes the understanding of what makes a “good microbiota host” critical in the conceptualization of breeding programs aimed at improving productivity, quality and sustainability through the management of the holobiont (Wissuwa et al., 2009).

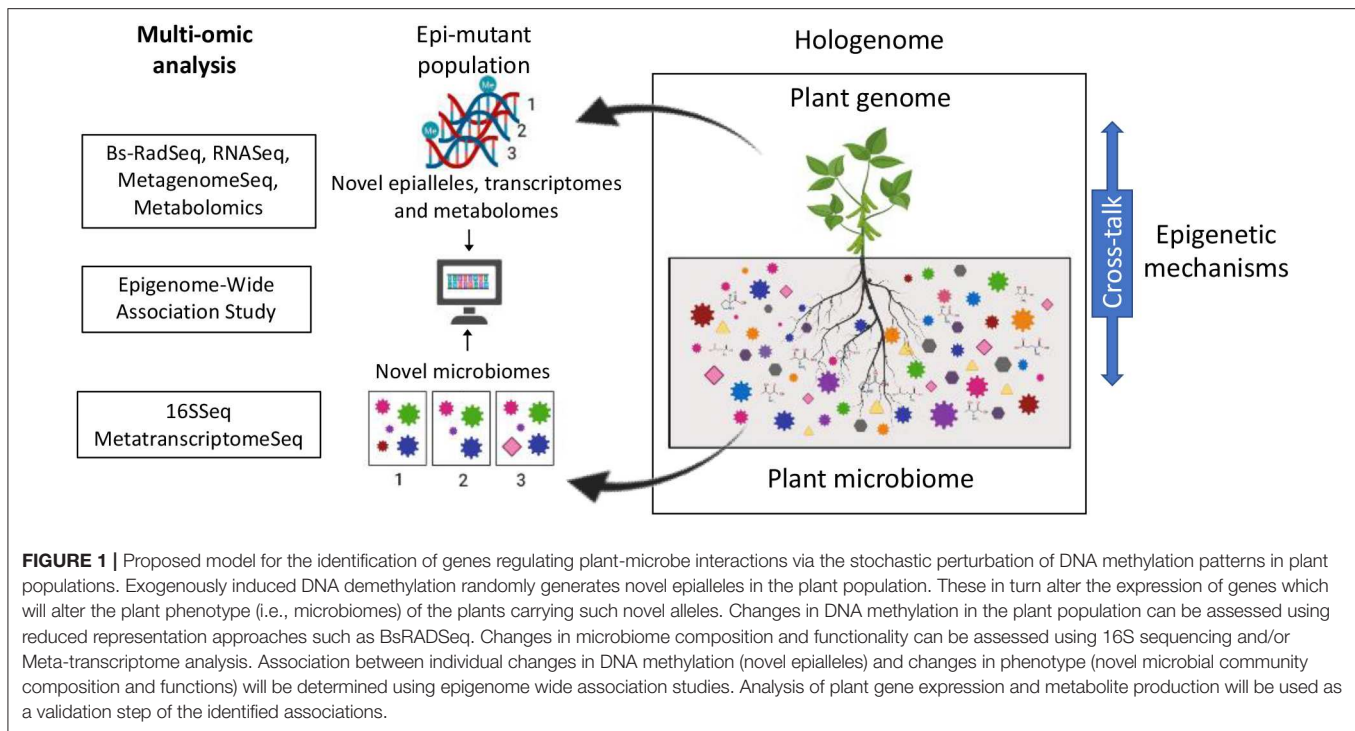
DRIVERS OF SOIL MICROBIOME COMPOSITION

The below-ground microbiota is considered the richer and more functionally active of all the plant's compartments and is consequentially the most intensely studied (Rodríguez et al., 2019). The structural and functional diversity of the plant microbiota fluctuate in response to environmental and host pressures, creating a biological feedback loop (for an extensive review see Vorholt, 2012).

Although abiotic cues, such as soil physical and chemical characteristics, climate, and spatial features, have traditionally considered the main drivers of the plant microbiota composition (Weckert, 2016; Delgado-Baquerizo et al., 2020), it is now well-established that host factors such as genotype (Bulgarelli et al., 2015; Chaluvadi and Bennetzen, 2018), developmental stage (Sugiyama et al., 2014; Wagner et al., 2016) and plant organ (Wagner et al., 2016) contribute to the shaping and maintenance of the plant's microbial communities (Rodríguez et al., 2019).

THE PLANT AS A DRIVER OF ITS OWN MICROBIOTA

The plant itself plays a key role in shaping the composition and relative abundance of microbial species in their rhizosphere through physical (e.g., root architecture) (Chaluvadi and Bennetzen, 2018; Saleem et al., 2018) and chemical mechanisms (i.e., exudation of small molecules that serve as growth substrates or signals for suitable microbial partners, and as antimicrobials or growth deterrents for other microbes) (Bais et al., 2006). Interestingly, the diversity of the microbial community sharply decreases with proximity to the plant



(bulk soil>rhizosphere>endophytic compartment) (Rodriguez et al., 2019). This observed decline in diversity suggests that plants impose a strong selective pressure on their immediate surroundings (Bais et al., 2006).

Historically, the plant's genotype has been recognized as one of the key factors mediating this selectivity. The genotype impacts the microbial community to promote plant growth, improve abiotic stress tolerance, facilitate pathogen defense (Jones et al., 2019). However, Wibowo et al. (2018) showed that genetically identical plants, displaying distinct epigenomes differentially alter their microbiota. Symbionts have been shown to provide beneficial selectable variation to their hosts through the modification of the epigenetic profiles (Gilbert et al., 2010; Gómez-Díaz et al., 2012). Moreover, multiple studies have highlighted the importance of epigenetic mechanisms in regulating the cross-talk between the host and its associated microbiota (Figure 1; Gómez-Díaz et al., 2012; Cheeseman and Weitzman, 2015; Wang et al., 2016; Zhu et al., 2016; Kumar et al., 2018). Enhancing this genome-microbiome communication could be the target for future breeding programs.

EPIMUTAGENESIS: A TOOL TO IDENTIFY GENES REGULATING HOST-MICROBIOME INTERACTIONS

Understanding what makes a plant a good host for its microbiota will be essential to harness the plant-microbiota complex for crop improvement. Identifying the genes that enable plants to regulate the assembly of a beneficial root microbiota is paramount for future breeding programs aimed at a sustainably improving productivity and produce quality of produce. Although, very

little is known about the molecular mechanisms regulating the assembly of plant microbiota, multiple studies have pointed at the importance of epigenetic mechanisms regulating the cross-talk between the host and its associated microbiota. We propose that capitalizing on the availability of epimutant populations as a platform for the identification of loci involved in the regulation of plant microbiota assemblies using epigenome wide association studies (EWAS) (Flanagan, 2015; Birney et al., 2016; Jullian Fabres et al., 2017; Figure 1). The rationale behind the use of an EWAS approach resides on the stochastic nature of the epimutations induced by the application of exogenous demethylating agents. This approach would generate a unique set of epialleles in each plant within the epimutant population, which could partially alter the plant's ability to direct the assembly of its microbiota. Identifying the genes regulating host/microbe interactions will provide with valuable targets for breeding aiming at producing crops capable of assembling healthier microbiotas. This in turn, has the potential to aid global efforts in addressing the challenge of feeding a growing population via the development of socially and environmentally responsible agricultural approaches (Mehrabi et al., 2018).

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

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Effects of Different Vegetable Rotations on Fungal Community Structure in Continuous Tomato Cropping Matrix in Greenhouse

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Intensive greenhouse vegetable cultivation aggravates continuous cropping, resulting in the disturbance of the microbial community structure and the diversity of the soil matrix. In this study, we evaluated the diversity of the rhizosphere matrix fungi in rotation and continuous cropping systems by using high-throughput sequencing analysis of substrates under 6-years of continuous tomato cropping and rotation with cabbage, bean, or celery in greenhouse pots. The results showed that fungal richness in the Chinese cabbage rotation treatment (B) was significantly lower than that of other treatments, and fungal diversities of treatment B and the bean rotation treatment (D) were significantly lower than that of continuous tomato cropping (CK). Contrastingly, the celery rotation treatment (Q) increased the fungal diversity and richness. Furthermore, a principal coordinate analysis showed that the fungal soil community structure of each rotation treatment was different from that of CK. The relative abundances of several harmful fungi (such as *Pseudogymnoascus*, *Gibberella*, and *Pyrenochaeta*) in control CK were significantly higher than those in rotation treatments. In addition, the matrix electrical conductivity, organic matter, total K, and available P in treatments B and D were significantly higher than those in control CK. Moreover, pH and total N of treatment Q were significantly higher than those of control CK. Most fungi were positively correlated with organic matter and available P and negatively correlated with pH. Therefore, rotation with celery could improve the abundance and diversity of fungi in continuous tomato cropping substrates and reduce the relative abundance of harmful fungi. These results indicated that the rotation of celery and tomato could effectively maintain the ecological balance of the substrate microenvironment and provide a more effective way to prevent the problems of continuous tomato cropping in greenhouse.

Keywords: tomato, continuous tomato cropping, crop rotation, organic ecotype soilless culture substrates, fungal community

INTRODUCTION

Continuous cultivation of greenhouse tomatoes lead to soil acidification and salinization, which seriously restricts the sustainable development of vegetable production in sunlight greenhouses (Liu et al., 2014). As a new method of vegetable crop cultivation, nutrient cultivation in a matrix has been gradually developed worldwide (Liu et al., 2009; Maboko et al., 2013). However, the nutrient

matrix might be affected after years of continuous cultivation, and substrates need to be replaced continuously, which would cause a large consumption of human and material resources. For example, Van Assche and Vangheel (1993) found that the substrate of continuous cultivation was easily produced pathogenic bacteria, which caused plant diseases. However, continuous cropping of a single crop often leads to an imbalance in soil microbial communities, which is manifested in the decrease of microbial diversity, increase of pathogenic bacteria, and decrease of beneficial bacteria (Lu et al., 2013; Li et al., 2014; Fu et al., 2017). For instance, several studies have reported that continuous cropping reduced many microbial species and organic matter in the soil, leading to the occurrence of soil-borne diseases (Brussaard et al., 2007; Liang et al., 2011; Han et al., 2016). Soil-borne diseases of plants are a perennial epidemic, usually causing diseases of plant roots and sometimes endangering the whole plant, and these diseases are easily affected by the soil environment and cultivation measures. The pathogens of soil-borne diseases presented in plants include fungi, bacteria, actinomycetes, and nematodes, among which fungi are often dominant; for example, *Fusarium oxysporum* caused Fusarium wilt in Solanaceae. Moreover, *Verticillium* has been demonstrated to be the agent of vascular wilt, and *Pyrenochaetalycopersici* is considered as the vector of root rot. However, little is known about the effects of continuous cropping on microorganisms in the substrate. Therefore, it is of great significance to study the changes of the fungal community in the nutrient matrix after rotation of different vegetables in order to explore the ways to prevent and control continuous cropping obstacles in tomatoes.

In continuous cropping systems, crop rotation can effectively increase crop yields and reduce the incidence and severity of soil-related factors (Tian et al., 2011; Wright et al., 2015). Studies have shown that crop rotation can increase the absorption of nutrients, increase crop yield, and significantly increase the content of organic carbon, total nitrogen, and the total microbial population in the soil (Tian et al., 2009; Tao et al., 2015; Jahan et al., 2016; Lee et al., 2016). To improve soil microbial diversity by rotation may require the use of specific crop combinations, which are expected to have a greater impact on soil microbial diversity. For example, in the substrate of Chinese cabbage–tomato rotations, some studies have found that total microbial biomass and bacterial count increased significantly compared to those of winter fallow substrate, while the number of fungi decreased, and the proportion of fungi to bacteria also decreased (Wei et al., 2012). Moreover, intercropping leek with celery has been reported to have many beneficial effects, such as reducing weeds and pests without hindering cultivation (Baumann et al., 2000, 2001). Additionally, the occurrence of bacterial wilt in tomatoes was largely controlled during tomato–cowpea intercropping (Jing, 1999).

At present, studies on the soil microbial community in continuous cropping systems have mainly focused on bacteria (Zhang et al., 2010; Jun et al., 2014; Zhao et al., 2014), and a large number of bacterial populations have been shown to contribute to antimicrobial disease prevention, plant growth, or systemically induced plant resistance (Garbeva et al., 2004; Weller et al., 2006; Rodrigo et al., 2011). However, studies also have shown that fungi,

as one of the key components of microbial populations, could affect the stability and fertility of soil structure and could also have a wide range of key ecological functions, including decomposition, parasitism, pathogenesis, and symbiosis (Penton et al., 2014). In China's intensive vegetable production system, the effects of long-term continuous cropping on soil quality, especially on genetic and functional diversity of fungi, have not been widely acknowledged in cultivation. As we all know, high-throughput sequencing is a powerful tool for the study of microbial communities and has been widely used in many studies (Tago et al., 2014; Sommermann et al., 2018).

Therefore, the purpose of this study was to use high-throughput sequencing method to study the change trend of fungal diversity and community structure in a continuous cropping substrate after rotation of different vegetables and tomato substrates that had been planted for 6 years and 12 crops. We made two hypotheses: (1) the long-term continuous cropping of tomato would increase the abundance of harmful fungi in the substrate, while the implementation of rotation of different vegetable/tomato would lead to the change of fungal diversity, and the change of fungal community in the substrate is closely related to the physical and chemical properties of the substrate because most of the rhizosphere microorganisms are based on carbon sources in the substrate; and (2) different types of rotation vegetables have different effects on the substrate fungal community, different vegetable roots have different abilities to absorb nutrients, some vegetables contain strong allelochemicals, and the response of the root fungal community to them will also be different. To address these points, we compared the composition of fungal community in the substrate of different vegetables (cabbage, bean, and celery) rotation and tomato continuous cropping, and we analyzed the correlation between the two in combination with the physical and chemical properties of the substrate. Finally, we evaluated which vegetable rotation is better for quality tomato cultivation.

MATERIALS AND METHODS

Experiment Design

The test substrate was an organic ecotype soilless culture substrate (the substrate formula was the mixture of slag: spent mushroom: cow manure: chicken manure: corn straw = 13:5:5:2:14). From June 2012 to June 2018, the continuous cropping experiment was conducted in the solar greenhouse of “Zongzhai non-cultivated land facility agricultural demonstration park” in Zongzhai Town, Suzhou District, Jiuquan City, Gansu Province, China (98° 20' ~ 99° 18' E, 39° 10' ~ 39° 59' N). It is a typical continental climate. The average sea level is 1360 m, and the annual average temperature is 7.3°C with an annual average precipitation of 176 mm and sees annual sunshine hours of 3033–3316 h. The continuous cropping vegetable is tomato (*Lycopersicon Esculentum* Mill.), which was planted twice a year. The overwintering crop is generally raised in September of that year, planted in October, collected in the first 10 days of February of the next year, and pulled in time in May. Summer and autumn crops are generally planted in

time according to the overwintering time. Most of them are planted in June, they are listed in the first 10 days of August, and they are planted in time in October. The experimental tomato variety was “Jingfan 501,” a pink fruit of infinite growth type, with a plant spacing of 45 cm, row spacing of 25 cm, and 30 plants in each plot. After continuous cropping, the substrates contained total K: 11.78 ($\text{g} \cdot \text{kg}^{-1}$), total P: 1.31 ($\text{g} \cdot \text{kg}^{-1}$), total N: 0.51 ($\text{g} \cdot \text{kg}^{-1}$), available P: 82.81 ($\text{mg} \cdot \text{kg}^{-1}$), available K: 63.17 ($\text{mg} \cdot \text{kg}^{-1}$), alkali-hydrolyzable N: 907.67 ($\text{mg} \cdot \text{kg}^{-1}$), EC: 1683.67 ($\mu\text{S} \cdot \text{cm}^{-1}$), and pH: 6.37.

The rotation experiment was conducted in the glass greenhouse of Gansu Agricultural University from August 2018 to March 2019. The rotation vegetables included cabbage (*Brassica pekinensis* Rupr.), bean (*Phaseolus vulgaris* Linn.), and celery (*Apium graveolens* L.). The continuous cropping substrates collected in the continuous cropping experimental site was transported to the greenhouse of Gansu Agricultural University and then put into a 19 cm \times 30 cm pot. The amount of matrix in each pot was 5 kg. The seedlings that had been raised in advance were moved into the basin, and the field management measures of each treatment were consistent with the local conventional management measures.

In the rotation experiment, there were three treatments: cabbage rotation (treatment B), bean rotation (treatment D), and celery rotation (treatment Q). The control was the continuous cropping tomato (control = CK), and each treatment had three repetitions.

Matrix Sampling

The substrate samples were collected after rotation plant seedling pulling (March 2019), and 12 samples were selected for each treatment. After removing the 0–5 cm surface matrix and gently shaking off the matrix around the root system, the matrix adhered to the root surface was brushed off for collection and immediately stored as rhizosphere in an ice box. Subsequently, these samples were divided into two parts, one part was air-dried to determine the physical and chemical properties of the substrate, and the other part was used to extract substrate microbial DNA. There were four treatments in this experiment, and each treatment took three samples for DNA extraction, leaving a total of 12 samples.

Determination of Matrix Physicochemical Properties

Physical and chemical properties of the substrate were determined following Bao's (2000) method. The pH value of the substrate water suspension (1:5) was determined by glass electrode (PHS-3E; Shanghai Jingke, China). The conductivity of the substrate was mixed in the ratio of the substrate (water = 1:5), placed on the oscillator, vibrated for 30 min, and then filtered. The readings are measured by inserting DSJ-308A conductivity meter of Shanghai Jingke (DSJ-308A, Shanghai Jingke, China) into the filtrate. The content of organic matter in the matrix was assessed by the potassium dichromate method. Alkali-soluble N, available P, and available K were determined by the alkali diffusion method, molybdenum blue colorimetric method, and flame photometry, respectively. Total N, total P, and total K were

TABLE 1 | Physicochemical properties of substrates treated with different treatments.

Treatments	pH	EC ($\mu\text{S} \cdot \text{cm}^{-1}$)	Organic matter ($\text{g} \cdot \text{kg}^{-1}$)	Total N ($\text{g} \cdot \text{kg}^{-1}$)	Total P ($\text{g} \cdot \text{kg}^{-1}$)	Total K ($\text{g} \cdot \text{kg}^{-1}$)	Available P ($\text{mg} \cdot \text{kg}^{-1}$)	Available K ($\text{mg} \cdot \text{kg}^{-1}$)	Alkaline N ($\text{mg} \cdot \text{kg}^{-1}$)
B	6.87 \pm 0.04b	1215.33 \pm 6.06a	104.49 \pm 2.19b	1.83 \pm 1.06b	1.3 \pm 0.01a	12.73 \pm 0.23b	73.95 \pm 0.72a	35.5 \pm 0.46c	858.08 \pm 5.34b
D	6.73 \pm 0.06c	957.67 \pm 4.41b	113.59 \pm 2.19a	2.57 \pm 0.23b	1.32 \pm 0.05a	14.5 \pm 0.15a	70.87 \pm 0.14b	46.83 \pm 0.12a	861.47 \pm 2.44b
Q	7.07 \pm 0.01a	413.33 \pm 2.03d	94.44 \pm 1.83c	2.33 \pm 0.23b	1 \pm 0.01b	9.9 \pm 0.06d	66.45 \pm 0.48c	14.47 \pm 0.15d	880.25 \pm 5.05a
CK	6.76 \pm 0.01c	926.33 \pm 1.45c	97.29 \pm 2.81c	6.23 \pm 0.46a	1.26 \pm 0.06a	11.47 \pm 0.12c	65.07 \pm 1.65c	37.47 \pm 0.49b	833.58 \pm 7.45c

EC, electrical conductivity; pH, acidity, and alkalinity; B, D, Q, and CK are defined in Figure 1. Treatments B, D, and Q refer to crop rotation with Chinese cabbage, kidney bean, and celery, respectively. Control CK is continuous cropping with tomato.

TABLE 2 | The richness and diversity index of fungi community in different rotation vegetables and continuous tomato were analyzed and observed.

Treatments	Observed species	Shannon	Simpson	Chao1
B	200.33 ± 21.93c	2.95 ± 0.42c	0.64 ± 0.02b	281.87 ± 26.46b
D	267.67 ± 7.64b	3.47 ± 0.1b	0.70 ± 0.04b	296.55 ± 62.93ab
Q	295 ± 21.93a	4.65 ± 0.25a	0.95 ± 0.02a	366.74 ± 21.58a
CK	282.33 ± 6.81ab	5.06 ± 0.09a	0.90 ± 0.02a	332.85 ± 34.85ab

Values are presented as means ± standard deviation ($n = 3$). Different letters indicate statistically significant differences ($P < 0.05$). B, D, Q, and CK are defined in **Figure 1**.

determined by the Kjeldahl method, molybdenum antimony colorimetric method, and flame spectrophotometer (FP6410, Shanghai, China), respectively.

DNA Extraction

Total DNA of substrate microorganisms was extracted from 0.5 g mixed soil samples using an EZNA® Soil DNA Kit (OMEGA, Bio-Tek, Norcross, GA, United States) according to the manufacturer's protocols. Each composite substrate sample was extracted in triplicate, and the extracted DNA solutions were pooled.

Quantitative PCR and Illumina MiSeq Sequencing

The purified DNA was used as a template, and the fungal ITS2 region PCR amplification was carried out using primers fITS7 (5'-GTGARTCATCGAATCTTG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Bokulich et al., 2013). The 25 μ L reaction system consisted of 12.5 μ L Phusion Hot Start Flex 2 \times Master Mix, 2.5 μ L forward primer, 2.5 μ L reverse primer, 50 ng template DNA, and dd H₂O added up to volume. The PCR reaction was carried out on an ABI GeneAmp® Model 9700 (Applied Biosystems, Foster City, CA, United States). The amplification conditions involved pre-denaturation at 98°C for 30 s, denaturation at 98°C for 10 s, annealing at 54°C for 30 s, extension at 72°C for 45 s, for a total of 35 cycles, and a final extension at 72°C for 10 min. Each sample was prepared in three replicates and the final PCR amplification product was detected by 1% agarose gel electrophoresis and purified by using an AxyPrep DNA Gel Extraction Kit (AxyGenBioSciStates, Union City, CA, United States) using quantitative fluorescence. The purified PCR products were quantified by Quant-iT PicoGreen dsDNA Assay Kit on Promega QuantiFluor fluorescence quantitative system. The qualified library concentration should be more than 2 nM. After diluting the qualified on-line sequencing libraries (the index sequence is not repeatable), they were mixed according to the required sequencing amount according to the corresponding proportion, and transformed into a single chain by NaOH for on-line sequencing; using the MiSeq sequencer for 2 \times 250 bp double terminal sequencing, the corresponding reagent is MiSeq Reagent Kit v2500 cycles.

Processing of Sequenced Data

The original data obtained by sequencing was an image file. After base calling, the resulting file was saved in a fastq format. QIIME (version 1.17; Caporaso et al., 2010) was used to filter the quality of the fastq file. PEAR (version 0.9.6; Zhang et al., 2014) and

Vsearch (version 2.3.4; Rognes et al., 2016) software were used to splice the two terminal sequences of the original data and filter the chimeric sequence. By using a Vsearch (version 2.3.4; Rognes et al., 2016) algorithm, the sequences with a similarity greater than 97% were clustered, and then the OTU representative sequences obtained by clustering analysis were compared with the RDP database (version 11.5; Cole et al., 2009, 2014) and UNITE database (version 7.2; Nilsson et al., 2019) to get the species annotation results of all OTUs.

Statistical Analysis

Statistical analyses of data were performed using the R packages Stats and Vegan (version 2.3-5; Oksanen et al., 2016). Alpha-diversity indices (Shannon index, Simpson index, Chao1 index, and a number of observed species) were calculated using QIIME (alpha_diversity.py). For β -diversity analysis, a cluster analysis (sample clustering by Bray-Curtis distance) was used to show the similarity between samples, and weighted UniFrac distance measurement (based on system development structure) is used to generate PCoA map to further evaluate the similarity between community members of the samples (Lozupone et al., 2006). According to the sample species abundance table, a Kruskal Wallis non-parametric test and Dunet t test were used to judge whether there was significant difference between different groups, and multiple comparison corrections were done with Benjamini-Hochberg FDR. Generally, $P < 0.05$ was considered as the significant difference (R Core Team, 2017). A redundancy analysis (RDA) was performed by the RDA function in the "vegan" package in "R" (version 2.1.3), which was used to study the effect of physical and chemical properties of rhizosphere matrix on the composition of rhizosphere matrix fungal community.

One-way analysis of variance (ANOVA) and Least Significant Difference (LSD) were applied to evaluate the effects of different tillage methods on physical and chemical properties of substrate.

RESULTS

Effects of Rotating Different Vegetables on Physicochemical Properties of Tomato Continuous Cropping Substrate

After the rotation of different vegetables, the substrate pH and alkaline-N of treatments B and Q were significantly higher than those of control CK (**Table 1**). Compared to those of control CK, substrate pH and alkaline-N increased by 1.63 and 2.93%, respectively, in treatment B and by 4.59 and 5.6%, respectively,

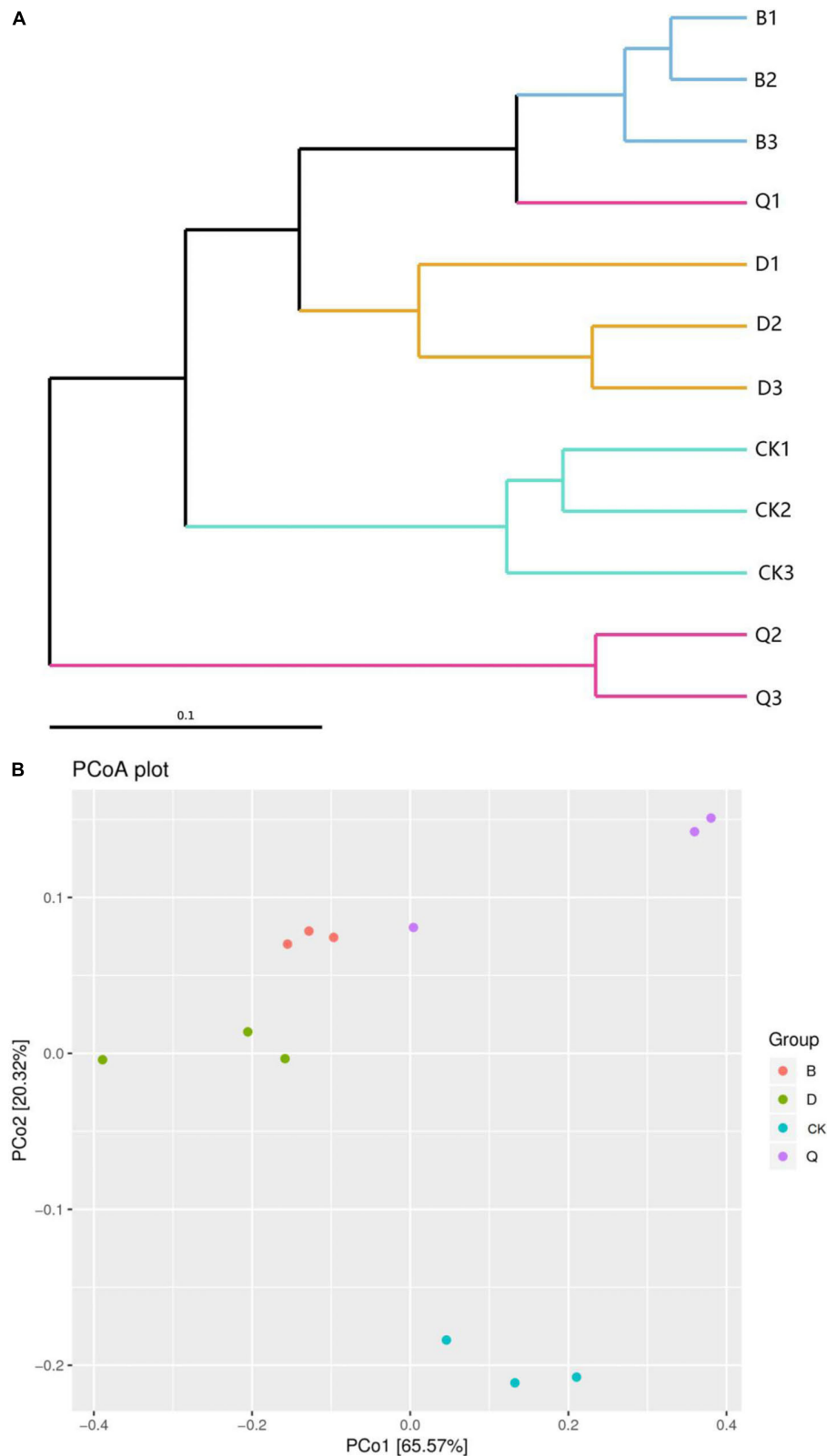
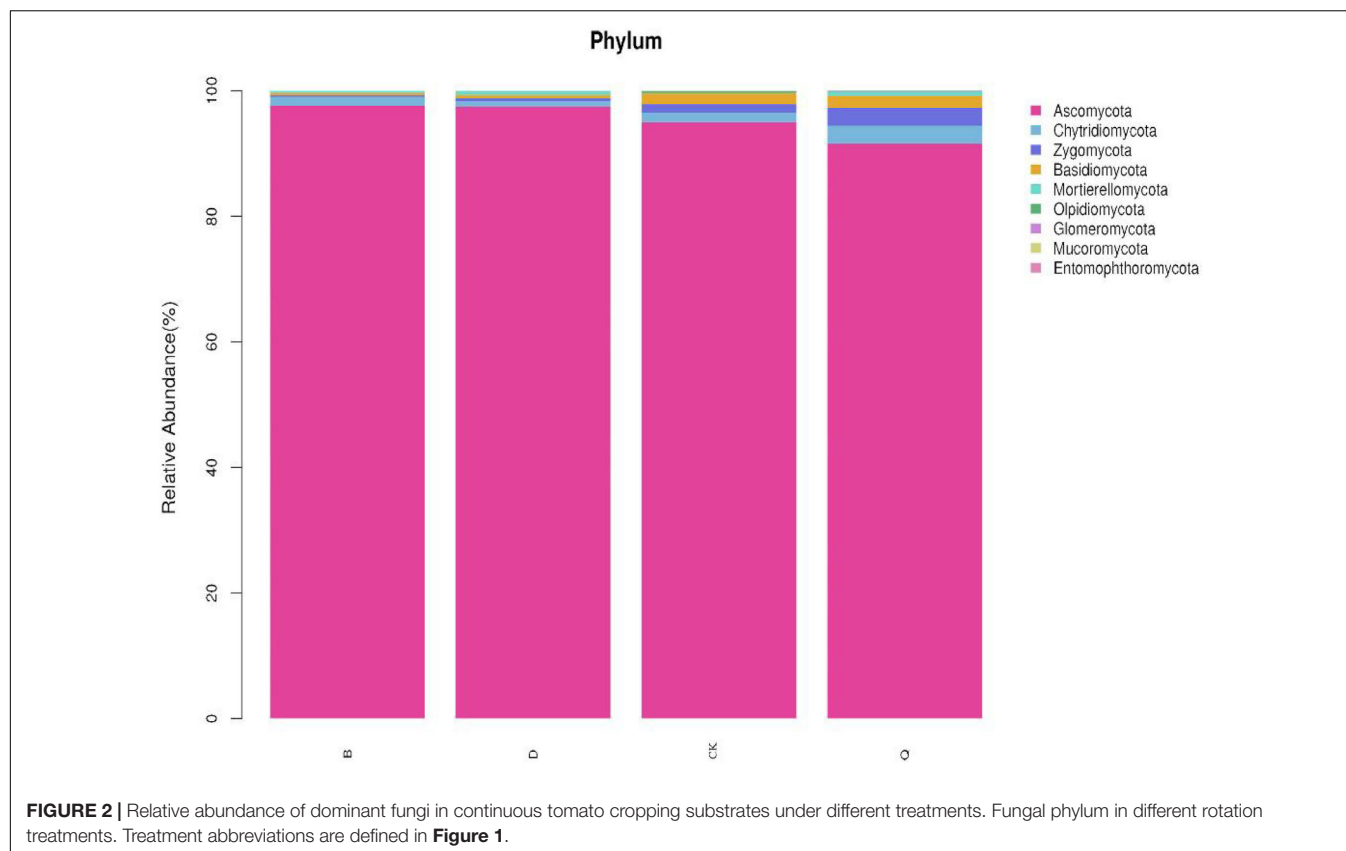


FIGURE 1 | Hierarchical clustering **(A)** and principal coordinates analysis **(B)** of fungal communities in tomato cropping substrate based on different treatments. Treatments B, D, and Q refer to crop rotation with Chinese cabbage, kidney bean, and celery, respectively. Control CK is continuous cropping with tomato.



in treatment Q. The EC, organic matter, total K, and available P of the B and D treatment were significantly higher than those of control CK. In treatment B, EC, organic matter, total K, and available P increased by 31.2, 7.4, 10.99, 13.65%, respectively, while in treatment D they increased by 3.38, 16.75, 26.42, and 8.91%, respectively, compared with the control (**Table 1**). In treatment Q, EC, total P, total K, and available K were significantly lower than those in treatment F, by 55.38, 20.63, 13.69, and 61.38%, respectively.

Effects of Different Vegetable Rotations on the Diversity of Fungi in Tomato Continuous Cropping Substrate

Fungal α -Diversity

Substrate sample abundance (observed species and Chao1 index) and diversity (Shannon and Simpson indices) are shown in **Table 2**. The Chao1 index and observed species mainly reflect the number of OTUs in the sample. Treatment B had the lowest observed species and Chao1 index, while treatment Q had the highest values. These results indicate that the fungi in treatment B had the lowest abundance. Fungi in treatments Q and control CK had the highest and second highest abundances, respectively. Shannon and Simpson indices also reflect the number of species in the sample and the average or uniformity of species abundance in the sample. Treatments B and D had significantly lower Shannon and Simpson indices compared with control CK (**Table 2**).

Fungal β -Diversity

Hierarchical clustering was used to analyze β -diversity of fungal communities in the continuous tomato cropping system (**Figure 1A**). The hierarchical clustering resolved the fungal community into four clusters, one composed of group B, one of group D, one of group Q, and one of CK. Groups B and D were clustered together and separate from the control CK. In addition, fungal community from treatment Q differed the most from those in treatments B, D, and control CK.

The Unifrac weighted PCoA based on OTUs also clearly showed the differences among different vegetable rotations and continuous tomato cropping samples. PCo1 and PCo2 explained 65.57 and 20.32% of the total variability of fungal data, respectively. The fungal community in the sample series of continuous tomato cropping (CK) was clearly separated from the other nine samples by PCo2. The fungal community members of the cabbage (B) and the bean (D) rotation matrix samples were the most similar (**Figure 1B**). The unweighted unifrac algorithm showed similar results, but, for the sake of clarity, only the weighted Unifrac-PCoA plot is shown here.

Effects of Different Vegetable Rotations on Fungal Community Composition in Tomato Continuous Cropping Substrate

There were differences in fungal community composition among treatments. At the phylum level, the dominant fungal phyla for treatments B and D were *Ascomycota*, with 97.62 and 97.47%

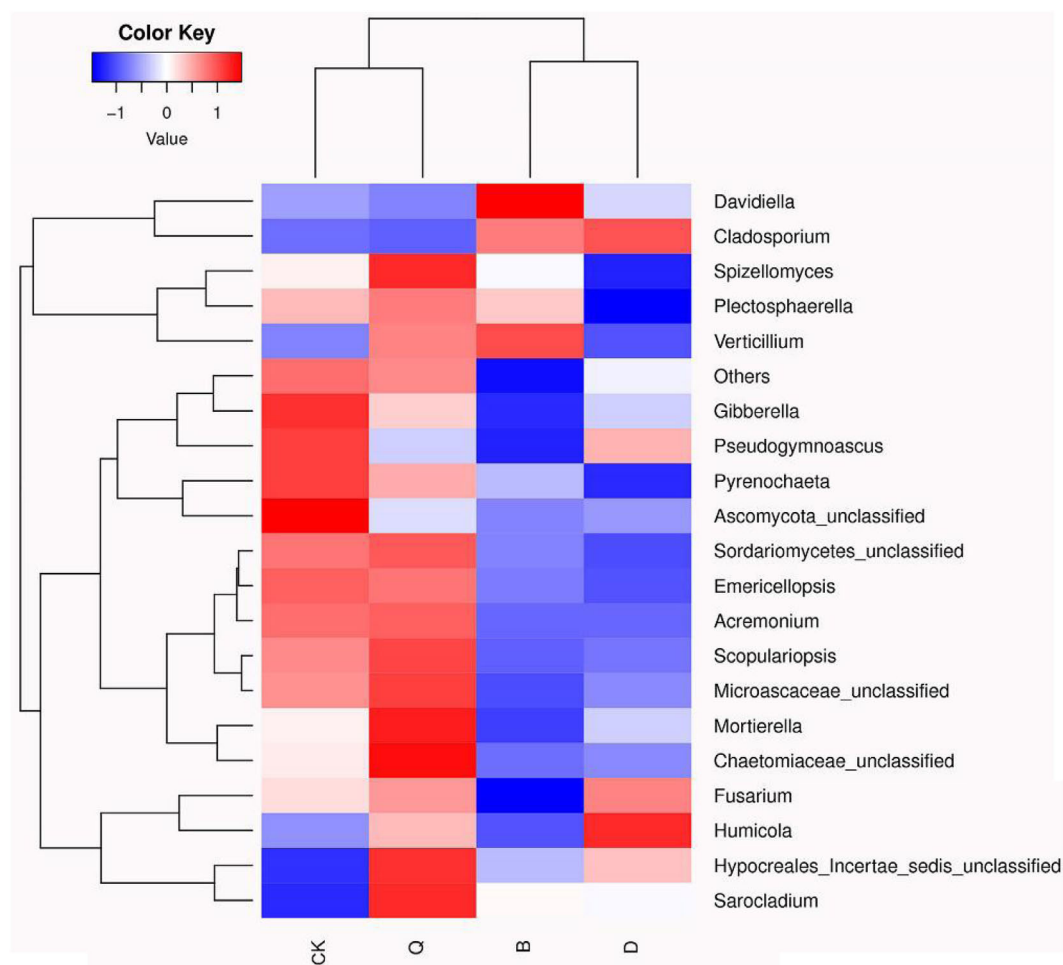


FIGURE 3 | Heatmap analysis of fungal communities in continuous tomato cropping substrates under different treatments. The color of relative abundance of the community changed from blue to red, indicating that the relative abundance of the community changed from low to high. Treatment abbreviations are defined in Figure 1.

relative abundance, respectively, and *Chytridiomycota*, with 1.41 and 0.91% relative abundance, respectively (Figure 2). The dominant fungal phyla in treatments Q and control CK were *Ascomycota*, *Chytridiomycota*, *Zygomycota*, and *Basidiomycota*, with relative abundances of 95.00, 1.55, 1.34, and 1.69%, respectively, in treatment Q, and 91.58, 2.87, 2.86, and 1.89%, respectively, in control CK. Compared with control CK, *Ascomycota* relative abundance in treatments B and D increased by 2.76 and 2.6%, respectively, but it decreased by 3.6% in treatment Q. Compared with control CK, *Chytridiomycota*, *Zygomycota*, and *Basidiomycota* relative abundances decreased by 9.03, 82.09, 78.11%, respectively, in treatment B, and by 41.29, 64.93, and 72.19%, respectively, in treatment D. Contrastingly, *Chytridiomycota*, *Zygomycota*, and *Basidiomycota* in treatment Q increased by 85.16, 113.43, and 11.83%, respectively, compared with control CK.

The fungal community relative abundance was further identified by heatmap analysis. According to the similarity

comparison, the colonial structure of fungi in all treatments was divided into four groups (Figure 3). Treatments B and D were clustered into one group, and Q and CK were clustered into the other, indicating that there were significant differences in the community structure among the communities. The abundances of *Gibberella*, *Pseudogymnoascus*, *Pyrenochaeta*, and *Ascomycota_unclassified* were significantly higher in control CK than in treatment B, D, and Q. However, in treatment B, *Davidiella*, *Cladosporium*, and *Verticillium* abundances were higher than those in control CK. In treatment D, *Cladosporium* and *Humicola* abundances were higher than those in control CK.

As shown in Table 3, 19 fungi with significant difference between continuous cropping of tomato and continuous cropping of cabbage, bean and celery were selected for *t*-test ($P < 0.05$) (Supplementary Tables S1, S2). The abundance of *Cladosporium*, *Verticillium*, *Sarocladium*, *Davidella*, and *Hypocreales-incertae-sedis-unclassified* was

significantly higher than CK, while that of *Pseudogymnoascus*, *Microascaeae-unclassified*, *Gibberella*, *Chaetomiaceae-unclassified*, *Ascomycota-unclassified*, *Pyrenochaeta*, *Acremonium*, *Fusarium*, *Emericellopsis*, *Sordariomycetes*, and *Scopulariopsis* was significantly lower than CK. In D treatment, the abundance

TABLE 3 | Comparison of fungal genus in different vegetable rotations and continuous tomato cropping substrates (t-test).

Genus	log ₂ FC B/CK	p-value (*p < 0.05)
<i>Cladosporium</i>	1.17	0.0495*
<i>Pseudogymnoascus</i>	-7.36	0.0495*
<i>Verticillium</i>	5.52	0.0495*
<i>Microascaeae_unclassified</i>	-1.28	0.0495*
<i>Gibberella</i>	-2.05	0.0495*
<i>Chaetomiaceae_unclassified</i>	-0.64	0.0495*
<i>Ascomycota_unclassified</i>	-3.36	0.0495*
<i>Pyrenochaeta</i>	-2.58	0.0495*
<i>Acremonium</i>	-0.74	0.0495*
<i>Sarocladium</i>	1.09	0.0495*
<i>Fusarium</i>	-2.21	0.0495*
<i>Davidiella</i>	0.88	0.0495*
<i>Emericellopsis</i>	-1.31	0.0495*
<i>Sordariomycetes_unclassified</i>	-1.84	0.0495*
<i>Scopulariopsis</i>	-2.09	0.0495*
<i>Hypocreales_Incertae_sedis_unclassified</i>	1.36	0.0495*

Genus	log ₂ FC D/CK	p-value (*p < 0.05)
<i>Cladosporium</i>	1.34	0.0495*
<i>Plectosphaerella</i>	-3.08	0.0495*
<i>Pseudogymnoascus</i>	-2.01	0.0495*
<i>Microascaeae_unclassified</i>	-1.02	0.0495*
<i>Gibberella</i>	-1.24	0.0495*
<i>Ascomycota_unclassified</i>	-3.16	0.0495*
<i>Humicola</i>	5.55	0.0495*
<i>Pyrenochaeta</i>	-4.04	0.0495*
<i>Emericellopsis</i>	-1.48	0.0495*
<i>Sordariomycetes_unclassified</i>	-2.27	0.0495*
<i>Scopulariopsis</i>	-1.94	0.0495*
<i>Hypocreales_Incertae_sedis_unclassified</i>	2.61	0.0495*

Genus	log ₂ FC Q/CK	p-value (*p < 0.05)
<i>Pseudogymnoascus</i>	-4.33	0.0495*
<i>Gibberella</i>	-0.77	0.0495*
<i>Ascomycota_unclassified</i>	-2.57	0.0495*
<i>Mortierella</i>	1.34	0.0495*
<i>Humicola</i>	3.12	0.0495*
<i>Pyrenochaeta</i>	-1.08	0.0495*
<i>Sarocladium</i>	2.19	0.0495*
<i>Hypocreales_Incertae_sedis_unclassified</i>	4	0.0495*

*Significant at the 0.05 probability level. B, D, Q, and CK are defined in Figure 1. FC in log₂ FC is fold change, which represents the ratio of the expression amount between two samples (groups). After taking the logarithm based on 2, it is log₂ FC.

of *Cladosporium*, *Humicola*, and *Hypocreales-incertae-sedis-unclassified* was significantly higher than CK, and the abundance of *Plectosphaerella*, *Pseudonymnosacus*, *Microascaeae-unclassified*, *Gibberella*, *Ascomycota-unclassified*, *Pyrenochaeta*, *Emericellopsis*, *Sordariomycetes-unclassified*, and *Scoreriopsis* was significantly lower than CK. In Q treatment, the abundance of *Mortierella*, *Humicola*, *Sarocladium*, and *Hypocreales-incertae-sedis-unclassified* was significantly higher than CK, while that of *Pseudonymnoascus*, *Gibberella*, *Ascomycota-unclassified*, and *Pyrenochaeta* was significantly lower than CK.

Effects of Environmental Factors on Fungal Community Distribution in Continuous Tomato Cropping Substrate

RDA was used to analyze the relationship between the structure (relative abundance) of 10 fungal communities and the physicochemical properties of their substrate matrices (Figure 4). The fungal community in each treatment matrix was classified by two axes, which explained 82.47% of the total variability. Abundant fungal genera in control CK were positively correlated with organic matter, alkali-soluble N, and total K and negatively correlated with total N and pH. Contrastingly, those in treatment Q were positively correlated with EC, total P, available P, and available K, while those in treatment B were positively correlated with total N, and those in D treatment were positively correlated with total N and pH.

DISCUSSION

With increasing years of continuous cropping, the soil organic matter, total N, available P, available K, and alkali-soluble N contents all showed a continuous downward trend (Li et al., 2017), while crop rotation or intercropping can effectively alleviate the soil nutrient decline and imbalance caused by single crop continuous cropping (Latif et al., 1992; Long et al., 1999). Our results showed that substrate EC, organic matter, total K, and available P were significantly higher after rotation with cabbage and kidney bean than in continuous tomato cropping (Table 1), which indicated that the rotation of Chinese cabbage or bean could change the physical and chemical properties of the substrate of continuous cropping of tomato and significantly improve the nutrient supply of the substrate of continuous cropping of tomato. In the study of Costa and Crusciol (2016), it was also proven that rotation could increase the input of organic carbon in soil. But the substrate pH and alkali-N of rotation celery were significantly higher than that of tomato continuous cropping (Table 1). Some researchers indicated that the proportion of fungi in the soil would decrease with the decrease in soil pH in boreal forest acid soil (Högberg et al., 2003) or alkaline soil (Alfaro et al., 2017), which indicated that rotation celery could increase the proportion of fungi in the continuous cropping substrate.

Crop continuous cropping can alter the structure of fungal community structure in soil (Xiong et al., 2015; Liu et al., 2019). In our research results, the PCoA of the matrix fungal community structure of the continuous tomato cropping that

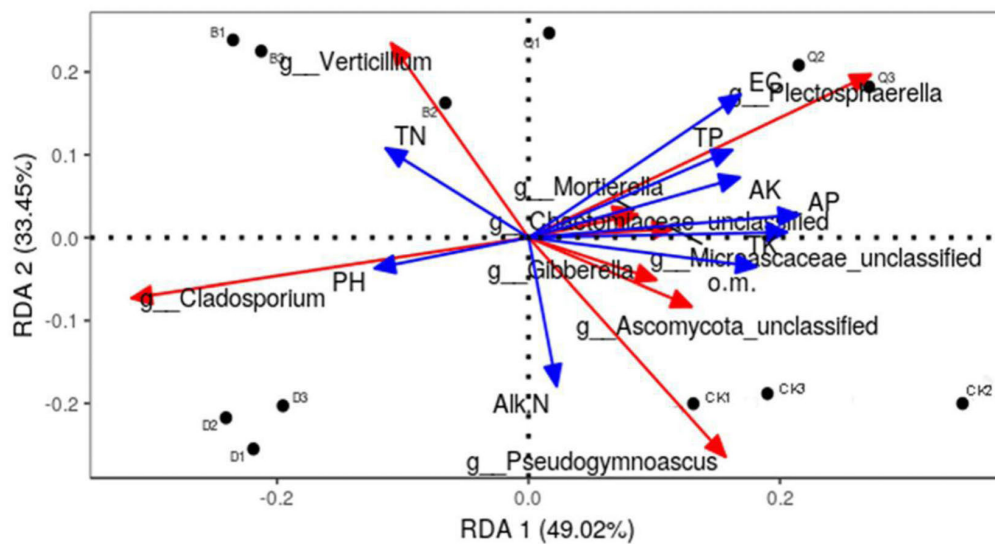


FIGURE 4 | Effect of environmental factors on the distribution of fungal communities in continuous tomato cropping substrates. Dots represent matrix samples; blue arrows represent the physical and chemical properties of the matrix; and red arrows represent the matrix microorganisms. The angle between the influencing factors (between the factors and the microorganisms) indicate a positive correlation between two factors (acute angle) and a negative correlation (obtuse angle), and the arrow length represents the strength of the effect for each factor. EC, matrix conductivity; pH, matrix pH; o.m., matrix organic matter; TN, total nitrogen; TP, total phosphorus; TK, total potassium; AP, available phosphorus; AK, available potassium; Alk N, alkali nitrogen. Treatment abbreviations are defined in **Figure 1**.

was subjected to vegetable rotation identified a clear separation of the communities (**Figure 1B**), which was also confirmed by the hierarchical clustering (**Figure 1A**). That is to say, the use of different cultivation techniques can also cause significant differences in fungal genetic communities in the soil, leading to characteristic changes in fungal community structure (Lupwayi et al., 1998; Brussaard et al., 2007; Ghimire et al., 2014). Rotation of different vegetables can have different effects on the richness and diversity of fungi in continuous cropping matrix. Rotation of celery could improve the richness and diversity of fungi in continuous cropping matrix, while rotation of cabbage and bean can reduce the richness and diversity of fungi in continuous cropping matrix (**Table 2**). Celery is a kind of vegetable with strong allelochemicals. Rotation with crops with stronger allelochemicals can effectively improve the richness and diversity of soil fungi (Ding et al., 2018). It has also been shown that continuous cropping can increase the fungal richness of crop rhizosphere soil (Meng et al., 2012; Zhou and Wu, 2012). Rotation can effectively reduce the amount of culturable fungi in soil (Wei et al., 2012). In our study, rotation cabbage and bean treatment reduced the richness and diversity of fungi in tomato continuous cropping matrix (**Table 2**). This may due to the different root exudates of rotation soil, which can reduce the self-toxicity of tomatoes, inhibit the propagation of pathogenic bacteria, and improve the microecological environment of soil (Alvey et al., 2003; Zhu and Fox, 2003). It is an important discovery that rotation of different vegetables will have opposite effect on the diversity and richness of fungi in continuous cropping matrix.

Fungi can decompose soil organic matter and play an important role in the terrestrial ecosystem (Abed et al.,

2013; Peay et al., 2013; Acosta-Martínez et al., 2014). Among them, *Chytridiomycota*, *Zygomycota*, and *Basidiomycota* are the main media of organic matter decomposition in most terrestrial ecosystems (James et al., 2006; Richardson, 2009). Compared with tomato continuous cropping (CK), rotation celery (Q) increased the relative abundance of *Chytridiomycota*, *Zygomycota*, and *Basidiomycota* (**Figure 2**). Qin et al. (2017) also found that the relative abundance of *Zygomycota* and *Basidiomycota* in potato continuous cropping soil under ridge and furrow mulching cultivation mode was higher than that under flat ridge mulching cultivation mode. This indicated that rotation celery could increase the relative abundance of beneficial fungi in tomato continuous cropping substrates.

In addition, as shown in the fungal community heatmap, *Gibberella* and *Pyrenochaeta* abundances in cabbage, kidney bean, and celery rotations were significantly reduced compared with those of continuous tomato cropping (**Figure 3**). *Gibberella* is a well-known pathogen associated with diseases of several crops including rice, sugar cane, and corn (Marasas et al., 2006; Mohd et al., 2008; Hsuan et al., 2011). It has been reported that sugarcane-soybean intercropping can also reduce the relative abundance of *Gibberella* in soil (Lian et al., 2018). Furthermore, *Pyrenochaetalycopersici* is a soil-borne fungus that causes root rot of tomato wood. After infection with pathogens, tomato roots form lesions and wrinkles, and finally form a cork structure (Jones et al., 1989). With increasing continuous tomato planting time, pathogens accumulate in the soil and the incidence of *Pyrenochaetalycopersici* is multiplied (Last et al., 2010). The use of biological control, such as treatment of tomato plants with *Trichoderma viride* 18/17 SS, *Streptomyces* spp. AtB42, and

Bacillus subtilis M51 PI, can effectively prevent the growth of *Pyrenochaetalycopersici* (Fiume and Fiume, 2008). The rotation method used in the present study alleviated the soil-borne disease caused by continuous tomato cropping. We found that rotation of cabbage, kidney beans, and celery can significantly reduce *Pyrenochaeta* abundance in continuous tomato cropping substrate (Table 3).

Rotation is a good practice to protect vegetable production. By changing the soil microenvironment, it can effectively improve soil physical and chemical properties, regulate soil fertility, and increase crop yield (Jahan et al., 2016; Lahouar et al., 2016). Several studies have shown that environmental factors shape fungal communities and structures (Liu et al., 2015; Lahouar et al., 2016). In the present study, we used RDA plots to show the interaction between physicochemical properties of the substrate and 10 fungal communities of interest. The most abundant taxa were found to be strongly negatively correlated with matrix pH and total N (Figure 4). Our findings are consistent with other studies, showing that soil pH is a very important factor for building microbial communities (Rousk et al., 2010; Jun et al., 2014; Ding et al., 2018). The EC value of substrate not only affects the growth of rhizosphere, but also affects the development of fungi. Our study shows that the EC of the substrate after planting celery is 413.33 $\mu\text{S}/\text{cm}$ (Table 1), and the study of Li et al. (2010) also showed that celery can grow normally within the EC Value of 200–400 $\mu\text{S}/\text{cm}$. In our study, the EC of substrate was negatively correlated with the richest taxa (Figure 4), which is consistent with previous research results (Rousk et al., 2010; Zhao et al., 2014; Ding et al., 2018). In contrast, the most abundant taxa were positively correlated with the matrices total P, total K, available P, available K, organic matter, and EC (Figure 4). Our findings are consistent with previous studies showing that soil physicochemical properties have a strong impact on soil microbial communities and structures (Rousk et al., 2010; Fernández-Calviño and Bååth, 2016; Hietala et al., 2016).

CONCLUSION

In this experiment, the effects of Chinese cabbage, kidney bean, and celery rotation on the physical and chemical properties, fungal diversity, and community structure of continuous tomato cropping rhizosphere were studied. Rotating different vegetables improved the physical and chemical properties of the substrate under greenhouse continuous tomato cropping. Rotating cabbage and kidney bean reduced the diversity and

abundance of fungi, which changed the fungal community structure of the substrate, while celery rotation increased fungal diversity and abundance of continuous cropping and also reduced the relative abundance of harmful fungal genera. Therefore, from the perspective of maintaining the balance of the substrate microbial ecological environment, we believe that the rotation of celery can better solve the problems arising from continuous tomato cropping.

DATA AVAILABILITY STATEMENT

The datasets (SRP253823) for this study can be found in the NCBI Sequence Read Archive (<https://identifiers.org/ncbi/insdc.sra:SRP253823>).

AUTHOR CONTRIBUTIONS

JY and JL designed the work. LJ, NJ, and LN performed the work. LJ analyzed the data. JX, XX, LH, ZT, and YW revised the manuscript. All authors approved the final version of this 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/fmicb.2020.00829/full#supplementary-material>

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Rhizosphere Soil Fungal Communities of Aluminum-Tolerant and -Sensitive Soybean Genotypes Respond Differently to Aluminum Stress in an Acid Soil

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Different soybean genotypes can differ in their tolerance toward aluminum stress depending on their rhizosphere-inhabiting microorganisms. However, there is limited understanding of the response of fungal communities to different aluminum concentrations across different genotypes. Here, we used metabarcoding of fungal ribosomal markers to assess the effects of aluminum stress on the rhizosphere fungal community of aluminum-tolerant and aluminum-sensitive soybean genotypes. Shifts in fungal community structure were related to changes in plant biomass, fungal abundance and soil chemical properties. Aluminum stress increased the difference in fungal community structure between tolerant and sensitive genotypes. *Penicillium*, *Cladosporium* and *Talaromyces* increased with increasing aluminum concentration. These taxa associated with the aluminum-tolerant genotypes were enriched at the highest aluminum concentration. Moreover, complexity of the co-occurrence network associated with the tolerant genotypes increased at the highest aluminum concentration. Collectively, increasing aluminum concentrations magnified the differences in fungal community structure between the two studied tolerant and sensitive soybean genotypes. This study highlights the possibility to focus on rhizosphere fungal communities as potential breeding target to produce crops that are more tolerant toward heavy metal stress or toxicity in general.

Keywords: rhizosphere fungal community, aluminum toxicity, soybean genotypes, metabarcoding, network

INTRODUCTION

Aluminum (Al) toxicity is one of the most widespread problem in acidic soils, affecting approximately 40% of the arable land worldwide (Ma et al., 2001; Pierluigi et al., 2008). In acidic soils with pH values below five, insoluble forms of Al are turned into soluble Al^{3+} ions (Kinraide, 1991; Da Mota et al., 2008). Many studies have reported that Al^{3+} with high phytotoxicity causes

inhibition of nitrate reductase activity and disruption of nitrogen reduction and assimilation (Zhao and Shen, 2018). Moreover, the absorption and utilization of other soil elements such as phosphorus, potassium and iron by plant roots are also affected by Al stress (Pfeffer et al., 1986; Delhaize and Ryan, 1995; Pineros and Kochian, 2001). Therefore, increased concentration of soluble Al can lead to inhibition of plant root growth and, thus, reduction in crop yield (Kochian, 1995; Kong et al., 1997; Exley, 2012; Riaz et al., 2018).

There have been several reports on possible mechanisms of plants to increase tolerance toward high Al^{3+} concentrations, among which the chelation of Al^{3+} through organic acids such as malic acid, oxalic acid, or citric acid excreted by plant roots is considered to be one of the vital mechanisms (Ma et al., 2001). The different levels of Al tolerance vary significantly among genotypes, largely because of different types and quantities of secreted organic acids (Miyasaka et al., 1991; Wu et al., 2018). For instance, more organic acids are excreted by Al-tolerant (Al-T) soybean genotypes when compared with Al-sensitive (Al-S) genotypes, leading to chelation of more Al^{3+} (Yang et al., 2010). However, the increased amounts of organic acids not only regulate Al^{3+} concentrations in soil (Silva et al., 2004), but also shapes the microbial community composition at the root-soil interface through providing nutrients (Jones et al., 1996; Bürgmann et al., 2005).

Microorganisms greatly contribute to plant health and productivity (Mendes et al., 2013; Li et al., 2014a,b). When subjected to environmental stress, plants have the potential to recruit specific microbes to the root system to alleviate the stress (Rodriguez et al., 2019). For example, some plant-growth-promoting bacteria (PGPB) in the soil such as *Klebsiella*, *Serratia*, and *Enterobacter* have the capacity to form Al^{3+} -siderophore complexes and improve P-uptake efficiency to cope with Al stress (Mora et al., 2017). Previous studies have investigated the structure of rhizosphere bacterial communities in Al-T and Al-S plants and suggested that Al-T genotypes recruit certain bacterial species that help mitigating Al toxicity (Yang et al., 2012a; Wang et al., 2013; Lian et al., 2019). However, these studies have focused on rhizosphere bacteria as key players, ignoring that fungal species play important roles in nutrient cycling and stress tolerance (Kawai et al., 2000; Peltoniemi et al., 2012). For instance, some fungi, such as *Penicillium* and *Aspergillus* have been shown to improve Al-tolerance by producing organic acids and at the same time provisioning plants root with nitrogen and phosphorus to promote growth and increase vitality (Kiers et al., 2011).

In this study, differences in rhizosphere fungal community structure of cultivated soybean genotypes with different tolerance levels to Al were assessed using high-throughput DNA sequencing of the internal transcribed spacer (ITS) region, and correlated with plant growth and chemical soil properties. Based on the higher adaptability of Al-tolerant soybean genotypes to Al toxicity, we hypothesized that (1) fungal diversity of Al-T genotypes is higher when compared to Al-S genotypes, and (2) the response of fungal community structure to aluminum between Al-T and Al-S soybean genotypes is different, and these differences increase with increasing Al concentration.

MATERIALS AND METHODS

Soil Source and Plant Materials

The soil used in the pot experiment was collected from an agricultural field in Suixi County (110°25'N, 21°32'E), Guangdong Province, China, in June 2017. The chemical properties are provided in **Supplementary Table S1**. The soybean (*Glycine max* L.) cultivars used in this study included the Al-tolerant genotypes HuaChun2 and Lee as well as the Al-sensitive genotypes LiuDou1 and Young (Hanson and Kamprath, 1979; Hanson, 1991; Wen, 2007).

Experimental Description and Rhizosphere Soil Collection

A completely randomized block design was set up in a greenhouse of the South China Agricultural University in Guangzhou, China. Before the experiment, the soil was air-dried and sieved with a 4 mm mesh size. Soybeans were seeded into 2 kg soil per pot (150 mm height × 200 mm top diameter and 150 mm bottom diameter). Six seeds of equal size were planted in each pot and germinating soybeans were subsequently removed to obtain two plants per pot after eight days of growth. Aluminum sulfate $\text{Al}_2(\text{SO}_4)_3$ was used as Al source. Three different Al concentrations ranging from 0 (none) to 0.2 (low) and 0.4 (high) g Al^{3+} kg⁻¹ soil were applied as treatments. Simultaneously, the same levels of Al concentrations were also applied without planting of soybean (no plant controls, CK). Each treatment in the experiment was installed in three replications. The experimental conditions of daytime temperatures ranged from 28 to 32°C, and night time temperatures ranged from 16 to 20°C. Soil moisture was kept at 80% of the moisture level in the field by weighting and watering.

Because Al toxicity is affecting the flowering stages, six rhizosphere soil samples (three replications per cultivar) at 0 (zero), 0.2 (low) and 0.4 (high) g kg⁻¹ Al^{3+} concentrations were collected at 40, 50, and 65 days after planting, which represented the flowering stages at the three different Al concentrations, respectively. All rhizosphere soil samples were collected by gently shaking the plant root to remove loosely attached soil, and then the soil adhering to the root system was transported to an aseptic bag filled with 30 ml of phosphate-buffered saline and processed for molecular microbial community analysis as previously described (Shi et al., 2015). Two grams of rhizosphere soil was taken from each sample, placed in a sterilized microcentrifuge tube and stored at -80°C for DNA extraction. The remaining soil sample was stored at 4°C until measuring the chemical properties.

Soil Properties Measurement

Soil pH was measured in aqueous solution using a FE20-FiveEasyTM pH meter (Mettler Toledo, Columbus, United States). Total carbon (TC) and nitrogen (TN) were determined by a vario MAX CN Elemental Analyser (Elementar Analysensysteme, Hanau, Germany). Total soil potassium (TK) was measured by inductively coupled plasma-atomic emission spectrometry on an ICPS-7500 (Shimadzu, Kyoto, Japan).

(Lian et al., 2019). Total soil phosphorus (TP) was determined by digesting with $\text{H}_2\text{SO}_4\text{-HClO}_4$ as previously described (Huang et al., 2011) and measured by a continuous flow analytical system (Skalar, Breda, Netherlands). The titrimetric method was used to evaluate soil exchangeable H^+ and Al^{3+} (Abreu et al., 2003). Soil nitrate (NO_3^- -N) and ammonium (NH_4^+ -N) were extracted with 2 mol L^{-1} KCl, and then assayed by a continuous flow analytical system (Skalar, Breda, Netherlands) as previously described (Jiang et al., 2017). Soil available phosphorus (AP) was determined by molybdenum-antimony colorimetric method (Sun et al., 2015).

Molecular Genetic Analyses

Rhizosphere soil DNA was extracted using the Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, United States) as specified in the manufacturer's instructions. The internal transcribed spacer region ITS1 of the fungal ribosomal operon was amplified using primer ITS1 (CTTGGTCATTAGAGGAAGTAA; Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC; White et al., 1990) with a unique six nt barcode at the 5' end. PCR amplification for sequencing was carried out in a volume of $30 \mu\text{L}$ with $15 \mu\text{L}$ of Phusion High-Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, United States), $0.2 \mu\text{M}$ of forward and reverse primers, and 10 ng template DNA. PCR reaction cycling conditions were $1 \times (60 \text{ s}, 98^\circ\text{C}), 30 \times (10 \text{ s}, 98^\circ\text{C}; 30 \text{ s}, 50^\circ\text{C}; 60 \text{ s}, 72^\circ\text{C})$, and a final elongation cycle at 72°C for 5 min . Quantitative PCR (qPCR) was done using the same primers and according to the protocol described previously (Yao et al., 2017). Briefly, each PCR reaction contained $10 \mu\text{L}$ of SYBR Premix Ex TaqTM (Takara, Dalian, China), $1.0 \mu\text{L}$ of 10 mM forward and reverse primers, $1.0 \mu\text{L}$ of soil DNA, and $7.0 \mu\text{L}$ of sterilized water. qPCR was performed in an ABI 7900 system following a program that started with initial denaturation at 95°C for 45 s , followed by 32 cycles of 95°C for 15 s , 58°C for 20 s , 72°C for 20 s , and one final cycle of 45°C for 10 min for cooling. Sequencing libraries were generated from PCR products using NEB Next UltraTM DNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA, United States) according to the manufacturer's protocol. Libraries were paired-end sequenced on an Illumina MiSeq platform using $2 \times 300 \text{ bp}$ chemistry (Illumina Inc., San Diego, CA, United States). Raw sequences were deposited in the NCBI short-read archive under the accession numbers PRJNA561469.

Bioinformatic Processing

Raw sequence data were processed using QIIME v1.19.1 (Caporaso et al., 2010). In brief, reads shorter than 200 bp and average quality score below 20 were removed, and paired-end reads were merged into full-length amplicon sequences with FLASH (Magoč and Salzberg, 2011). Potentially chimeric sequences were detected by running the UCHIME algorithm (Edgar et al., 2011). The CD-HIT program was used to cluster OTUs at 97% sequence identity (Li and Godzik, 2006). Taxonomic assignments of OTU representative sequences was

done against the UNITE database¹ (Abarenkov et al., 2010) using the RDP naïve Bayesian classifier (Wang et al., 2007). All the samples were randomly resampled to the same sequence depth ($29,169$ sequences per sample), in order to reduce the influence of sequencing depth on treatment effects. In addition, we filtered the OTU table to remove rare OTUs (less than 0.001% abundance), sparse OTUs (OTUs that did not occur in at least three samples), and outlier OTUs defined as OTUs that showed a ratio of more than $10:1$ from the highest to the second highest value.

Statistics Analyses

Alpha-diversity metrics, i.e., Chao1 richness estimator and Shannon diversity index, were calculated with the QIIME software. Non-metric multidimensional scaling (NMDS; Kruskal, 1964) based on Bray-Curtis dissimilarities, non-parametric multivariate analysis of variance (PERMANOVA; Anderson, 2001), canonical correspondence analysis (CCA; Braak, 1986), and Mantel test (Mantel, 1967) were conducted in R (R Development Core Team, 2006) using the functions *metaMDS*, *adonis*, *cca*, and *mantel* of the “vegan” package (Oksanen et al., 2013). Canonical analysis of principal coordinates (CAP; Anderson and Willis, 2003) was performed using the CAPdiscrim function in the R package “BiodiversityR” (Kindt and Coe, 2005). The relative abundance of fungal phyla was visualized by the R package “circlize” (Gu et al., 2014). Differences between treatments in soil properties and the relative abundances of fungal genera being associated with Al-T genotypes were assessed with two-way analysis of variance (ANOVA) in Genstat (Version 13.0), followed by Duncan's multiple range test ($P < 0.05$).

The association strength of each OTU and higher level taxa with a particular genotype \times Al-concentration group or group combination was determined using correlation-based indicator species analysis (De Cáceres and Legendre, 2009) with all possible site combinations (De Cáceres et al., 2010) using the *multipatt* function in the R package *indicspecies* (De Cáceres and Legendre, 2009). *P*-value correction for multiple testing was performed using the false discovery rate correction according to Storey (2002) using the R package *qvalue* (Storey et al., 2015). A bipartite association network was generated based on the indicator results, which were the taxa that were identified in the indicator species analysis, to visualize positive associations of particular OTUs with specific treatments or treatment combinations as described previously (Hartmann et al., 2015) using the Allegro Fruchterman-Reingold algorithm in CYTOSCAPE 3.8 (Shannon et al., 2003).

Co-occurrence networks were utilized to assess the relationships between fungal OTUs with a relative abundance $>0.1\%$. Pairwise correlations between OTUs were obtained by calculating Spearman correlation coefficients using the R package “psych” (Revelle, 2017), and correlations with $r > 0.8$ and $P < 0.05$ were included in the network. Co-occurrence networks visualization were constructed for each plant genotype and Al treatment using Gephi v.0.9.2 (Bastian et al., 2009). Topological properties of the networks were calculated to elucidate community structure differences across genotypes and

¹<http://unite.ut.ee>

Al treatments. Hubs were defined as OTUs in the network that show high degree, high betweenness centrality and high closeness centrality (Berry and Widder, 2014; Agler et al., 2016).

RESULTS

Soybean Biomass, Soil Fungal Abundance and Diversity

The biomass of all soybeans decreased with Al concentration, but significant differences between tolerant (Al-T) and sensitive (Al-S) genotypes only occurred at the highest Al concentration (Figure 1A). Fungal abundance varied from 4.2 to 13.3×10^7 ITS1 copies g^{-1} dry soil, showing a significant increase at the low Al concentration and a significant decrease at the high Al concentration when compared to the control (Figure 1B, $P < 0.001$). At all Al concentrations, Al-T genotypes had significant higher fungal abundance than Al-S genotypes (Figure 1B). There were no significant differences in fungal alpha

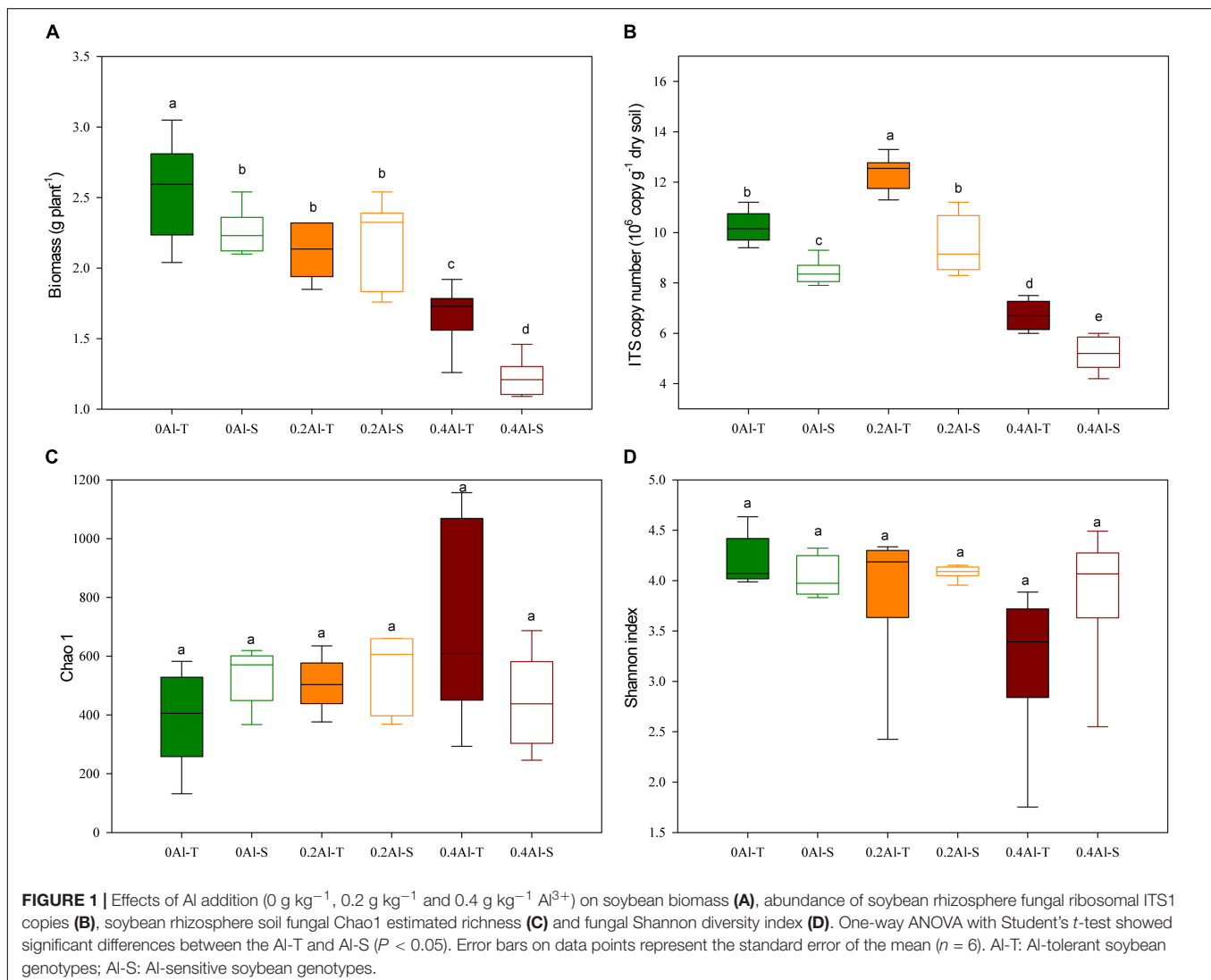
diversity (e.g., Chao1 richness and Shannon diversity index) across the different Al concentrations and between the sensitive and tolerant genotypes (Figures 1C,D).

Soil Chemical Properties

The chemical properties of all soil samples are shown in **Supplementary Table S1**. Exchangeable Al^{3+} and H^+ significantly increased and pH decreased with increasing Al concentration ($P < 0.05$). At the highest Al concentration, exchangeable Al^{3+} was significantly higher in pots with Al-S compared to Al-T genotypes. Available phosphorus, NO_3^- -N and NH_4^+ -N were significantly lower in pots with Al-S genotypes at high Al concentration.

Rhizosphere Fungal Community Structure

A total of 2,019,383 quality-filtered fungal ITS1 sequences were obtained. Number of reads ranged from 29,169 to 59,719 with a mean read count of $44,875 \pm 9139$. Fungal communities



showed distinct compositions between rhizosphere and the unplanted soil (**Supplementary Figure S1**). The rhizosphere fungal communities changed with increasing Al concentration (**Figure 2A** and **Table 1**). Sensitive and tolerant genotypes harbored significantly different communities at all treatment

levels, but separation was strongest at the high Al concentration (**Figure 2B** and **Table 1**).

Eight fungal phyla were identified in the dataset. The community was dominated by Ascomycota (mean relative abundance of $86.29 \pm 10.94\%$), followed by Mucoromycota

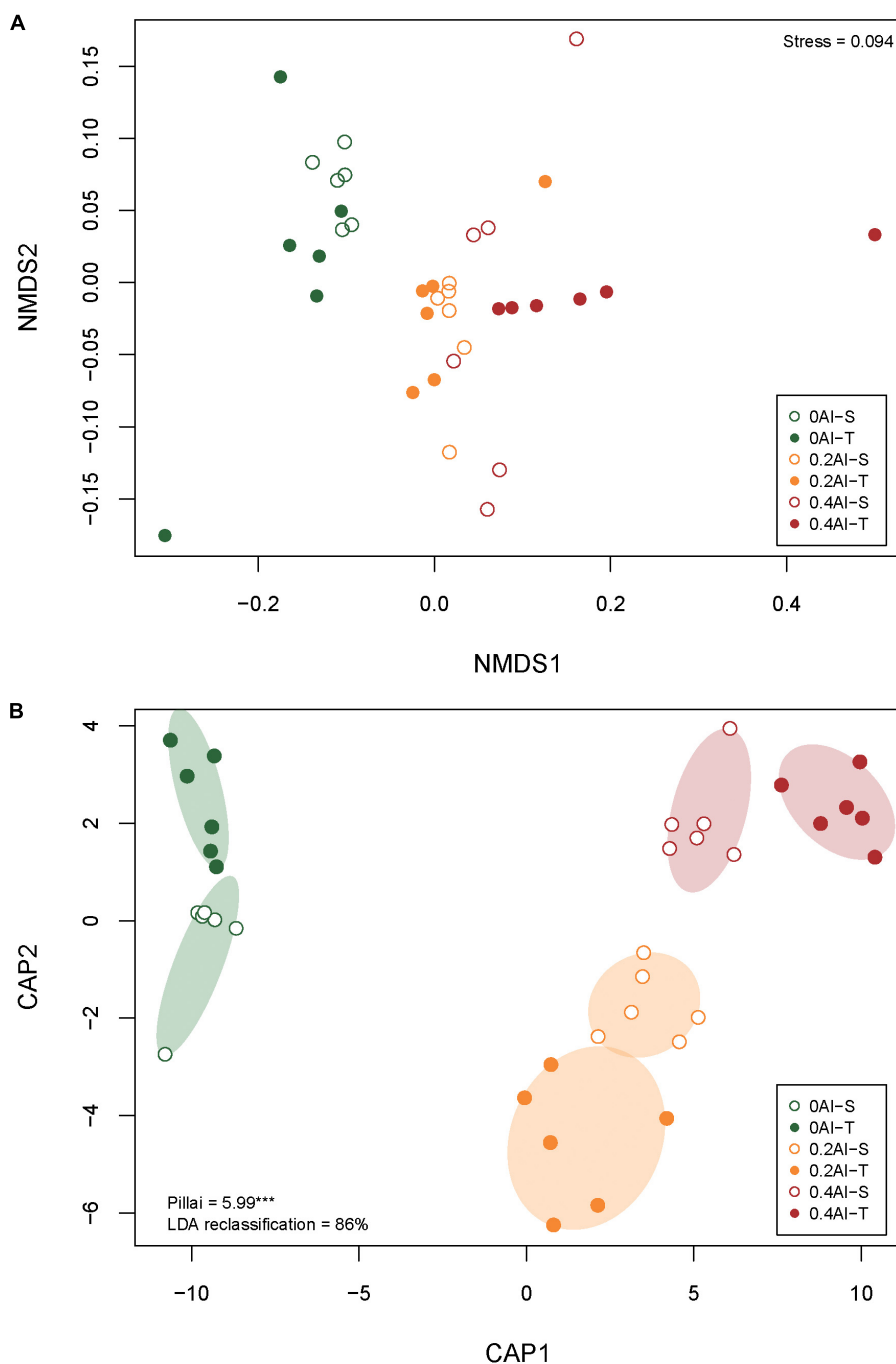


FIGURE 2 | Non-metric multidimensional scale (NMDS) (**A**) and canonical analysis of principal coordinates (CAP) (**B**) based on Bray-Curtis dissimilarities showing differences in rhizosphere fungal community structures at 0 g kg^{-1} , 0.2 g kg^{-1} , and 0.4 g kg^{-1} Al concentrations. The stress value for the NMDS as well as Pillai's trace and the leave-one-out re-allocation success rate of the linear discriminant analysis for the CAP are provided in the plot corners. Al-T: Al-tolerant soybean genotypes; Al-S: Al-sensitive soybean genotypes.

TABLE 1 | Effects of aluminum concentration and soybean genotypes on fungal community structure assessed by permutational multivariate analysis of variance (PERMANOVA).

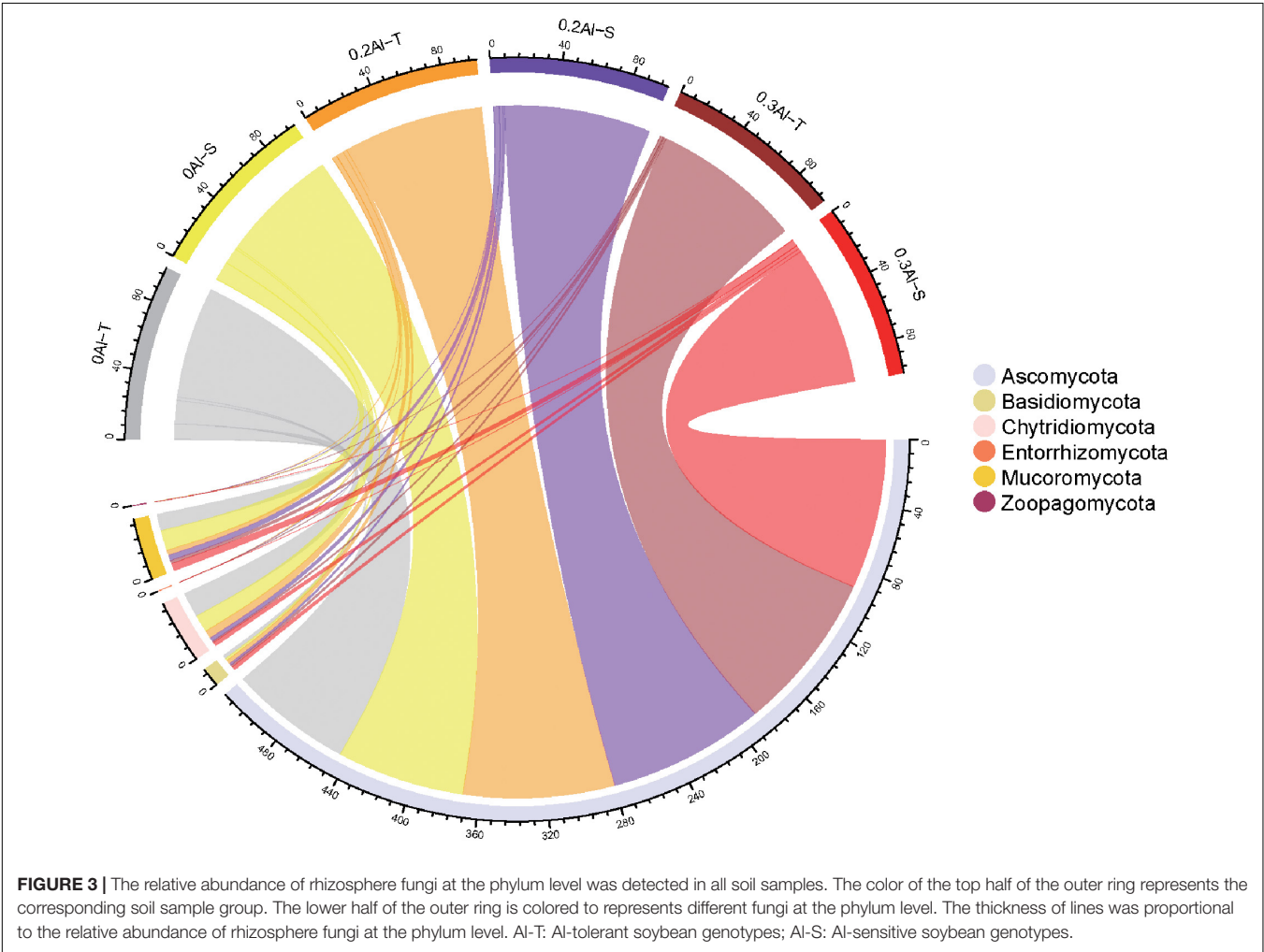
Factor	F	R ²	P
Al	10.5	0.325	0.0001
trait ^a	2	0.032	0.0307
genotype	2.1	0.066	0.0083
Al:trait	2.6	0.08	0.0012
Al:genotype	2.1	0.127	0.001
Pairwise comparison	F	R ²	P
0Al-T ^b vs 0Al-S ^c	1.7	0.145	0.009
0.2Al-T ^b vs 0.2Al-S ^c	1.5	0.132	0.016
0.4Al-T ^b vs 0.4Al-S ^c	2.4	0.196	0.009

^aSensitive versus tolerant, ^bAl-tolerant soybean genotypes, ^cAl-sensitive soybean genotypes.

(5.88 ± 4.63%), Chytridiomycota (6.0 ± 8.3%), Basidiomycota (1.78 ± 1.3%), Zoopagomycota (0.04 ± 0.07%) and Entorrhizomycota (0.01 ± 0.02%) (Figure 3). For these six

fungal phyla, no significant differences in relative abundance were observed between Al-T and Al-S genotypes at the different Al concentrations.

Fungal taxa that significantly changed between the sensitive and tolerant genotypes across the different Al concentrations were identified using indicator species analysis and visualized using a bipartite association network (Figure 4). A total of 163 out of 618 OTUs (26%) representing 61% of the sequences were significantly ($q < 0.1$) associated to one or more treatment groups (Figure 4 and Supplementary Table S2). Among these OTUs, 74 were most strongly associated with only one treatment (Figure 4), confirming the basic distinctness of the communities in all six treatments. A total of 10 and seven OTUs were most strongly associated with the Al-S genotypes under low and high Al concentrations, respectively, whereas seven and 22 OTUs were associated with the Al-T genotypes under the low and high Al concentrations, respectively (Figure 4). Only OTU58 (*Penicillium janthinellum*) was associated with cross-combination of the Al-T genotype under the low and high Al concentrations (Figure 4). Abundant genera (>1%) that associated with Al-T genotypes included *Penicillium*,



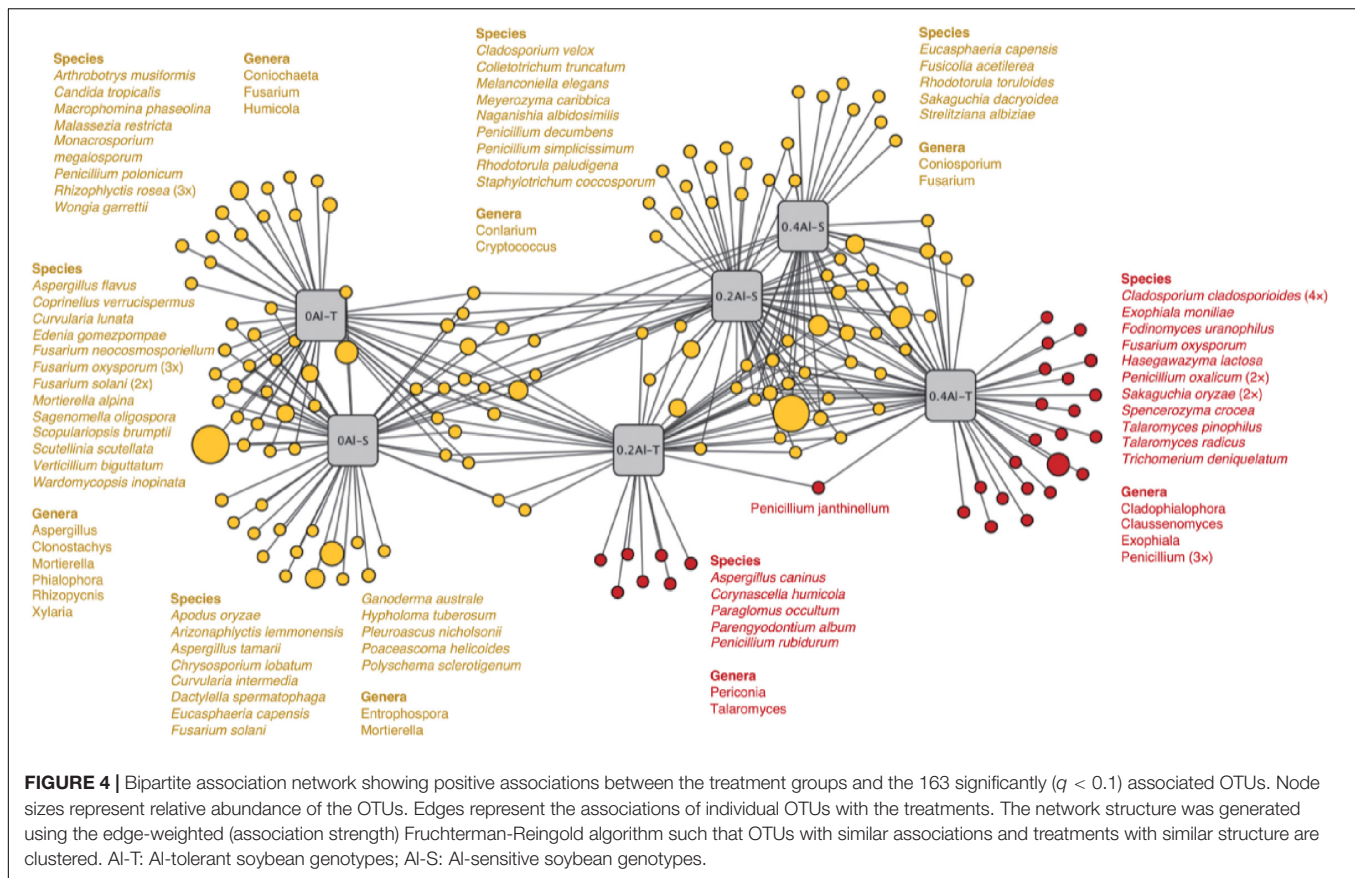
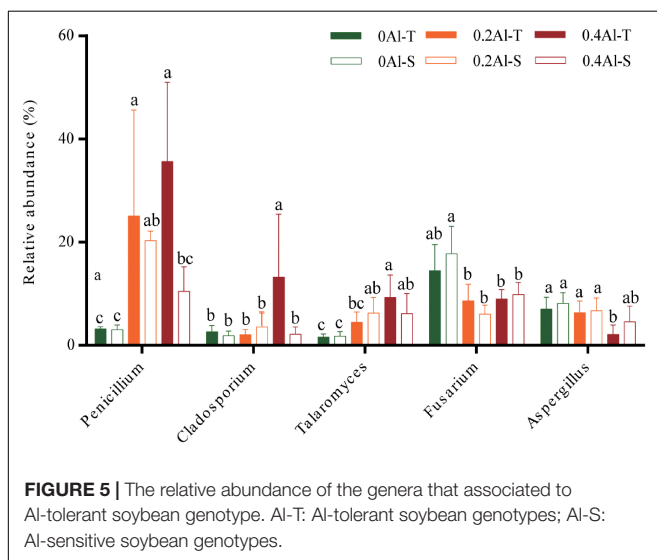


FIGURE 4 | Bipartite association network showing positive associations between the treatment groups and the 163 significantly ($q < 0.1$) associated OTUs. Node sizes represent relative abundance of the OTUs. Edges represent the associations of individual OTUs with the treatments. The network structure was generated using the edge-weighted (association strength) Fruchterman-Reingold algorithm such that OTUs with similar associations and treatments with similar structure are clustered. Al-T: Al-tolerant soybean genotypes; Al-S: Al-sensitive soybean genotypes.

Cladosporium and *Talaromyces* and increased with increasing Al concentration (Figure 5). Conversely, *Aspergillus* and *Fusarium* decreased with increasing Al concentration, while revealing no significant difference between the tolerant and sensitive genotypes (Figure 5).



Networks Analysis of Rhizosphere Fungal Communities

Fungal co-occurrence network structure of the two genotypes changed with increasing Al concentration. At zero Al addition, network structure in term of positive correlation edges, average node degree, graph density and modularity were similar between the two genotypes (Figures 6A,B and Table 2). At low Al concentration, number of negative correlation edges and modularity was higher for Al-S than Al-T genotypes, while positive correlation edges were more frequent for Al-T (Figures 6C,D and Table 2). At high Al concentration, the Al-T genotypes had higher average node degree, as well as positive correlations when compared to the Al-S genotypes, but the average path length and modularity were lower (Figures 6E,F and Table 2). Hub OTUs were identified by calculating node degree, closeness centrality and betweenness centrality for all nodes in the network (Table 3). For example, OTU48 (*Aspergillus*) and OTU208 (*Talaromyces*) were identified as central hubs for the Al-T genotype under high Al concentration (Table 3).

Soil Characteristics Were Linked to Changes of Fungal Communities

CCA and mantel testing were performed to identify correlations between soil characteristics and fungal community structure, indicating that fungal community structures were significantly correlated with specific soil characteristics such as available

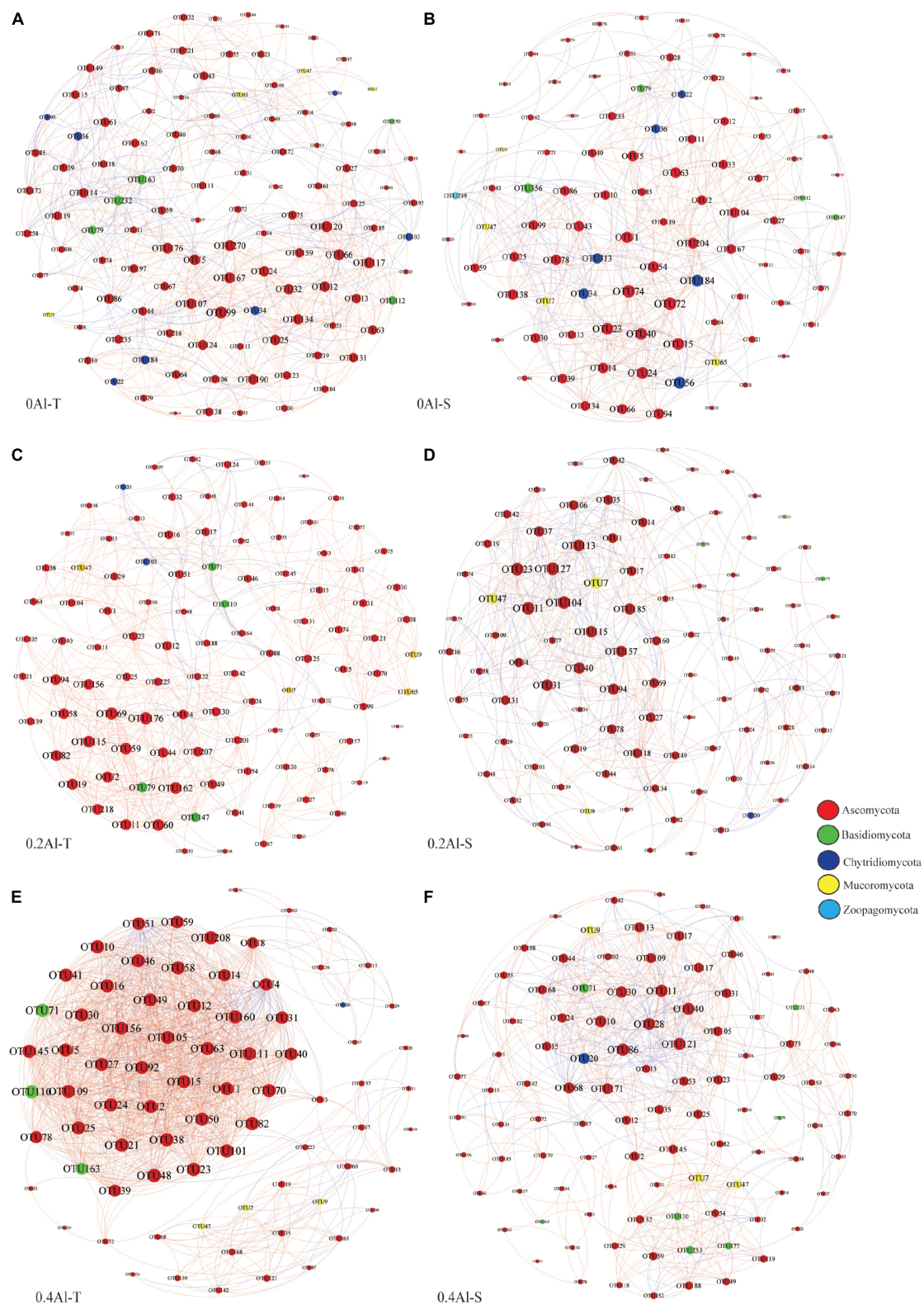


FIGURE 6 | Co-occurrence network of the rhizosphere fungal community for different Al concentrations and soybean genotypes, i.e., 0 Al-T (**A**), 0 Al-S (**B**), 0.2 Al-T (**C**), 0.2 Al-S (**D**), 0.4 Al-T (**E**) and 0.4 Al-S (**F**). Nodes represent OTUs colored-coded by phyla and scaled proportional to the number of connections (node degree). Connections were drawn at $r > 0.8$ (positive correlations, red) or $r < -0.8$ (negative correlations, blue) and $P < 0.05$. Al-T: Al-tolerant soybean genotypes; Al-S: Al-sensitive soybean genotypes.

TABLE 2 | Topological characteristics of rhizosphere fungal networks across the different Al concentrations and soybean genotypes.

Network metrics	0Al-T ^a	0Al-S ^b	0.2Al-T ^a	0.2Al-S ^b	0.4Al-T ^a	0.4Al-S ^b
Number of nodes	116	95	108	101	78	103
Number of edges	812	582	662	567	1138	732
Number of positive correlations	551	439	600	374	1026	541
Number of negative correlations	261	143	62	193	112	191
Network diameter	6	10	7	8	9	8
Interconnecting piece	2	1	3	2	3	3
Graph density	0.122	0.13	0.12	0.11	0.37	0.13
Average node degree (avgK)	14	12.25	12.26	11.23	29.17	14.21
Average weighted node degree	4.91	5.91	9.74	3.52	39.31	6.96
Average clustering coefficient (avgCC)	0.62	0.66	0.64	0.67	0.86	0.68
Average path length (APL)	3.01	3.19	3.17	3.50	2.44	3.10
Modularity (M)	1.48	1.02	0.67	2.96	0.18	1.18

^aAl-tolerant soybean genotypes. ^bAl-sensitive soybean genotypes.

phosphorus, exchangeable H⁺, exchangeable Al³⁺, NH₄⁺-N, NO₃⁻-N, and pH (**Supplementary Figure S2** and **Table 4**). In addition, TC also correlated with fungal community structure of the Al-T genotypes, which shifted with increase of Al concentration along CCA1 (**Supplementary Figure S2A**).

DISCUSSION

The aim of this study was to reveal the effects of Al stress on the rhizosphere fungal community structure of aluminum sensitive (Al-S) and tolerant (Al-T) soybean genotypes. The Al tolerant genotype harbored more abundant and structurally different fungal communities when compared to the sensitive genotype (**Figures 1, 2**). This finding supports the hypothesis that the plant, besides directly secreting more organic acid to chelate Al, might also recruit specific fungal species to the rhizosphere that themselves secrete organic acids for Al detoxification (Kochian, 1995; Ma et al., 1997; Yang et al., 2012b). Thus, different soybean genotypes secrete different amounts and types of organic acids that cause different response to Al toxicity (Ryan et al., 2001; Kochian et al., 2015). However, fungal alpha-diversity showed no difference among the treatments, which is in contrast with our first hypothesis. This result indicated that fungal diversity was stable in the rhizosphere and was not affected by Al stress and soybean genotypes in this study. Notably, Al stress tended to increase difference in fungal community composition between the tolerant and sensitive genotypes, which is in accordance to what has been observed for bacteria (Lian et al., 2019).

Al stress reduced fungal abundance indicating that Al inhibited the growth of soil fungi (He et al., 2012). Moreover, Al stress altered the rhizosphere fungal community composition of both genotypes (**Figure 2**), which is consistent with previous studies suggested that Al affects fungal community structure (Vosátka et al., 1999; He et al., 2012). However, the observation that fungal community structure differed between the genotypes even without Al stress is in contrast with the study by Wang et al. (2009) reporting that fungal communities of three soybean genotypes were not different at the same growth stage. This discrepancy could be explained by the fact that different genotypes were investigated, different soil types were tested,

and fungal communities were assessed using lower-resolution molecular methods.

Several studies have shown that Al tolerant genotypes can secrete more organic acids to chelate more Al ions (Kochian, 1995; Ma et al., 1997; Innes et al., 2004; Yang et al., 2012b). Based on the indicator species analysis, we have revealed that certain fungal genera significantly associated with the tolerant genotypes at high Al concentration. The comparisons among the treatments has identified several fungal taxa that have increased in relative abundance in the rhizosphere of the tolerant plant, including *Penicillium*, *Cladosporium* and *Talaromyces*. *Penicillium* has previously been shown to be highly tolerant to Al stress and therefore reduce Al toxicity by secreting organic acids and increasing soil pH (Zhang et al., 2002). Previous study has also reported that *Penicillium* can promote plant growth via increasing nutrient status of plants (Vessey and Heisinger, 2001). *Cladosporium* has been shown tolerant toward heavy metals, and could transfer phosphorus to the plant and promote plant growth under phosphorus deficiency, thereby cope with the Al toxicity (Bewley, 1980; Shao and Sun, 2007; Hiruma et al., 2016). *Talaromyces*, which is close to *Penicillium* and has initially been described as a sexual state of *Penicillium* species, are also known to be tolerant toward heavy metals (Yilmaz et al., 2014; Nam et al., 2019). Thus, the tolerance of soybean to Al toxicity may be closely related to the presence of these species.

A considerably large fraction of the community, representing 26% of the OTUs, responded significantly to Al addition and plant genotype (**Figure 4**). It has been suggested that aluminum contamination and soybean genotypes can affect entire clades of the rhizosphere microbial community structure by changing fundamental factors such as nutrition availability (Xu et al., 2009; Yang et al., 2012a). It is worth noting that some OTUs, e.g., OTU490 (*Penicillium decumbens*) and OUT 240 (*Penicillium simplicissimum*) associated with Al-S genotype under low Al concentration were affiliated to the genus *Penicillium*. Considering that this genus was significantly increased with Al stress and showed a higher relative abundance under the tolerant genotypes, it might contribute to Al tolerance with both sensitive and tolerant genotypes, and this contribution might be different for the two genotype groups (**Figure 4**).

TABLE 3 | Topological characteristics of hubs observed in rhizosphere soils across the different Al concentrations and soybean genotypes.

Genotype	Phylum	Class	Order	Family	Genus	Species	OTU	Degree	Closeness centrality	Betweenness centrality
OAL-T ^a	Ascomycota	Sordariomycetes	Microascales	Microascaceae	<i>Cephalotrichum</i>	<i>Unclassified Cephalotrichum</i>	OTU5	23	0.40	316.14
	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	<i>Nigrospora</i>	<i>Nigrospora oryzae</i>	OTU176	23	0.40	316.14
OAL-S ^b	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	<i>Fusarium</i>	<i>Fusarium oxysporum</i>	OTU72	24	0.41	193.58
	Ascomycota	Sordariomycetes	Glomerellales	Glomerellaceae	<i>Colletotrichum</i>	<i>Colletotrichum chlorophyti</i>	OTU74	24	0.41	193.58
0.2Al-T ^a	Ascomycota	Sordariomycetes	Glomerellales	Plectosphaerellaceae	<i>Plectosphaerella</i>	<i>Unclassified Plectosphaerella</i>	OTU69	24	0.36	135.15
	Ascomycota	Sordariomycetes	Trichosphaeriales	Trichosphaeriaceae	<i>Nigrospora</i>	<i>Nigrospora oryzae</i>	OTU176	24	0.36	135.15
0.2Al-S ^b	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	<i>Corynascella</i>	<i>Corynascella humicola</i>	OTU157	22	0.38	458.28
	Ascomycota	Sordariomycetes	Capnodiales	Cladosporiaceae	<i>Cladosporium</i>	<i>Cladosporium cladosporioides</i>	OTU11	26	0.35	129.68
0.4Al-T ^a	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	<i>Aspergillus</i>	<i>Aspergillus niger</i>	OTU48	46	0.56	155.33
	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	<i>Talaromyces</i>	<i>Talaromyces pinophilus</i>	OTU208	45	0.49	261.89
0.4Al-S ^b	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	<i>Fusarium</i>	<i>Unclassified Fusarium</i>	OTU86	32	0.43	243.72
	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	<i>Cladosporium</i>	<i>Cladosporium cladosporioides</i>	OTU11	31	0.43	220.02

^aAl-tolerant soybean genotypes. ^bAl-sensitive soybean genotypes.**TABLE 4** | Analysis of the correlation (*r*) and significance (*P*) values between environmental factors and fungal communities by Mantel test. Al-T: Al-tolerant soybean genotype; Al-S: Al-sensitive soybean genotype.

	<i>r</i> (Al-T ^a)	<i>P</i> (Al-T ^a)	<i>r</i> (Al-S ^b)	<i>P</i> (Al-S ^b)	<i>r</i> (High-Al)	<i>P</i> (High-Al)
pH	0.499	0.004	0.624	0.004	−0.031	0.582
Exchangeable H ⁺	0.554	0.004	0.356	0.010	−0.033	0.636
Exchangeable Al ³⁺	0.611	0.004	0.359	0.012	−0.008	0.636
TC	0.309	0.013	−0.059	0.759	0.156	0.272
TN	−0.114	0.821	−0.112	0.288	0.043	0.534
C:N	−0.026	0.636	0.094	0.337	0.261	0.209
TK	−0.018	0.636	−0.058	0.710	0.216	0.287
TP	−0.018	0.636	−0.096	0.821	−0.064	0.636
AP	0.418	0.004	0.734	0.004	0.314	0.240
NH ₄ ⁺ -N	0.300	0.015	0.472	0.004	−0.033	0.636
NO ₃ [−] -N	0.532	0.004	0.589	0.004	−0.002	0.2474

^aAl-tolerant soybean genotypes. ^bAl-sensitive soybean genotypes.

Soil chemical properties, such as available phosphorus, NH₄⁺-N, NO₃[−]-N, exchangeable H⁺, exchangeable Al³⁺, and pH, were significantly correlated with shifts in fungal community structure of both genotypes (Table 4 and Supplementary Figure S2), and all these chemical properties were significantly associated with changing Al concentrations (Supplementary Table S1). These results suggested that the impacts of Al stress on the fungal communities might be directly linked to the alteration of soil chemical properties and vice versa.

Co-occurrence networks showed substantial structural differences between the Al tolerant and sensitive genotypes at both low and high Al concentrations. At the high Al concentrations, the fungal network of the tolerant genotypes revealed more negative correlations and lower modularity, which could be interpreted as representing increased inter-species competition according to network theory (Saavedra et al., 2011; Fan et al., 2018). Moreover, most fungal OTUs in Al-T genotypes are connected by positive links are considered to be unstable; in such a network, fungal OTUs may generated co-fluctuations and positive feedback along with environmental changes (Coyte et al., 2015; Vries et al., 2018). Besides, fungal hubs might also play an important role in mediating Al toxicity. For example, potential hub OTU48 was assigned to *Aspergillus*, which can produce organic acids that might alleviate Al toxicity by forming complexes with Al (Kawai et al., 2000).

In conclusion, aluminum stress had no effect on fungal diversity, but increased differences in fungal community structure between the sensitive and tolerant genotypes with increasing aluminum concentrations. Fungal genera such *Penicillium*, *Cladosporium*, and *Talaromyces* increased with increasing Al concentration and were enriched under the tolerant genotypes at high Al concentration. A more complex structure in fungal co-occurrence networks was found for the tolerant genotypes at high Al concentrations. However, to what extent these “enriched” fungal taxa have an impact on Al detoxification is not yet known and subject to future, more

mechanistic experiments. These experiments also need to be carried out in different soil types and under different climatic conditions in order to evaluate the universality of the findings. This study highlights the possibility that rhizosphere fungi involved in Al detoxification can be used as breeding target.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI Bioproject, under accession number PRJNA561469.

AUTHOR CONTRIBUTIONS

TL and HN designed the research. TL, QS, YL, AS, and ZC performed the research. QS, TL, and MH analyzed the data and wrote the manuscript. All authors have read and approved the manuscript as submitted.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01177/full#supplementary-material>

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Soil Sickness in Aged Tea Plantation Is Associated With a Shift in Microbial Communities as a Result of Plant Polyphenol Accumulation in the Tea Gardens

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In conventional tea plantations, a large amount of pruned material returns to the soil surface, putting a high quantity of polyphenols into the soil. The accumulation of active allelochemicals in the tea rhizosphere and subsequent shift in beneficial microbes may be the cause of acidification, soil sickness, and regeneration problem, which may be attributed to hindrance of plant growth, development, and low yield in long-term monoculture tea plantation. However, the role of pruning leaf litter in soil sickness under consecutive tea monoculture is unclear. Here, we investigated soil samples taken from conventional tea gardens of different ages (2, 15, and 30 years) and under the effect of regular pruning. Different approaches including liquid chromatography–mass spectrometry (LC-MS) analysis of the leaf litter, metagenomic study of root-associated bacterial communities, and *in vitro* interaction of polyphenols with selected bacteria were applied to understand the effect of leaf litter-derived polyphenols on the composition and structure of the tea rhizosphere microbial community. Our results indicated that each pruning practice returns a large amount of leaf litter to each tea garden. LC-MS results showed that leaf litter leads to the accumulation of various allelochemicals in the tea rhizosphere, including *epigallocatechin gallate*, *epigallocatechin*, *epicatechin gallate*, *catechin*, and *epicatechin* with increasing age of the tea plantation. Meanwhile, in the tea garden grown consecutively for 30 years (30-Y), the phenol oxidase and peroxidase activities increased significantly. Pyrosequencing identified *Burkholderia* and *Pseudomonas* as the dominant genera, while plant growth-promoting bacteria, especially *Bacillus*, *Prevotella*, and *Sphingomonas*, were significantly reduced in the long-term tea plantation. The qPCR results of 30-Y soil confirmed that the copy numbers of bacterial genes per gram of the rhizosphere soil were significantly reduced, while that of *Pseudomonas* increased significantly. *In vitro* study showed that the growth of catechin-degrading bacteria (e.g., *Pseudomonas*) increased and plant-promoting

bacteria (e.g., *Bacillus*) decreased significantly with increasing concentration of these allelochemicals. Furthermore, *in vitro* interaction showed a 0.36-fold decrease in the pH of the broth after 72 h with the catechin degradation. In summary, the increase of *Pseudomonas* and *Burkholderia* in the 30-Y garden was found to be associated with the accumulation of catechin substrates. In response to the long-term monoculture of tea, the variable soil pH along with the litter distribution negatively affect the population of plant growth-promoting bacteria (e.g., *Sphingomonas*, *Bacillus*, and *Prevotella*). Current research suggests that the removal of pruned branches from tea gardens can prevent soil sickness and may lead to sustainable tea production.

Keywords: indirect allelopathy, catechins, soil sickness, monoculture problems, plant polyphenol

INTRODUCTION

Plant soil feedbacks can alter the composition and structure of the soil microbial community and nutrient homeostasis as a result of all the interactions between plants and soil organisms, affecting soil fertility and plant growth (Kaur et al., 2009; Bever et al., 2010; van de Voorde et al., 2012; Baxendale et al., 2014). Soil disease or replanting disease is plant-soil negative feedback caused by consecutive planting of a single crop or its related species on the same field, resulting in a reduction in crop yield and quality (Muller, 1966; Huang et al., 2013; Zhao et al., 2015, 2016; Wu L. et al., 2016). Long-term monoculture not only impedes the growth and production of many annual crops, trees, and shrubs in orchards but also causes replanting and regeneration problems (Canals et al., 2005). Therefore, it is pertinent to understand the mechanisms underlying soil sickness associated with long-term monoculture practice, to explore the allelochemical interaction with soil microbiota, and to provide a solution for maintaining a sustainable agro-ecosystem.

Degradation of autotoxins or allelochemicals by microorganisms has been reported in previous studies (Bever et al., 2010; Weidenhamer et al., 2013). Several cinnamic and benzoic acids were initially detected in the Cecil Ap horizon soil, and after a few days, they were not detected in amended soil (Blum et al., 2000). Phenolics, being the most abundant plant metabolites, are thought to control the rates of soil organic matter decomposition and can be applied as a tool to evaluate the soil dynamics and ecosystem functioning (Min et al., 2015). Hence, the effects of soil microbiota are critical to the fate of plant phenolic compounds, including other potential allelochemicals found in the soil (Bever et al., 2010; Ehlers, 2011).

Polyphenols enter the soil in the form of leachates from above plant parts as plant residues and litter (Hättenschwiler and Vitousek, 2000). Contributions related to these input pathways, mainly underground flows, have a limited understanding. In most of the less disturbed terrestrial ecosystems, plant growth and net primary productivity depend on recycled nutrient availability, while external nutrient inputs contribute little to the total requirement. The rate-limiting steps in the nutrient cycle, such as climate, substrate quality (litter), and decomposing microbes, are decisive at influencing soil microbial mineralization (Heal, 1997). Polyphenols influence

soil microbial activity and soil physicochemical properties, indicating interactions with nutrient cycling, thereby modifying the flux and pool of soil nutrients available to microbes and/or plants (Hättenschwiler and Vitousek, 2000). Several studies suggest that through specific mechanisms, soil macrofauna increases the biodegradation and mineralization of soil organic matter (Wardle and Lavelle, 1997) and high concentrations of polyphenols could limit such fauna in terms of activity and abundance (Neuhauser, 1978). Consequently, the direct effect of polyphenols on soil fauna is hard to explain owing to the complexity of soil food webs and the co-variability of other compounds.

Tea is an important economic crop, widely cultivated in Southeast Asia and China (Chen and Lin, 2015) and contains several phenolic compounds throughout the tea plant, especially in the leaves, accounting for 18–34% of the dry weight of the leaves (Wan, 2003). In tea plantation systems, the conventional management approach, especially pruning, is employed twice a year in terms of keeping tea bushes in the best shape, improving tea quality, increasing yield, and even inhibiting diseases and pests (Yilmaz et al., 2004; Maudu et al., 2010). The trimmed tea leaves and branches are rich in nutrients and are often amended into tea gardens to improve the soil physicochemical properties, soil organic matter, and nutrient availability (Weeraratna et al., 1977). However, some studies have demonstrated that the decomposition of accumulated residues and litter produces allelochemicals, mainly phenolic compounds, flavonoid, and alkaloids, which, in turn, inhibit the microbial activities in the rhizospheric soil. The indirect allelopathy of pruned tea leaves, especially polyphenols on soil sickness in long-term tea cropping systems, needs further consideration. The objectives of this study were to inspect the impact of tea litter and its polyphenols on (a) root-related bacterial communities in terms of structure and composition, (b) ratooning or regeneration problems related to soil sickness, and (c) growth and quality parameters of tea plants in response to continuous tea cultivation. Assuming that polyphenols represent a possible allelochemical substance, they regulate the tea soil bacterial community feedback processes, which leads to the progressive imbalance during long-term tea monocropping. In addition, changes in microbial community structure and composition play an important role in soil sickness as compared to the direct allelopathy.

MATERIALS AND METHODS

Site Overview

Current experiment on tea plantation was carried out at Fujian Agriculture and Forestry University tea fields, Fuzhou City, Fujian Province, China (latitude: 26°05'09.60'' N; longitude: 119°14'03.60'' E) (27°43' N, 118°72' E). The area has a subtropical monsoon climate, with average annual temperature and rainfall (20–25°C, and around 900–1362 mm, respectively).

Soil Sampling

Rhizosphere soil along with roots was taken from different aged tea gardens (2, 15, and 30 years) and bulk soil (CK) from the same fields was used as control. The location, agronomic management practices, and environmental conditions of the tea gardens were the same. To overcome error began by spatial heterogeneity, five randomly taken soil samples from 15 sampling locations were combined into one replication, and three replications were carried out for each sample. In order to deliver soil samples to the laboratory, all soil samples were immediately stored in a sterile icebox (Lin et al., 2013). For the analysis of soil enzymes and microbes, a part of each soil sample and roots were stored at –80°C, and the remainder was air-dried for physicochemical attributes. With little modification in the method proposed by Edwards et al. (2015), we determined the microbial fauna in 2-Y, 30-Y, and CK soil. Shortly, the bacterial communities were obtained from tea root compartments, e.g., RS, rhizosphere (soil firmly adhered to the root surface); RP, rhizoplane (suite of microbes present on the root surface by sonication); and ES, endosphere (interior of the same plant roots after sonication).

Quality Parameters, Growth Index, and Yield Determination

Theophylline (TPY), theanine (TNN), and the total polyphenol (TPP) content of the uppermost leaves taken from three plants grown in each tea garden, i.e., 2-Y, 15-Y, and 30-Y, was determined by the method proposed by Peng et al. (2008) for quality determination. To examine the total chlorophyll content of the third leaf, eight plants were randomly selected from each garden and values were recorded using an instrument (SPAD-502 Plus). Further, using the US portable CO₂ gas analyzer (CID-301), the net photosynthetic rate (Pn) of the third leaf was measured. Three replicates for each sample were taken to perform Pn. One hundred buds' weight (fresh and dry) was estimated in grams for each of three biological replications.

Soil Enzyme Activities Analysis

With a slight modification, the activity of soil polyphenol oxidase and peroxidase was determined by the method previously adopted (Perucci et al., 2000; Saiya-Cork et al., 2002; Xu et al., 2015). Briefly, 1 g of soil was added to a 100-ml flask containing 10 ml of 1% pyrogallol before incubation at 300°C for 2 h, and then 35 ml of ether was added into the flask and then shaken for 15 min on a thermostat oscillator at 250°C. Moreover, peroxidase activity was determined using the same procedure along with the

addition of 2 ml of a 0.5% H₂O₂ solution, and the final absorbance was recorded at 430 nm.

Leaf Litter Biomass Determination and Identification and Quantification of Allelochemicals

Standard Materials and Their Calibration Curves

In order to quantify and identify concentration of allelochemicals in leaf litter samples, ≥98% pure catechin, epigallocatechin (EGC), epicatechin gallate (ECG), epicatechin (EC), epigallocatechin gallate (EGCG), taxifolin (TF), and protocatechuic acid (PCA) were purchased as external standards from Cayman Chemical (1180 E. Ellsworth Road Ann Arbor, MI, United States). Formic acid and LC-MS grade methanol were also purchased from Sigma-Aldrich (St. Louis, MO, United States). For a stock solution preparation, 1 mg of every standard was suspended in 1 ml of 99.9% methanol and 0.1% by volume of formic acid. Serial dilutions were made using the same solvent as the stock solution, with a dilution range of 1.25–10 µg/ml in order to obtain a calibration curve. Each solution was distinctly injected with a 10-µl aliquot for HPLC-ESI-MS analysis. With all correlation coefficients > 0.998, excellent linearity was achieved within the calibration range (Supplementary Figures S1, S2).

Allelochemicals Determination From Leaf Litters

The leaf litter biomass that comes on the soil surface from each tea plant per pruning was determined in grams (Supplementary Figure S3). Leaf litters were collected randomly from three tea plants in each tea garden and were weighed by a digital balance. By adopting the method of Wang et al. (2013) with slight modifications, the allelochemicals concentration in leaf litter was determined by HPLC-ESI-MS. We took 5 g of the litter and fermented it for 3 days in 15 ml of a solution (containing 50% methanol and 0.1% acetic acid) at room temperature (25°C), and after three times sonication, it was vortexed at 150 rpm for 24 h. By vaporization at ultra-pressure, the methanol extract was air-dried and the crude extract was passed through a C-18 column (open column). After centrifugation at 4500 rpm for 10 min, the supernatant was then shifted to a sample collection vessel for liquid chromatography (LC).

The Chromatographic Conditions for Allelochemical Determination

Following this, HPLC-ESI-MS was carried out using a T3 RP-18 column (100 × 2.1 mm; 5 µm; Waters, Milford, MA, United States), eluted with buffer A (0.1% acetic acid) and buffer B (100% methanol) at a flow rate of 300 µl/min at 25°C. Initially, the column was eluted with 95% buffer B, followed by a linear increase in buffer A to 35% from 0 to 10 min, and further maintained in 90% buffer A until 10.50 min. Then, a linear increase in buffer B to 95% was maintained. Finally, the column was maintained in 95% buffer B for up to 19 min. The total time for running one sample was 19 min. The negative ionization mode was selected to perform mass spectrometry at a temperature of 100°C, and ion scans were carried out at low-energy collision (20 eV) using nitrogen as the collision gas. All the data from HPLC-ESI-MS were processed

to determine the mean concentrations of the selected catechin compounds in each sample, by using Bruker Daltonics Data analysis software (version 4.0).

The Metagenomic Analysis of the Root-Associated Bacterial Communities

Soil DNA Extraction

Fresh soil samples were passed through a 2-mm mesh sieve and then 0.5 g was taken in 2-ml Eppendorf tubes for DNA extraction. DNA was extracted using a BioFast Soil Genomic DNA Extraction Kit (Bioer Technology Co., Ltd., Hanzhou, China) according to the manufacturer's instructions. In order to estimate the quality and concentration of soil DNA, NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, United States) was used. DNA was diluted to 1 ng/μl using sterile water accordingly to concentration.

PCR Amplification

Primers 515F/806R with the barcode were used in order to amplify the distinct region of 16S rRNA gene (16S V4). All PCR reactions were conducted with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR conditions were (95°C for 3 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s, with a final extension at 72°C for 10 min) (GeneAmp 9700, ABI, United States). PCR reactions were carried out in triplicate in 20-μl mixture having 2 μl of 2.5 mM deoxyribonucleoside triphosphate (dNTPs), 4 μl of 5 × Fast Pfu buffer, 0.4 μl of Fast Pfu polymerase, 0.4 μl of every primer (5 μM), and template DNA (10 ng) (TransGen catalogue no. AP221-02). PCR products were purified with Qiagen Gel Extraction Kit (Qiagen, Germany).

Library Preparation and Illumina Hi Sequencing

Purified PCR products were sent to Novogene Bioinformatics Technology Co., Ltd., (Beijing, China) for high-throughput sequencing. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, United States) following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina HiSeq2500 platform and 250-bp paired-end reads were generated.

Processing and Analyzing of Data

Paired-End Reads Assembly and Quality Control

Paired-end reads was assigned to samples based on their unique barcode and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH (V1.2.7)¹ (Magoč and Salzberg, 2011), and the splicing sequences were called raw tags. Quality filtering on the raw tags were performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013), according to the QIIME(V1.7.0)² (Caporaso

et al., 2010). The tags were compared with the reference database(Unite Database)³ using UCHIME algorithm (UCHIME Algorithm⁴) (Edgar et al., 2011) to detect chimera sequences, and then the chimera sequences were removed (Haas et al., 2011).

OTU Cluster and Species Annotation

Sequence analysis was performed by Uparse software (Uparse v7.0.1001)⁵ (Edgar, 2013). Sequences with ≥97% similarity were assigned to the same OTUs. Representative sequence for each OTU was screened for further annotation. For each representative sequence, the Unite Database⁶ (Kõljalg et al., 2013). was used basing on Blast algorithm, which was calculated by QIIME software (Version 1.7.0)⁷ to annotate taxonomic information.

Heat Map and Graph Construction

Heat map was preceded by ggplot2 package in R software (Version 2.15.3).

Quantitative PCR Analysis of Total Bacteria in Ratooning Monoculture Tea Soil

A quantitative PCR (qPCR) assay was conducted to determine the size of bacterial population in the rhizosphere soil of different aged tea plantations. The Universal primer set Eub338/Eub518 was used to estimate the bacterial community size. qPCR was performed according to the method of Wu H. et al. (2016).

qPCR Analysis of *Pseudomonas* and *Bacillus* Population in Ratooning Monoculture Tea Soil

A qPCR assay using the primer sets Psf/Psr (Chen et al., 2017) was also conducted to determine the copy number of *Pseudomonas* in tea rhizosphere soil after different years of monoculture. However, for *Bacillus* genus, we used the method of Wu H. et al. (2016). The 15-μl PCR reaction contained 7.5 μl of 2 × SYBR green I Super Real Premix (TransGen, Beijing, China), 0.5 μl of each primer (10 μM), 1 μl of 20 ng/μl template DNA, and 5.5 μl of RNase-free H₂O. Serial dilutions of plasmid DNA were set as standard curve.

In vitro Interactions of Allelochemicals With Selected Model Bacteria

Preparation of LB Medium

LB powder, 5 g, was mixed in 200 ml of ultrapure water in a 250-ml Erlenmeyer flask, covered the top with aluminum foil, and autoclaved at 121°C for 15 min.

For LB agar plates preparation, 3 g (1.5%) of bacteriological agar was added to 200 ml of LB liquid medium (5 g broth

¹<http://ccb.jhu.edu/software/FLASH/>

²<http://qiime.org/index.html>

³<https://unite.ut.ee/>

⁴http://www.drive5.com/usearch/manual/uchime_algo.html

⁵<http://drive5.com/uparse/>

⁶<https://unite.ut.ee/>

⁷http://qiime.org/scripts/assign_taxonomy.html



FIGURE 1 | Replanting problems in continuous monoculture tea garden soil. 2-Y, 15-Y, and 30-Y indicate tea garden soil in which tea was planted continuously for different years (2, 15, and 30 years).

powder in 200 ml of ddH₂O) in a 2500 ml Erlenmeyer flask and autoclaved at 121°C for 15 min. After autoclaving, when medium was cooled to ~50°C, 200 µl of pimarin antifungal (0.06 g/l methanol) was added to 200 ml of media. The LB agar media was poured into the petri plates.

Preparation of Soil Serial Dilution

Five grams of soil was added to 45 ml of ddH₂O in a 50-ml Erlenmeyer flask. The soil samples were diluted up to 10⁻³ times. Soil suspension (60 µl) of 10⁻² and 10⁻³ dilutions was subjected to each hard-nutrient agar plate surface. The soil suspension was spread until it was equally distributed on hard agar plate. Last, the plates were incubated at 30°C for 24–72 h, and the colonies that show dark zone surrounding them were separated from a mixed bacterial community in the culture.

Sub-Culturing of Bacteria

Already prepared LB agar plates were used to get each purified bacterial colony. For this, divide the plates under sides by drawing equal numbers of squares to each plate and label it by giving specific numbers and then pick each colony carefully by sterilizing the pipette tip and attaching it to each label square. The plates were incubated at 30°C for 24–72 h again. These colonies represent each bacterial strain.

Enrichment and Preservation of Bacteria

The sterilized LB liquid broth was prepared and 5-ml broth solutions were distributed in sterilized 5-ml tubes. After that, one colony into each tube was suspended and incubated at 30°C for 72 h. To preserve each bacterium, put 1 ml of bacterial suspension in a sterilized Eppendorf

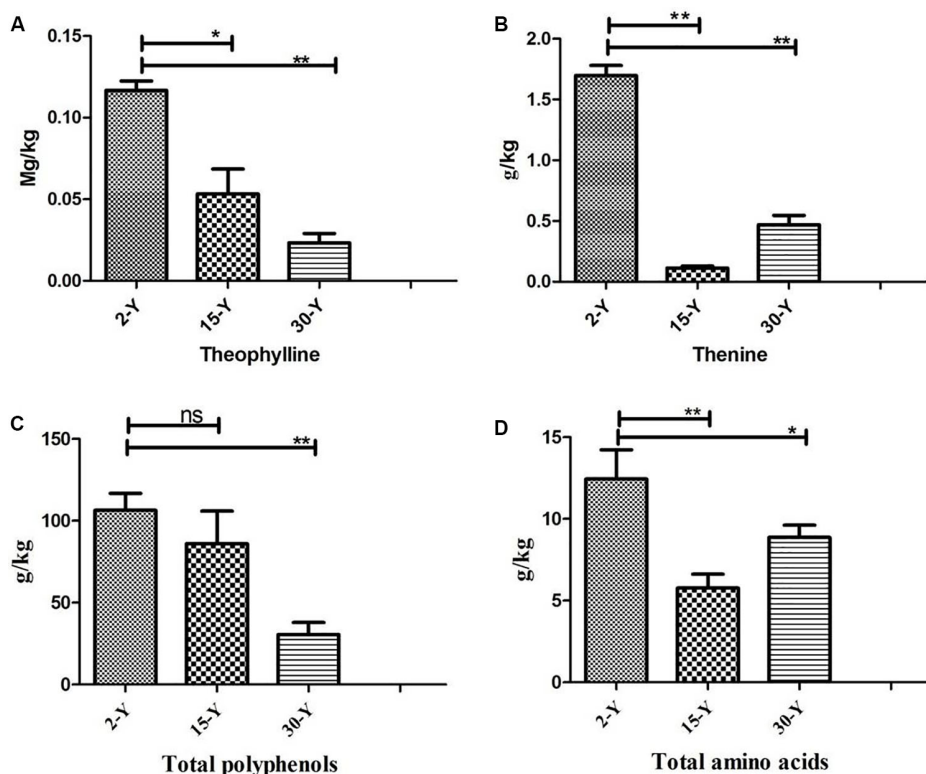


FIGURE 2 | Quality parameters of tea leaves from tea plantations of different ages. (A) Theophylline, (B) theanine, (C) total polyphenols, and (D) total free amino acids. Stars in the column show significant differences (LSD test, $P < 0.05$, $n = 3$). 2-Y, 15-Y, and 30-Y indicate tea gardens planted continuously for different years (2, 15, and 30).

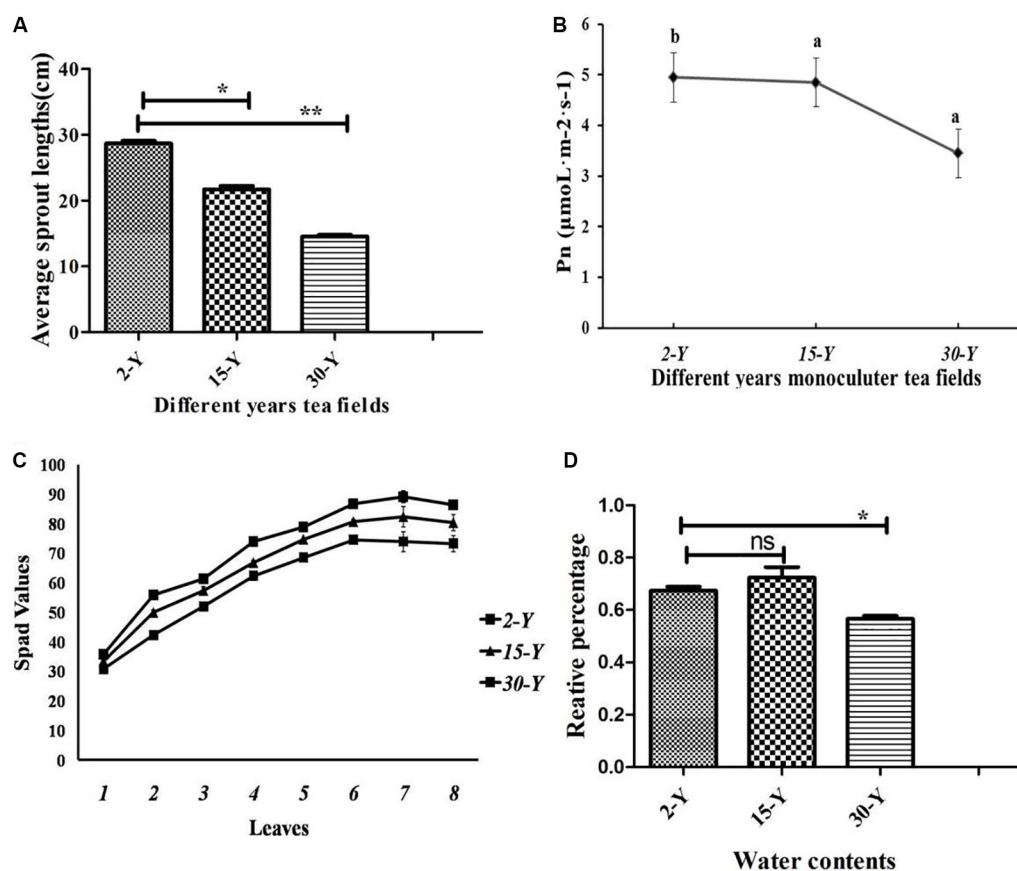


FIGURE 3 | Physiological characteristics of tea leaves. **(A)** Length of new tea sprouts ($P < 0.05$, $n = 5$), **(B)** photosynthetic rate (Pn) of the third tea leaf in young sprout ($P < 0.05$, $n = 3$), **(C)** content of tea leaf chlorophyll with young sprouts from bottom to top ($P < 0.05$, $n = 8$), **(D)** water contents in five leaves. Stars in the column show significant differences (LSD test, $P < 0.05$, $n = 5$) under different fields. 2-Y, 15-Y, and 30-Y indicate tea gardens planted continuously for different years (2, 15, and 30).

tube and add an equal amount of glycerol (1:1) to it and preserve these colonies at -20°C for further use. DNA was extracted from each labeled bacterial suspension. The DNA were sent to the company for 16s RNA sequencing to identify key microbes.

The Influence of Identified Allelochemicals on the Growth of Selected Model Microbes

The LB Liquid broth was diluted six times and 5 ml was distributed in each glass tube; all tubes were sterilized at 121°C for 20 min. After autoclaving, the broth was amended with an appropriate amount of identified allelochemical stock solution that was filtered through a $0.22\text{-}\mu\text{m}$ filtration membrane. The final concentrations were 0, 1.25, 2.5, 5, and $10\text{ }\mu\text{g/ml}$, respectively, for each allelochemical. The control was set in the same amount of ddH₂O instead of allelochemical. Each treatment was repeated three times. Fifty microliters of each labeled bacteria was added to each tube, which has been already activated. All tubes were placed in a thermostatic shaker at 30°C and 200 rpm for 72 h. Further, $200\text{ }\mu\text{l}$ of bacterial culture was taken in a 96-hole enzyme label plate (Thermo Scientific Multiskan Mk3, Shanghai, China). To obtain the appropriate

bacterial growth, the OD at 600 nm was regularly checked (Wu H. et al., 2016).

Biotransformation of Catechin and Its Effect on Soil pH

The LB liquid broth was diluted six times to minimize LB nutrient effect on bacterial growth and 5 ml was distributed in each glass tube. All the tubes were sterilized at 121°C for 20 min. After precooling, $50\text{ }\mu\text{l}$ of each labeled bacteria was added to each tube that has been already activated. The broth was amended with an appropriate amount of EC stock solution that was filtered through a $0.22\text{-}\mu\text{m}$ filtration membrane. After growing bacteria, an appropriate amount of broth was centrifuged at 13,000 rpm for 5 min at 4°C . The supernatant was taken for pH determination and for identification of their metabolites by LC-ESI-MS at 24, 48, 72, 96, and 120 h, respectively (Wang et al., 2013).

Statistical Analysis

All the experiments included three replications and repeated at least two times. The effects of the different treatments were analyzed by LSD test using SPSS 19.0, and the graphs were displayed using GraphPad Prism 5 software.

RESULTS

The Performance of Tea Plants in Consecutively Ratooned Monoculture Soil

Tea plants grown in soil taken from different tea plantations (15-Y and 30-Y) showed weak growth, wilting, chlorosis, and regeneration obstacles compared to tea plants grown in the recently established tea garden (2-Y) soil (**Figure 1**). In addition, compared to the 2-Y garden, tea quality parameters, especially theophylline (TPY), total polyphenols (TPP), theanine (TNN), and total amino acids (TAA), were significantly lower in old gardens (15-Y and 30-Y) as shown (**Figures 2A–D**). Regarding physiological and growth parameters, continuous tea cropping significantly reduced net photosynthetic rate (Pn), new tea sprout's length, and chlorophyll content of the third leaf (**Figures 3A–D**). **Supplementary Table S1** shows the tea yield obtained from the tea gardens with different planting histories. In comparison with 2-Y gardens, the old gardens (15-Y and 30-Y) significantly reduced 100 buds fresh and dry weight (**Supplementary Table S1**). These results indicate that tea quality and productivity are negatively affected by long-term tea monoculture.

Analysis of Soil Enzyme Activities in Different Age Monoculture Tea Plantations

The activity of phenol oxidase peroxidase were promoted by 13.78, 14.92, and 51.52% in 2-, 15-, and 30-Y tea plantations, respectively, as compared to CK. Likewise, the activity of peroxidase was sharply increased by 11.58, 10.12, and 17.88%, respectively, in 15- and 30-Y tea plantations as compared to CK (**Supplementary Table S2**).

Leaf Litter Biomass Input Into Tea Gardens and Analysis of Their Allelochemicals

Our results indicated that in each pruning turn, about 615, 838, and 969.67 g/plant leaf litters were returns into 2-Y, 15-Y, and 30-Y garden, respectively (**Supplementary Figure S3**). Five compounds including EGC, EGCG, EC, catechin (\pm C), and ECG, were identified in the leaf litters in both the newly planted tea gardens (2-Y) and the continuously monocultured tea gardens (15-Y and 30-Y) (**Figure 4**). Moreover, in the continuous monoculture tea gardens (15-Y and 30-Y tea gardens) the input of EGC, \pm C, EC, EGCG, and ECG allelochemicals were high as compared to young tea plantations (2-Y).

Response of Bacterial Genera Under Continuous Monoculture Soil

Heat map analysis displayed that *Acidobacteria* (*Candidatus_koribacter*) and *Proteobacteria* (*Rhodocyclus*, *Agrobacterium*, *Caulobacter*, *Hydrogenophaga*, and *Methylibium*) were the dominant bacterial phyla in CK (**Figure 5A**). The genera (e.g., *Pseudomonas*, *Burkholderia*, *Salinispora*, and *Helicobacter*)

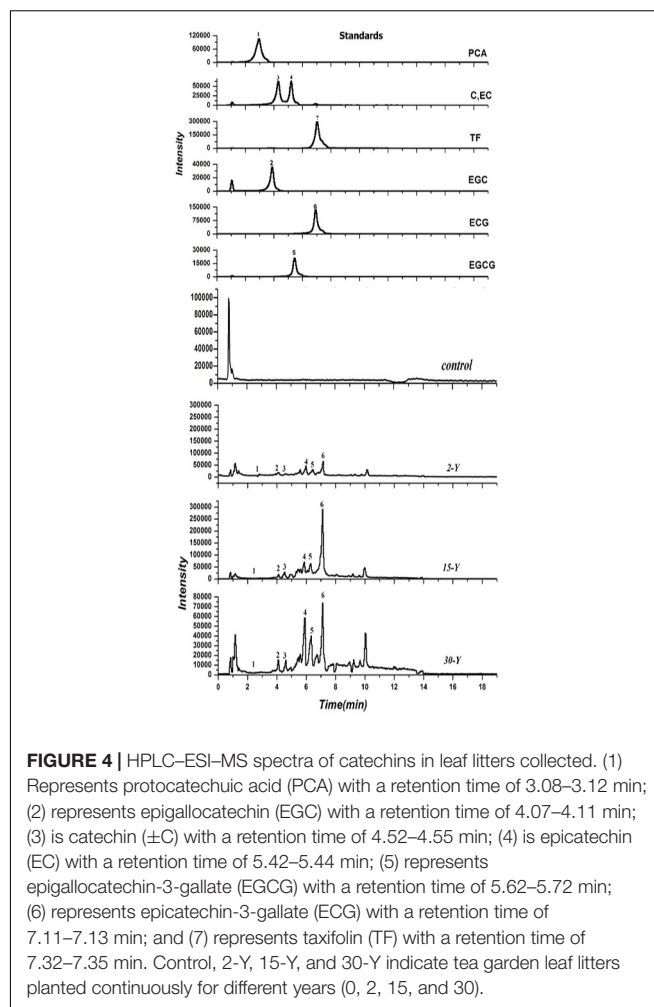


FIGURE 4 | HPLC-ESI-MS spectra of catechins in leaf litters collected. (1) Represents protocathechuic acid (PCA) with a retention time of 3.08–3.12 min; (2) represents epigallocatechin (EGC) with a retention time of 4.07–4.11 min; (3) is catechin (\pm C) with a retention time of 4.52–4.55 min; (4) is epicatechin (EC) with a retention time of 5.42–5.44 min; (5) represents epigallocatechin-3-gallate (EGCG) with a retention time of 5.62–5.72 min; (6) represents epicatechin-3-gallate (ECG) with a retention time of 7.11–7.13 min; and (7) represents taxifolin (TF) with a retention time of 7.32–7.35 min. Control, 2-Y, 15-Y, and 30-Y indicate tea garden leaf litters planted continuously for different years (0, 2, 15, and 30).

were dominant in RS30, whereas *Devosia*, *Coprococcus*, *Opitutus*, *Sphingomonas*, *Prevotella*, *Allobaculum*, *Bacteroides*, and *Oscillospira* were dominant in RS2. In RP2, the dominant genera included *Candidatus_solibacter*, *Opitutus*, *Devosia*, *Sphingomonas*, and *Rhodoplanes*, while in RP30, the dominant groups found were of *Flavobacterium*, *Novosphingobium*, *Polaromone*, *Pedobacter*, *Janthinobacterium*, *Paenibacillus*, *Escherichia*, and *Bifidobacterium*, respectively. Similarly, *Chitinophaga*, *Steroidobacter*, *Bradyrhizobium*, and *Methylibium* were predominant in ES2, and *Bradyrhizobium*, *Halomonas*, and *Bacillus* were predominant in ES30. Stamp analysis verified that *Salinispora*, *Pseudomonas*, and *Burkholderia* were dominant genera in both rhizoplane (RP30) and rhizosphere (RS30) compartments of old tea garden (30-Y) as compared with Bulk soil (CK) and fresh soil tea plantation (2-Y) rhizosphere (RS2) and rhizoplane (RP2) (**Figures 5B–D**, respectively).

Abundance of Total Bacteria and *Pseudomonas* and *Bacillus* Genera by Colony-Forming Units' qPCR Methods

Employing high-throughput sequencing, colony-forming units, and qPCR, we characterized the entire bacterial community

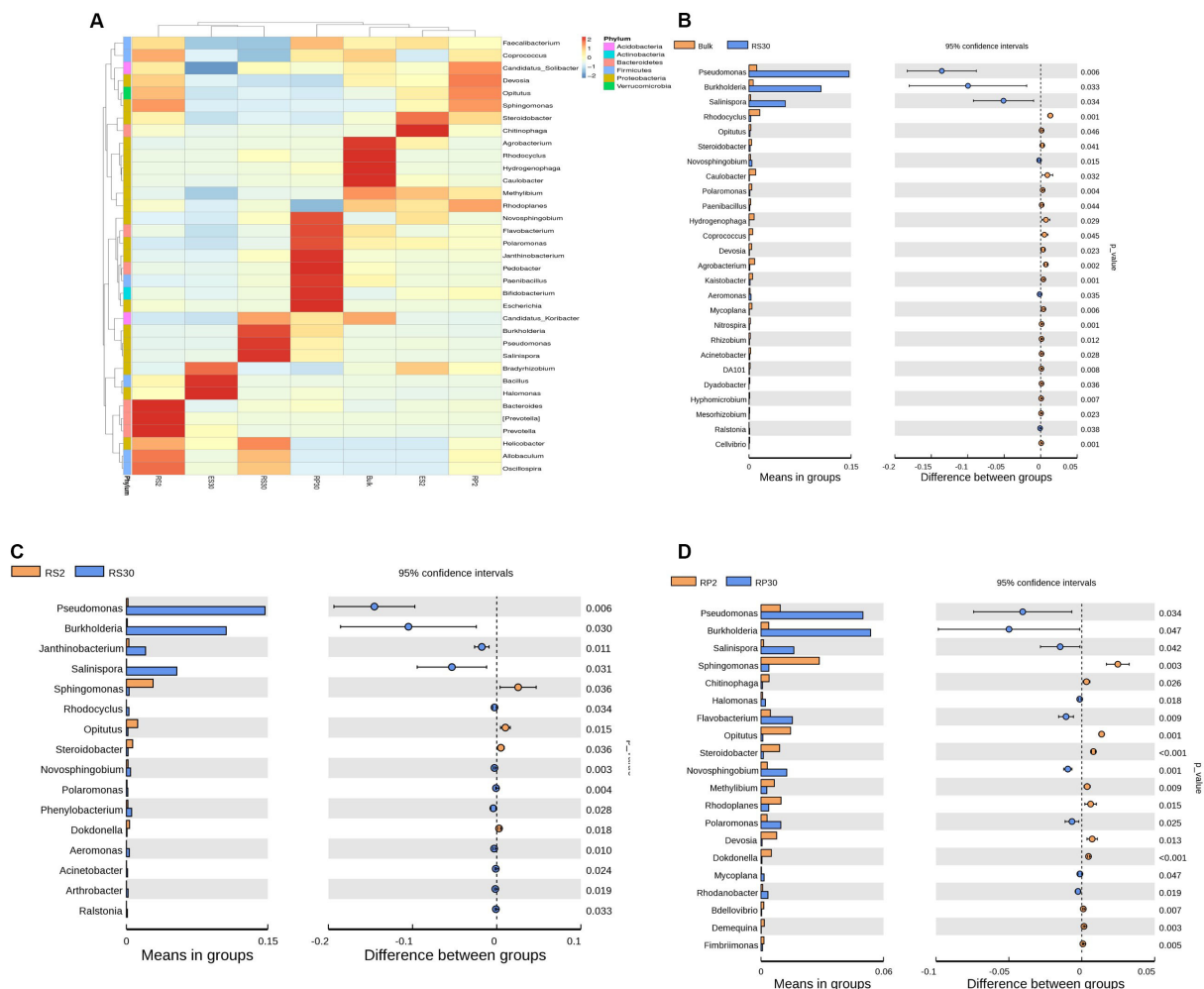


FIGURE 5 | (A) Heat map showing the distribution of the 35 most abundant genera. Bulk soil (CK), rhizosphere (RS2 and RS30), rhizoplane (RP2 and RP30), and endosphere (ES2 and ES30) of tea gardens continuously cropped for 2 and 30 years, respectively (LSD test, $P < 0.05$, $n = 3$). **(B)** Error bar plots displaying significant difference of most abundant genera among in Bulk (CK) and 30-Y rhizosphere (RS30) tea plantation (t -test, $P < 0.05$, $n = 3$). **(C)** Error bar plots displaying significant difference of most abundant genera in the 2-Y rhizosphere (RS2) and 30-Y rhizosphere (RS30) tea plantation (t -test, $P < 0.05$, $n = 3$). **(D)** Error bar plots displaying significant difference of most abundant genera in 2-Y rhizoplane (RP2) and 30-Y rhizoplane (RP30) tea plantation (t -test, $P < 0.05$, $n = 3$). The points explain differences among ("CK and RS30"), ("RS2 and RS30"), and ("RP2 and RP30") (red, green, and blue bars, respectively); the values on the right-hand side display the P -values derived from the t -test error bar plots.

composition and structure, including *Pseudomonas* and *Bacillus* in the CK, 2-Y, and 30-Y tea gardens. These results indicated that within 30-Y, the entire bacterial population and plant growth-promoting bacterial genus (*Bacillus*) were reduced compared to 2-Y and CK soil, while the catechin degradation bacteria (*Pseudomonas*) was increased (Figure 6).

In vitro Interactions of Different Types of Allelochemicals With Model Growth-Promoting Bacteria

Based on the HPLC-LC/MS identification of different types of active catechins allelochemicals in the leaf litter, a series of single and mixed allelochemicals with the final concentration gradients (0, 1.25, 2.5, 5, and 10 $\mu\text{g/ml}$) were set to identify the

response of selected model growth-promoting bacteria such as *Bacillus* found in the tea garden. Our results determined that the different allelochemicals concentration significantly influenced the growth of selected growth-promoting bacteria. Moreover, as the concentration of these identified allelochemicals increased, the growth of *Bacillus* decreased (Figure 7).

In vitro Interactions of Different Types of Allelochemicals With Model Catechins Degrading Bacteria

Similarly, a series of single and mixed allelochemicals with the final concentration gradients (0, 1.25, 2.5, 5, and 10 $\mu\text{g/ml}$) were also set to identify the response of catechin degrading bacteria, *Pseudomonas* spp., which was dominant in the 30-Y

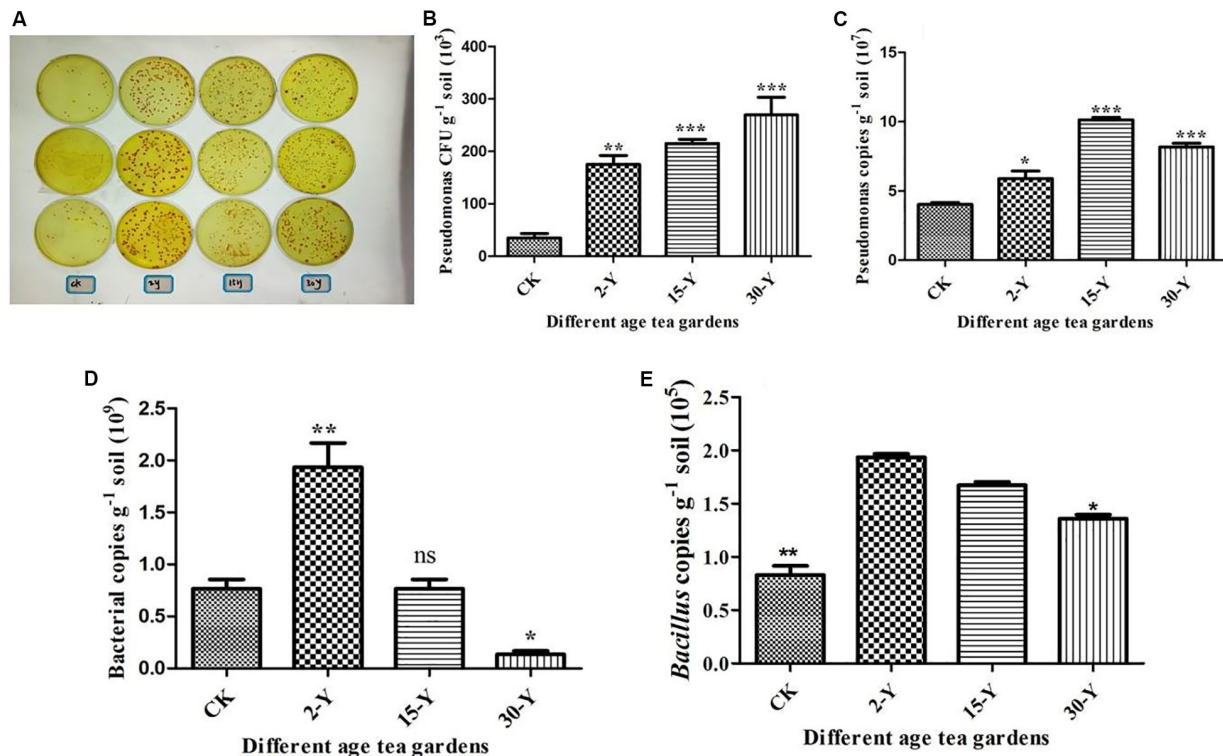


FIGURE 6 | Abundance of total bacteria, *Pseudomonas* and *Bacillus* genera by qPCR analysis. **(A)** *Pseudomonas* populations. **(B)** CFU of *Pseudomonas* per gram of soil. **(C)** The contents of *Pseudomonas* genera in tea rhizosphere soils after different years of monoculture by qPCR analysis using the primer sets Pstf/Psr (Tan and Ji, 2010). **(D)** The total bacterial contents by qPCR analysis using the primer set Eub338/Eub518. **(E)** The contents of *Bacillus* genera in tea rhizosphere soils after different years of monoculture by qPCR analysis (Wu H. et al., 2016). CK, 2Y, 15Y, and 30Y refer to bulk soil without planting any crop, newly planted 2-year garden, and replanted 15- and 30-year garden, respectively. Stars in the column show significant differences (LSD test, $P < 0.05$, $n = 3$).

consecutively monoculture tea garden. The results showed that the different allelochemicals concentration have no significant effect on the growth of *Pseudomonas* spp. Low concentration at 1.25 $\mu\text{g/ml}$ of $\pm\text{C}$ promoted the growth of *Pseudomonas* spp. However, the high concentration (10 $\mu\text{g/ml}$) of PCA and a mix of seven known allelochemicals significantly inhibit the growth of the selected model bacteria. It is therefore suggested that the dominant bacteria such as *Pseudomonas* used these leaf litters as a carbon substrate to some extent (Figure 8).

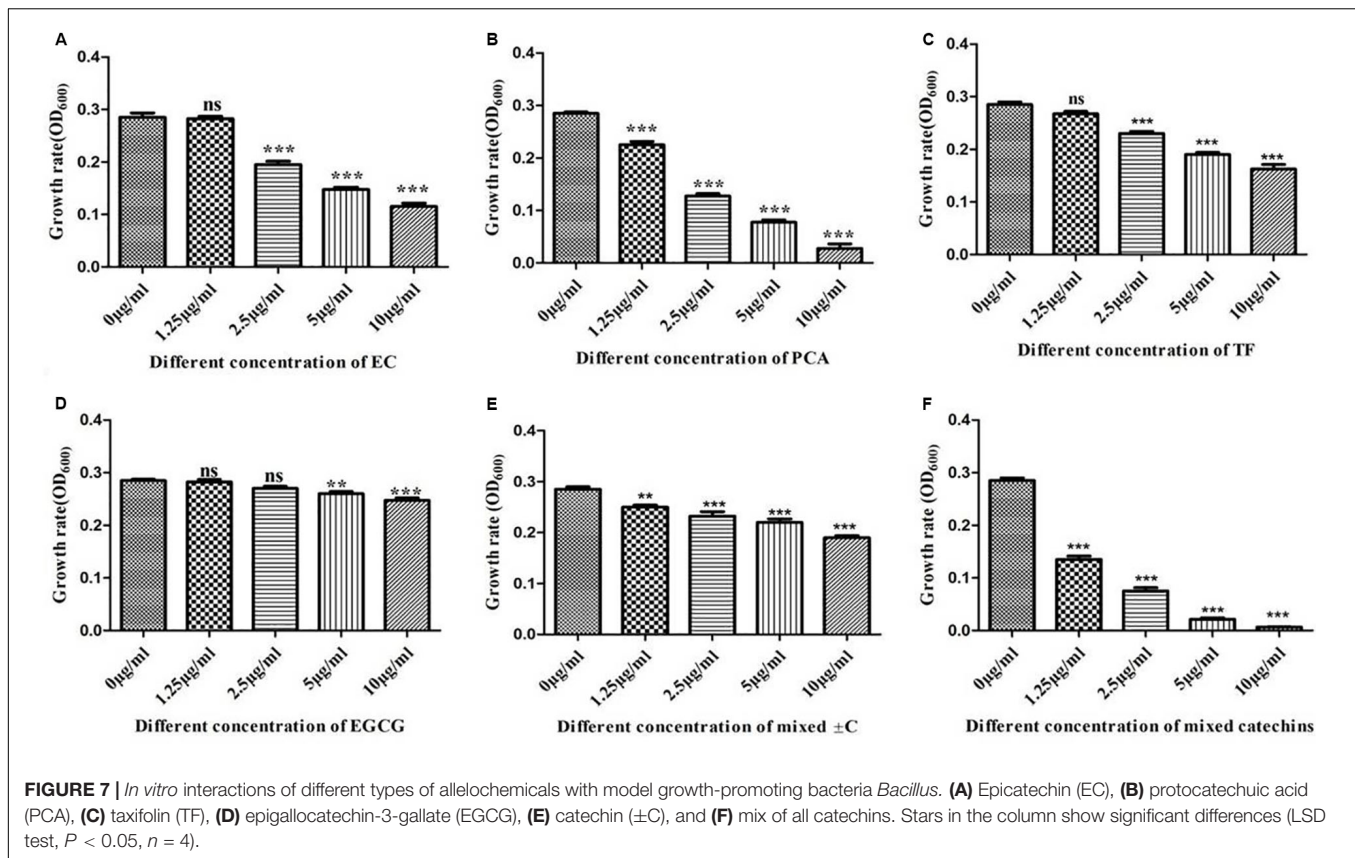
Biotransformation of Catechins and Their Effect on pH

In order to understand the role of the dominant bacterial populations in lowering the pH of the tea rhizosphere with the increasing tea planting age and consecutive monoculture problems, the dominant *Pseudomonas* was selected as a model bacterium. Epicatechin (EC) with the final concentration gradients 5 $\mu\text{g/ml}$ and activated *Pseudomonas* bacterium (50 μl) was subjected to six times diluted LB medium. After 48 and 72 h, the media were purified and filtered for pH and metabolite analysis. The LC-MS analysis results identified the compound that was PCA after 72 h (Figure 9A). Moreover, results showed that the pH of the media drops to 0.36-fold after 72 h (Figure 9B). These results suggested that the dominant bacteria identified in

30-Y tea plantation used leaf litter. After using these substrates, the bacteria convert these allelochemicals into different types of acids like PCA, which may change the pH of the tea rhizosphere soil over time.

DISCUSSION

Our results indicated that long-term monoculture has an adverse effect on tea quality (TNN, TPY, TPP, and TAA content) and physiological and growth parameters of tea plants compared to freshly grown tea plants, which is consistent with previous findings (Arafat et al., 2017; Jiang et al., 2019). Similarly, previous studies have shown that tea production, as well as quality, declines dramatically with increasing cultivation time (Illukpitiya et al., 2004; Arafat et al., 2019). Owing to shifts in soil physicochemical and biological properties, a long-term tea monoculture can lead to a “soil-sickness” or “replanting disease,” which has an adverse impact on tea productivity (Owuor, 1996; Huang et al., 2006; Utkhede, 2006; Kamau, 2007; Qu and Wang, 2008). The underlying factors of soil sickness are usually considered to be nutrient imbalances, autotoxins generation, and/or shift in soil microbial community structure and diversity (Zhao et al., 2015). Plant-associated microbial communities are also considered as a second genome of the plant and are essential for soil fertility



and plant health (Berendsen et al., 2012). The regulation of soil microbiota owing to allelochemicals interaction is closely associated with replanting disease in agricultural systems (Li et al., 2014; Liu et al., 2017).

Soil microbial biomass is commonly considered to be associated with soil fertility, including as a potential indicator of soil quality (Shengchun and Yinghua, 2003; Zhang and Zhang, 2003). Due to changes in soil management approaches, soil microbial dynamics shift faster than soil organic matter (Shengchun and Yinghua, 2003). Beneficial microbes, especially plant growth-promoting bacteria, are crucial for nutrient availability and plant growth (Acosta-Martínez et al., 2010; Cipollini et al., 2012; Huang et al., 2013). In the present study, we found *Burkholderia* and *Pseudomonas* as the dominant genera, while plant growth-promoting bacteria such as *Prevotella*, *Bacillus*, and *Sphingomonas* were significantly lower in the 30-Y tea garden. The qPCR results also confirmed that the bacterial density per gram of soil of 30-Y plantation was significantly lower compared to 2-Y. However, *Pseudomonas* population was selectively increased in 30-Y. It is generally believed that tea garden soils have an inhibitory effect on soil microorganisms due to high acidity and aluminum toxicity. We have previously reported that continuous planting of tea (30 years) has no significant effect on soil nutrients (N, P, and K). However, soil pH in continuous tea fields (30 years) was significantly lower than in the bulk and 2-year garden soil (2-Y) (Arafat et al., 2017).

Various effects of plant polyphenols have previously been observed under artificial laboratory conditions, indicating that they stimulate or inhibit (depending on the compound) the growth of the nitrifiers (Rice and Pancholy, 1973; Baldwin et al., 1983). Schimel et al. (1998), proving that plant polyphenols have a controlling effect on nutrient dynamics and species interaction in Alaskan taiga. The complexity of soil food webs, including the covariance of other compounds, makes it challenging to demonstrate the effects of polyphenols on soil biota directly. HPLC-LC/MS findings and *in vitro* interaction of the selected model growth-promoting bacteria with allelochemicals suggest that the various types of active catechins' allelochemical concentration as they enhanced, in turn, significantly suppressed *Bacillus* growth (Figure 7). *In vitro* analysis of soil microbial density by catechin showed inhibition and microbe suppression (Inderjit et al., 2009). Catechin-degrading bacteria (*Pseudomonas* spp.) were greater in terms of abundance in the 30-Y tea garden and different concentrations of allelochemicals did not significantly influence the growth of *Pseudomonas* spp. (Figure 8). At a lower concentration (1.25 μ g/ml of \pm C), the growth of *Pseudomonas* was increased; on the other hand, a high frequency (10 μ g/ml) of PCA and the combination of seven known allelochemicals significantly suppressed selected bacteria growth. These results suggest that dominant bacteria such as *Pseudomonas* utilized these catechins in leaf litter as a carbon substrate to a certain level. Wang et al. (2013) also showed that *Pseudomonas*

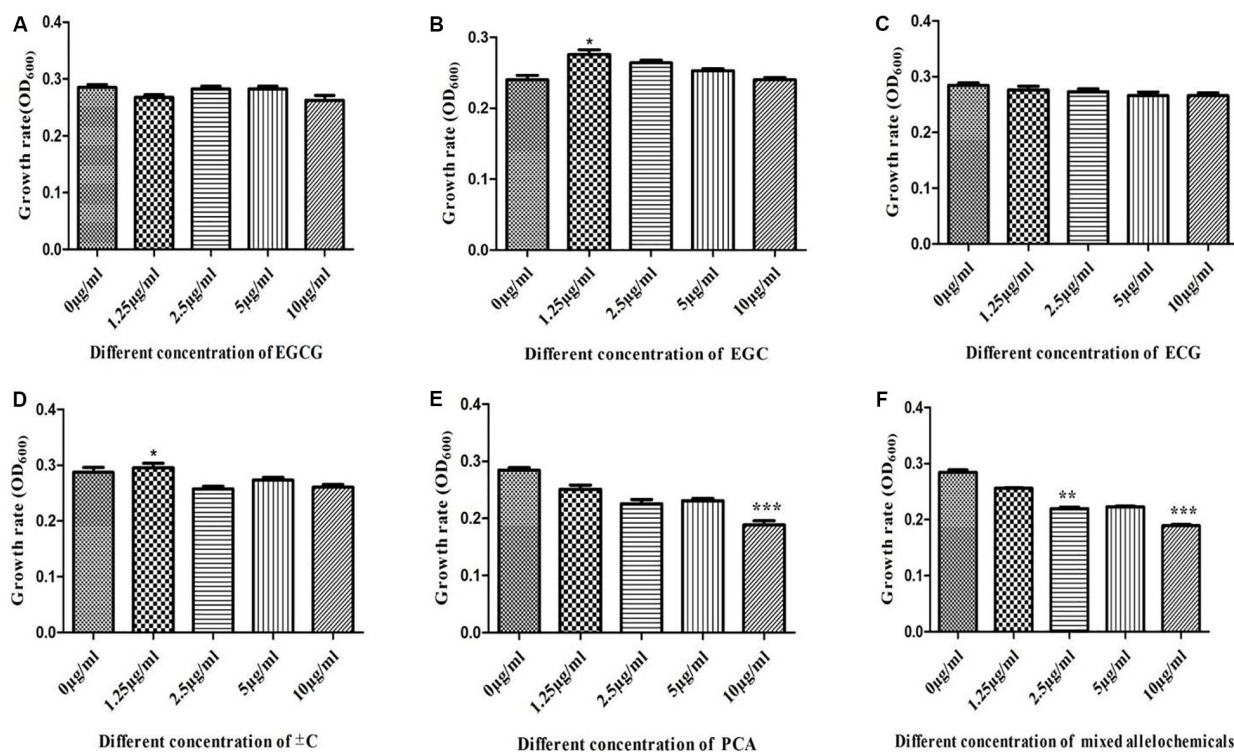


FIGURE 8 | *In vitro* interactions of different types of allelochemicals with model catechins degrading bacteria *Pseudomonas*. **(A)** Epigallocatechin-3-gallate (EGCG), **(B)** epigallocatechin (EGC), **(C)** epicatechingallate (ECG), **(D)** catechin (±C), **(E)** protocatechuic acid (PCA), and **(F)** mix of all catechins. Stars in the column show significant differences (LSD test, $P < 0.05$, $n = 4$).

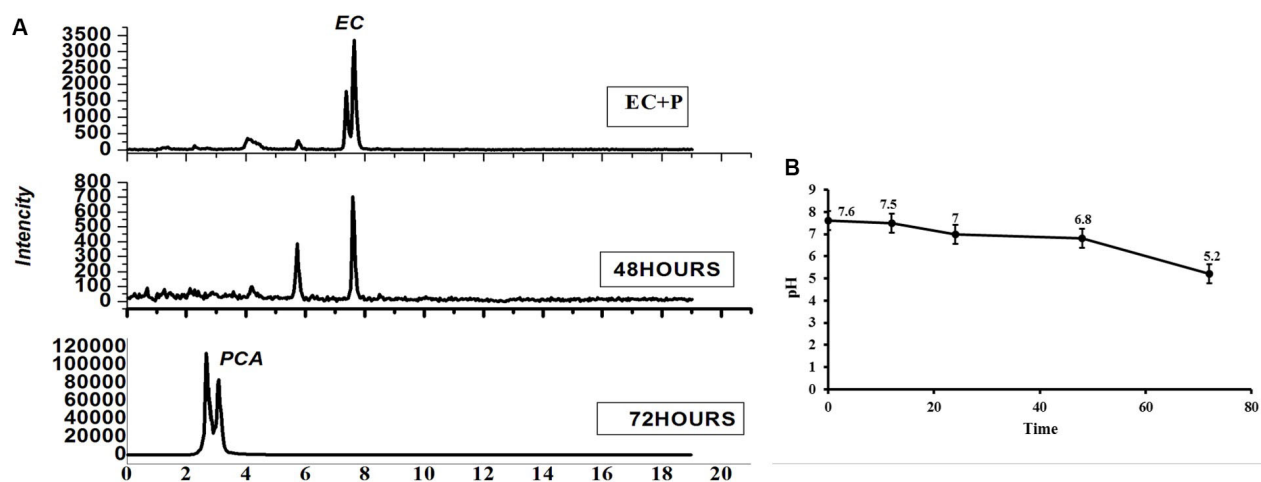


FIGURE 9 | Impact of catechin degradation on pH. **(A)** Catechin degradation, **(B)** effect on pH. EC + P represents Epicatechin (EC) + *Pseudomonas* (P) and PCA represents protocatechuic acid.

can use catechins as substrate and biotransformed it into most toxic allelochemicals. We aimed to examine if the dominant bacterial groups associated with tea roots in the 30-Y tea garden has a role in soil acidification. EC with the final concentration of 5 µg/ml, which can activate the growth of *Pseudomonas*, was investigated for biotransformation

and resulting metabolites. By LC-MS analysis, we detected compounds, especially PCA, and reduced the pH of the media (0.36 folds) after 72 h, indicating that abundant bacteria detected in the 30-Y garden converted the polyphenols in different types of acids (PCA, ferulic acids, etc.), which may lead to soil acidification.

CONCLUSION

Overall, long-term tea monocropping had a significant impact on the tea in terms of both quality and quantity, as well as soil regenerative capacity. We also studied the polyphenol interaction with microorganisms in tea residue and rhizosphere, which ultimately declined the plant growth-promoting bacteria. However, below a particular concentration gradient, no significant effect was observed on the growth of *Pseudomonas*. We found that *Pseudomonas* biotransformed polyphenols in various organic acids (PCA, TF, and EC) and changed soil pH, which may be the putative reason for altering the microbial structure and composition of tea rhizosphere during continuous tea planting. We recommend eliminating plant residues and trimmed materials from the tea plantation to avoid the accumulation of allelochemicals, which can delay soil acidification.

DATA AVAILABILITY STATEMENT

The data set has been submitted to the NCBI-Sequence Read Archive with the SRA accession: PRJNA626070. Temporary Submission ID: SUB7295720.

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AUTHOR CONTRIBUTIONS

WL, SL, and YA conceived the study. YA and IU wrote the manuscript. YA, YJ, and ZC performed field sampling and lab experiments. YA, IU, TC, and HZ performed the statistical analyses. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

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Pea (*Pisum sativum* L.) Plant Shapes Its Rhizosphere Microbiome for Nutrient Uptake and Stress Amelioration in Acidic Soils of the North-East Region of India

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Rhizosphere microbiome significantly influences plant growth and productivity. Legume crops such as pea have often been used as a rotation crop along with rice cultivation in long-term conservation agriculture experiments in the acidic soils of the northeast region of India. It is essential to understand how the pea plant influences the soil communities and shapes its rhizosphere microbiome. It is also expected that the long-term application of nutrients and tillage practices may also have a lasting effect on the rhizosphere and soil communities. In this study, we estimated the bacterial communities by 16S rRNA gene amplicon sequencing of pea rhizosphere and bulk soils from a long-term experiment with multiple nutrient management practices and different tillage history. We also used Tax4Fun to predict the functions of bacterial communities. Quantitative polymerase chain reaction (qPCR) was used to estimate the abundance of total bacterial and members of Firmicutes in the rhizosphere and bulk soils. The results showed that bacterial diversity was significantly higher in the rhizosphere in comparison to bulk soils. A higher abundance of Proteobacteria was recorded in the rhizosphere, whereas the bulk soils have higher proportions of Firmicutes. At the genus level, proportions of *Rhizobium*, *Pseudomonas*, *Pantoea*, *Nitrobacter*, *Enterobacter*, and *Sphingomonas* were significantly higher in the rhizosphere. At the same time, *Massilia*, *Paenibacillus*, and *Planomicrobium* were more abundant in the bulk soils. Higher abundance of genes reported for plant growth promotion and several other genes, including iron complex outer membrane receptor, cobalt-zinc-cadmium resistance, sigma-70 factor, and ribonuclease E, was predicted in the rhizosphere samples in comparison to bulk soils, indicating that the pea plants shape their rhizosphere microbiome, plausibly to meet its requirements for nutrient uptake and stress amelioration.

Keywords: conservation agriculture, residue management, proteobacteria, rhizosphere, tillage

INTRODUCTION

Intensive agriculture practiced to meet the food production needs of the ever-increasing population in the changing climate is posing a severe strain to soil and environmental health. Adopting climate-friendly practices appears to be the best strategy to achieve sustainability in agriculture (Rahi, 2017). Conservation agriculture (CA) is an evolved agroecosystem management approach for preserving and enriching the environmental resources while improving and sustaining the crop productivity amid possible environmental stresses (Das et al., 2014; Godfray and Garnett, 2014; Mango et al., 2017; Muzangwa et al., 2017). Comprehensively, CA requires the simultaneous application of viable crop rotations, minimal soil disturbances, and crop residue retention (Das et al., 2014, 2017; Kuotsu et al., 2014; Nichols et al., 2015). Incorporation of crop rotation and diversifying cropping systems with legume crops, including pea, lentil, and chickpea, is a key step toward CA, which increases the crop yield and decreases the impact of intensive cultivation on the environment (Laik et al., 2014; Gan et al., 2015).

The northeast region of India is ideally characterized by fragility, marginality, inaccessibility, and ecosystem diversity. Generally, the monocropping system of rice (*Oryza sativa* L.) cultivation is practiced in the northeast region of India (Das et al., 2018). Zero tillage cultivation of pea (*Pisum sativum* L.) has been considered beneficial to enhance the cropping intensity in the region (Das et al., 2018). Several strategies, including no-till, minimal tillage, and conventional tillage; *in situ* residue retention (ISRR); weed biomass (WB); green leaf manure (GLM) and farmyard manure; and rotation of peas and rice, are being tested on experimental farms of the ICAR Research Complex for North Eastern Hill Region, Umiam, Meghalaya, India (Das et al., 2014, 2017, 2018; Kuotsu et al., 2014). Although pea is cultivated under no-till and recommended dose of fertilizer and farmyard manure, it is likely that the residual effect of different tillage and nutrient application treatments followed for Kharif rice imminently influence pea growth and productivity during rabi season. The impact of reduced tillage intensity has been observed on the northeast India soils, as it potentially reverses the soil degradation and improves soil quality and crop productivity (Das et al., 2014; Kuotsu et al., 2014), but there are no reports on their impact on the soil microbial communities.

The majority of the soils in northeast regions of India are acidic (pH 5.0–6.0) and rich in organic carbon, which makes them deficient in available phosphorus, medium to low in available potassium, and highly rich in iron and aluminum (Thakuria et al., 2016). The acidic soils pose a severe challenge for pea cultivation due to the high availability of aluminum (Al), iron (Fe), and manganese (Mn), which can be toxic to the plants (Bojórquez-Quintal et al., 2017). The high availability of Fe and Al also leads to low availability of certain essential elements such as phosphorus, calcium, magnesium, and molybdenum (Vyas et al., 2007; Dambrine, 2018). Moreover, pea plants have high phosphorus requirements for nodule formation and its function and optimum photosynthesis (Powers and Thavarajah, 2019).

Microbial communities are vital indicators of soil quality and are highly sensitive to soil management practices

(Fierer et al., 2007; Sánchez-Cañizares et al., 2017). Previous studies have suggested that no-till alters microbial diversity and activity significantly when compared to conventional tillage (Degruene et al., 2017; Babin et al., 2019). Similarly, nutrient and residue management practices such as the application of chemical fertilizers often influence the endogenous microbial communities (Fierer et al., 2007). However, soil bacterial diversity is known to be affected by the numerous environmental factors including soil pH (Lauber et al., 2009; Cho et al., 2016; Sánchez-Cañizares et al., 2017), the chemistry of soil organic matter (Lupatini et al., 2017), plant species grown in the soil (Yamamoto et al., 2018), secretion of different root exudates by the plant species (Sasse et al., 2018; Huang et al., 2019), and processing of the soil before the cultivation of the plants (Moreno-Espindola et al., 2018). Plant root exudates primarily stimulate the microbial activity in the rhizosphere, resulting in increased microbial active biomass and abundance in the rhizosphere by several folds in comparison to surrounding bulk soil (Lugtenberg and Kamilova, 2009). During this process of increased microbial activity in plant rhizosphere, the selection of some specific microorganisms has been observed, leading to the buildup of plant-specific community in the rhizosphere (Hu et al., 2018). A recent study suggested that enrichment of members of bacterial families known for P-solubilizing, such as Rhizobiaceae, Enterobacteriaceae, Pseudomonadaceae, and Burkholderiaceae, has been observed in the pea rhizosphere (Harkes et al., 2020). In comparison to cereals, legumes pose a much stronger influence on the selection of rhizosphere microbiome (Hamel et al., 2018; Harkes et al., 2019, 2020).

Conservation agriculture and the introduction of pea as a rotation crop in the acidic soils of the North East Indian region have been proven profitable and advantageous to the farmers (Das et al., 2018). Therefore, it is important to decipher the structure of microbial communities under long-term CA to develop future strategies to improve the soil health in the region and expand pea cultivation. We hypothesize that different treatments of CA will influence the microbial communities in the acidic soils of North East India. We also hypothesize that the rotation crop plant (i.e., pea) shapes its rhizosphere microbiome to meet its nutrient uptake and stress tolerance requirements in the acidic and iron-rich soils of the North East Indian region.

MATERIALS AND METHODS

Sample Collection

Different tillage and residue management treatments are maintained for the last 8 years by alternatively cultivating rice followed by pea cultivation in the experimental fields of the ICAR Research Complex for NEH Region (950 m above mean sea level, 25°30'N latitude and 91°51'E longitude), located in Eastern Himalayan Region, India. The experimental site is characterized by a subtropical climate with mild winter and warm summer conditions. The average annual rainfall received is 2,000 mm. The maximum temperatures (25–32.3°C) were observed during July, and lower temperatures of 3°C to 14°C were recorded during the January–February months. Three different tillage

treatments, that is, no-till, minimum tillage, and conventional tillage, were practiced during the rice cultivation (Das et al., 2014). Six treatments of nutrient management including 100% NPK, 50% NPK, and 50% NPK with ISRR at 5 Mg ha⁻¹ (used after chopping into 10 cm pieces); 50% NPK with WB, that is, *Ambrosia artemisiifolia* (locally available weed) at 10 Mg ha⁻¹ on fresh weight basis used after chopping into ~10 cm size; 50% NPK with GLM, that is, *Tephrosia purpurea* (leguminous hedge plant grown in the fences and bunds) used at 10 Mg ha⁻¹ on fresh weight basis after chopping into ~10 cm size; and 100% organic treatment, that is, farmyard manure (at 5 t/ha) with rock phosphate (at 150 kg/ha) were maintained during rice cultivation. Cultivation of pea was undertaken as a rotation crop by following a no-till practice. Pea seeds were sown at a rate of ~80 kg/ha by opening narrow troughs of optimum depth with the help of manually operated furrow opener in between two rice lines, thus giving a row-to-row spacing of 20 cm for pea. The recommended dose of nutrients and seeds were placed in the furrow and covered with soil: Farm Yard Manure (FYM) mixture (2:1 ratio) for better seed and soil contact. As a whole, only one treatment was maintained as 100% organic treatment. In contrast, the remaining treatments were replaced with 50% inorganic treatment (20:60:40 N:P₂O₅: K₂O kg/ha), along with 50% of crop residues (WB, rice ISRR, GLM) incorporation. The N, P, and K for rice and pea were supplied through urea (46% N), single superphosphate (16% P₂O₅), and muriate of potash (60% K₂O), respectively. For microbial community analysis, bulk soil and pea rhizosphere samples were collected from each treatment plot. Bulk soil samples were collected in triplicates and pooled together from all the treatment plots. Three pea plants growing in the midrows of each plot were uprooted using a shovel. The whole root systems of the plants were separated from the loosely adhered soil by gentle shaking. The samples from each plot were pooled together in a sterilized polythene bag and transported to the laboratory under 4°C. All the samples were immediately processed for community DNA extraction.

Chemical Properties of Soil Samples

Three soil samples were obtained after harvest of pea from 0 to 15 cm soil depths from each plot using a soil auger and composited. Soil samples were air dried, grinded, and passed through 2 mm sieve and used for analyzing soil fertility parameters, such as soil pH determined in 1:2 soil water suspensions with the help of combined glass electrode on microprocessor-based pH meter (Jackson, 1973). Available N was estimated by the alkaline permanganate method (Subbiah and Asija, 1956), available P by Bray's extraction method (Bray and Kurtz, 1945) using spectrophotometer, and available K by neutral normal NH₄OAC extraction (Knudsen et al., 1982) using flame photometer. Soil organic carbon (SOC) concentration was determined by the Walkley and Black method (Nelson and Sommers, 2005). The total carbon was determined by the dry combustion method (Nelson and Sommers, 2005) using a Total Organic Carbon (TOC) analyzer (Elementer Vario Select, Langensfeld, Germany). The SOC was assumed to be equal to the total C with negligible inorganic C concentration as the soil pH was below 7 (Jagadamma and Lal, 2010).

Soil microbial biomass carbon (SMBC) was estimated by soil fumigation technique (Anderson and Ingram, 1993; Tabatabai, 1994). Soil microbial biomass nitrogen (SMBN) was estimated by soil fumigation method (Jimenez and Ladha, 1993). Soil biomass P is calculated from the difference between the amount of inorganic P (Pi) extracted by 0.5 (Spm) NaHCO₃ (pH 8.5) from fresh soil fumigated with CHCl₃ and the amount extracted from nonfumigated soil (Brookes et al., 1981). Soil dehydrogenase activity (DHA) was determined by the triphenyl formazan reduction method (Casida et al., 1964).

Isolation of Rhizosphere Soil, DNA Extraction, and Next-Generation Sequencing

Roots of pea plants were transferred to the 15 mL sterilized centrifuge tube and submerged in the 10 mL phosphate-buffered saline (PBS) to harvest rhizosphere soils. The tubes were subjected to sonication for 60 s, and roots were transferred to a fresh centrifuge tube filled with 10 mL PBS. The sonication step was repeated once again to remove the soil adhered to the roots. The process of washing in PBS and sonication was repeated three times to get the total rhizosphere soil into the PBS. The rhizosphere soil containing PBS was centrifuged at 5,000 × g for 10 min. The soil pellet was used for the extraction of rhizosphere metagenomic DNA. Total community DNA was extracted from bulk soil and rhizosphere soil samples using the DNeasy PowerSoil kit (Qiagen, Hilden, Germany) following the manufacturer's instruction. The concentration of resulting DNA was measured using NanoDrop-1000 (Thermo Fisher Scientific, Waltham, United States), and DNA concentration was normalized to 10 ng/μL. The freshly extracted DNA was used as the template for the amplification of the V4 region of the bacterial 16S rRNA gene using universal bacterial primers (Fadrosh et al., 2014). The amplicon sequencing (library preparation and sequencing) was performed on the Illumina Miseq platform according to the manufacturer's instructions (Illumina, Hayward, United States).

Bioinformatics and Statistical Analysis

Assembly of forward and reverse reads generated for each sample was carried out using FLASH (Fast Length Adjustment of SHort reads) (Magoc and Salzberg, 2011). Bacterial diversity analysis was done using a standard QIIME (v1.9.0) pipeline (Zhernakova et al., 2016) on the assembled sequences. These sequence reads were clustered into operational taxonomic units (OTUs) using UCLUST algorithm (Edgar, 2010) and SILVA database (Quast et al., 2012) by closed reference-based OTU picking method keeping 97% sequence similarity threshold. Representative sequences (repset) from each OTU were selected for taxonomic assignment. Statistical analysis of the alpha diversity across different groups was done using STAMP (Parks and Beiko, 2010). The differential abundance analysis of bacterial genera across the different study groups was also done using STAMP. The abundance of bacterial phylum and genus was represented using GraphPad Prism (GraphPad Software, La Jolla, CA, United States). Beta-diversity analysis of the bacterial

diversity present in the bulk and rhizosphere soil samples was done using the online tool microbiome analyst (Dhariwal et al., 2017). R language-based package ggtern, an extension of package ggplot2, was used to plot ternary diagrams of the differential abundant OTUs in the bulk soil and rhizosphere soil samples. The presence of the shared and unique bacterial genera across the bulk soil and rhizosphere samples was investigated using the online tool InteractiVenn¹. Operational taxonomic units-based predictive functional analysis of the bacterial community was done using the Tax4Fun package (Aßhauer et al., 2015) in R and KEGG database. Also, principal component analysis (PCA) based on the abundant predicted functions was performed in the PAST3 (Paleontological Statistics) software (Hammer et al., 2001).

Absolute Quantification of Bulk Soil and Pea Rhizosphere Bacteria

Quantitation of total bacteria and phylum Firmicutes was done using respective qPCR primers (Supplementary Table S1). Briefly, for each gene, 10 µL reactions (in triplicate) were set containing suitable pairs of primers (0.5 µM), 10 ng of metagenomic DNA and SYBR green master mix (Applied Biosystems Inc., Foster City, California, United States). The reactions were run on 7300 Real-time PCR system (Applied Biosystems Inc.) using the following PCR conditions: initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 10 s and 60°C for 1 min. Group-specific standard curves were generated from serial dilutions of a known concentration of individual PCR products. The amplification specificity of primers was checked by melt curve analysis performed at the end of qPCR cycles. The mean values of the three replicate were used for enumerations of tested gene copy numbers for each sample using standard curves.

Data Availability

The sequence data are made available at NCBI SRA submission with accession number SUB5624752 (Bioproject ID: PRJNA544901).

RESULTS

Chemical Properties of Soil

Soil pH (1:2.5) was found to be influenced by tillage and nutrient management practices at depth 0–15 cm. Application of 50% NPK + ISRR at 5 t/ha recorded slightly higher soil pH compared to 100% NPK. It was recorded that SOC and TOC at 0–15 cm soil depth were significantly varied by tillage and nutrient management practices (Table 1). Among the nutrient management practices, the higher SOC and TOC at 0–15 cm depth of soil were recorded under 50% NPK + WB, and it was at par with 50% NPK + GLM and FYM + WB + RP, whereas lower SOC and TOC contents at 0–15 cm depth of soil were recorded under 50% NPK and 100% NPK (no residue application). The interaction effect of tillage and nutrient management practices on SOC and TOC appeared significant. Among the nutrient management practices, SOC was observed to be the highest under MT with 50% NPK + GLM (2.89%) followed by NT with 50%

NPK + WB (2.83%). The lowest SOC at 0–15 cm was recorded under CT + 50% NPK (2.03%). TOC at 0–15 cm of the soil was higher under MT for FYM+WB+RP (3.25%) followed by NT + 50% NPK + GLM (3.24%) (Table 1).

The interaction effect of tillage and nutrient management practices on available N, P, and K content were significant (Table 1). Available N, P, and K contents were significantly higher under NT compared to MT and CT at both depths. Among the nutrient management practices, N recorded significantly higher under 50% NPK + GLM followed by 50% NPK + WB and 50% NPK + ISRR compared to 50% NPK, whereas available P recorded higher under 50% NPK + ISRR (11.5 kg/ha) at 0–15 cm. Available K at 0–15 cm of the soil was the higher under NT + 50% NPK + ISRR (235.2 kg/ha) followed by MT for 50% NPK + ISRR (225.5 kg/ha) (Table 1). Detailed results of other chemical properties of soils are provided in Supplementary Information.

Diversity Analysis of Bacterial Communities

A total of 2,686,925 raw sequence reads were generated for the bulk soil ($n = 18$) and rhizosphere soil ($n = 18$) samples using the Illumina Miseq sequencing platform. Among these sequences, 2,650,426 sequences (98.6%) were assembled using FLASH and clustered into 15,048 OTUs. Among these, 10,078 OTUs were represented by two or more than two sequences, whereas remaining OTUs were singletons.

Alpha diversity assessed by observed OTU, Shannon, Simpson, and Chao1 indices differed significantly between rhizosphere and bulk soils ($p > 0.05$) (Supplementary Figure S1). The values of diversity indices were always significantly higher in rhizosphere samples in comparison to bulk soils (Supplementary Figure S1). Tillage and residue management practices do not influence any of the alpha-diversity indices (Supplementary Figure S1). Both bulk soil and rhizosphere soil samples from the organic treatment exhibited the least intersample variation in Shannon and Simpson alpha-diversity indices in comparison to other residue management treatments (Supplementary Figures S1H,K).

The beta-diversity analysis based unweighted unifracs Principal Coordinates Analysis (PCoA) showed clustering of the bulk soil and rhizosphere soil sample into two distinct clusters (Figure 1). The total variation explained by the first was PCoA 34.8% (22% for axis 1 and 12.8% for axis 2), wherein all the bulk soil samples were on the positive side of axis 2 except two samples (B15 and B03), whereas all the rhizosphere soil samples were on the negative side of axis 2.

Taxonomic Composition of Bacterial Communities

A total of 71 bacterial phyla were detected in the bulk soil and rhizosphere samples. The highly abundant phyla include Proteobacteria (32.5%), Firmicutes (29.4%), Acidobacteria (9.03%), Actinobacteria (7.2%), Chloroflexi (4.7%), Nitrospirae (3.1%), Verrucomicrobia (2.7%), Thaumarchaeota (2.6%), Bacteroidetes (2.1%), and Planctomycetes (1.0%) and constituted 95% of the overall bacterial community (Figure 2A). A higher abundance of Firmicutes was recorded in bulk soil (~41.7%) in comparison to the rhizosphere (~17.8%).

¹<http://www.interactivenn.net>

TABLE 1 | Chemical properties of soils collected from different treatment plots.

Tillage	Soil pH				SOC (%)				TOC (%)			
Nutrient Inputs	ZT	MT	CT	Mean	ZT	MT	CT	Mean	ZT	MT	CT	Mean
100% NPK	4.58	4.58	4.65	4.60	2.66	2.47	2.28	2.47	3.16	3.13	2.95	3.08
50% NPK	4.55	4.49	4.58	4.54	2.41	2.34	2.03	2.26	3.12	3.06	2.66	2.95
50% NPK+ISRR	4.84	4.76	4.62	4.74	2.76	2.52	2.34	2.54	3.25	3.20	3.19	3.21
50% NPK+WB	4.73	4.71	4.70	4.71	2.83	2.50	2.42	2.58	3.21	3.18	3.18	3.19
50% NPK+GLM	4.66	4.61	4.75	4.67	2.82	2.89	2.72	2.81	3.24	3.23	3.16	3.21
FYM+WB+RP	4.72	4.71	4.62	4.68	2.80	2.78	2.67	2.75	3.17	3.25	2.91	3.11
Mean	4.68	4.64	4.65		2.71	2.58	2.41		3.19	3.18	3.01	
Variant	S.Em ±		C.D. (p = 0.05)		S.Em ±		C.D. (p = 0.05)		S.Em ±		C.D. (p = 0.05)	
Tillage	0.03		0.07		0.02		0.07		0.02		0.05	
Nutrient inputs	0.04		0.05		0.03		0.05		0.03		0.04	
Interactions	0.06		0.18		0.06		0.16		0.05		0.13	
	N (Kg/ha)				P (Kg/ha)				K (Kg/ha)			
	ZT	MT	CT	Mean	ZT	MT	CT	Mean	ZT	MT	CT	Mean
100% NPK	270.9	271.4	264.3	268.8	10.6	9.9	9.1	9.9	207.3	201.3	187.9	198.8
50% NPK	261.1	252.3	244.9	252.8	10.4	9.1	8.7	9.4	197.1	190.8	183.9	190.6
50% NPK+ISRR	307.2	291.2	281.6	293.3	11.5	10.8	10.4	10.9	235.2	225.5	208.9	223.2
50% NPK+WB	312.3	306.7	298.3	305.8	11.3	10.9	10.3	10.8	219.9	218.2	199.9	212.7
50% NPK+GLM	308.2	307.0	298.3	304.5	11.2	10.5	9.8	10.5	218.5	209.4	196.6	208.2
FYM+WB+RP	286.0	297.7	276.7	286.8	11.1	10.6	10.5	10.7	234.0	213.0	214.7	220.6
Mean	291.0	287.7	277.3		11.0	10.3	9.8		218.7	209.7	198.6	
Variant	S.Em ±		C.D. (p = 0.05)		S.Em ±		C.D. (p = 0.05)		S.Em ±		C.D. (p = 0.05)	
Tillage	1.4		4.1		0.1		0.3		3.9		11.3	
Nutrient inputs	2.0		2.9		0.2		0.2		5.6		7.9	
Interactions	3.5		10.0		0.3		0.7		9.7		27.7	

SOC, Soil Organic Carbon; TOC, Total Organic Carbon; N, Nitrogen; P, Phosphorus, K, Potassium; FYM, Farm Yard Manure; ISRR, in situ Rice Residue Retention; WB, Weed Biomass; GLM, Green Leaf manure; RP, Rock phosphate. Values in bold are the mean.

On the contrary, Proteobacteria were highly abundant in the rhizosphere (~43.9%) in comparison to bulk soil (~18.6%) samples (**Figure 2A**). At genera level, 24 bacterial genera, including *Bacillus*, *Nitrobacter*, *Pseudomonas*, *Paenibacillus*, and *Rhizobium*, were found dominant with a collective abundance of more than 0.5% (**Figure 2B**).

Comparative analysis at phylum and genus taxonomic ranks revealed the differences in the abundance of bacterial taxa in bulk soil and rhizosphere soil samples (**Figure 3**). Significantly higher abundance of Proteobacteria and Bacteroidetes was observed in pea rhizosphere samples in comparison to bulk soil (**Figure 3A**). The abundance of Firmicutes, Chloroflexi, Nitrospirae, and Planctomycetes was significantly higher in bulk soil samples over rhizosphere samples (**Figure 3A**). The abundance of genera *Rhizobium*, *Pseudomonas*, *Pantoea*, *Paenibacillus*, *Nitrobacter*, *Enterobacter*, and *Sphingomonas* was significantly higher in rhizosphere soils, whereas *Massilia*, *Paenibacillus*, and *Planomicrobium* were highly abundant in bulk soils (*t*-test, $P < 0.05$) (**Figure 3B**). Among the total of 917 bacterial genera reported in this study, 551 genera (60%) were common for bulk soil and rhizosphere soil samples (**Figure 3C**).

Rhizosphere samples showed a selection of 267 unique genera, whereas only 99 genera were exclusive to bulk soil samples.

Quantitative Analysis of Bacterial Community

The total bacterial abundance estimated by qPCR was statistically not different for the rhizosphere (8.9×10^9 copies/gm) and bulk soil (9.42×10^9 copies/gm) samples (**Figure 4A**). The abundance of phylum Firmicutes was significantly higher (3.76×10^9 copies/gm) in the bulk soil samples in comparison to the rhizosphere samples (1.78×10^9 copies/gm) (**Figure 4B**). The mean relative abundance of Firmicutes detected by 16S rRNA gene amplicon sequencing data was ~18% in the rhizosphere samples and ~42% in the bulk soil samples (**Figure 4C**).

Effect of Soil Management Practices on the Bulk Soil and Pea Rhizosphere Microbiome

Bulk soil microbial community showed significant differences in the abundance of *Pseudolabrys*, *Roseiarcus*, and *Tumebacillus*

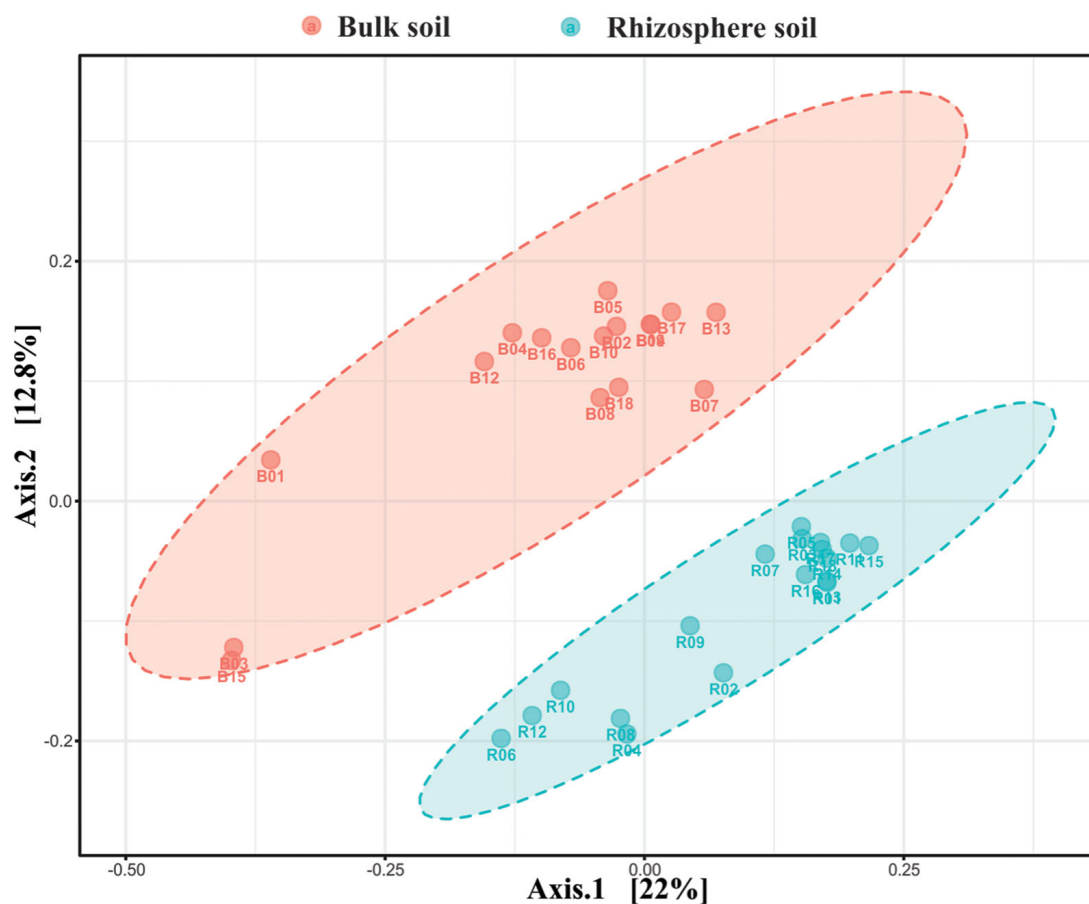


FIGURE 1 | PCoA biplot based on relative abundance of bacterial OTUs exhibiting the beta diversity among the bulk soil and pea rhizosphere samples.

across the residue management practices (**Figure 5A**). *Pseudolabrys* and *Tumebacillus* were highly abundant in samples with 100% NPK, 50% NPK, and organic treatments in comparison to remaining treatments. The abundance of *Roseiarcus* was higher in 50% NPK and organic treatment samples (**Figure 5A**). Significantly higher abundance of bacterial genera *Anaerolinea*, *Hydrogenispora*, and *Syntrophorhabdus* was recorded for the organic treatment among the rhizosphere samples (**Figure 5B**).

The impact of tillage history was also observed on the enrichment of specific OTUs in the bulk soil and rhizospheric soil (**Supplementary Figure S2**). A total of 66 OTUs belonging to 32 taxa were enriched in bulk soils with a history of minimum tillage. Specific enrichment of 12 OTUs belonging to four taxa was recorded for samples with conventional tillage, and only six OTUs belonging to four taxa were enriched in samples with zero tillage in bulk soils (**Supplementary Figure S2A**). Similarly, among the rhizosphere soil samples, significant enrichment of 295 OTUs of 96 taxa, 17 OTUs of the 13 taxa, and 82 OTUs of the 37 taxa was observed in the samples with conventional tillage, minimum tillage, and zero tillage history, respectively (**Supplementary Figure S2B**). Differences in the abundance of 11 genera were recorded in the rhizosphere sample across the history

of different tillage treatment (**Table 2**). All these genera showed higher abundance in the conventional tillage fields.

Correlations Between Soil Properties and Microbial Community Structure

Significant correlations were observed between the relative abundance of a few bacterial phyla and genera in both bulk and rhizosphere samples and soil properties (**Table 3** and **Supplementary Table S4**). However, the number of significant correlations was low in rhizosphere samples, in comparison to bulk soil samples. The relative abundance of Planctomycetes in bulk was a significantly negative correlation with six soil properties, including N, P, TOC, Ca, DHA, and SMBP (**Table 3**). In addition, a significant negative correlation was also observed between Chloroflexi and N, TOC, and Ca; Acidobacteria and N and DHA; Proteobacteria and Fe and Mn; and Actinobacteria and Fe, whereas Firmicutes showed a positive correlation with Mn in bulk soil. At the genera level, a significant negative correlation was recorded for the relative abundance of *Geobacter* and *Nitrobacter* in bulk soil with the majority of the soil properties, such as N, TOC, SOC, Ca, SMBC, SMBN, and SMBP; in case of *Geobacter*, pH values and P were also negatively

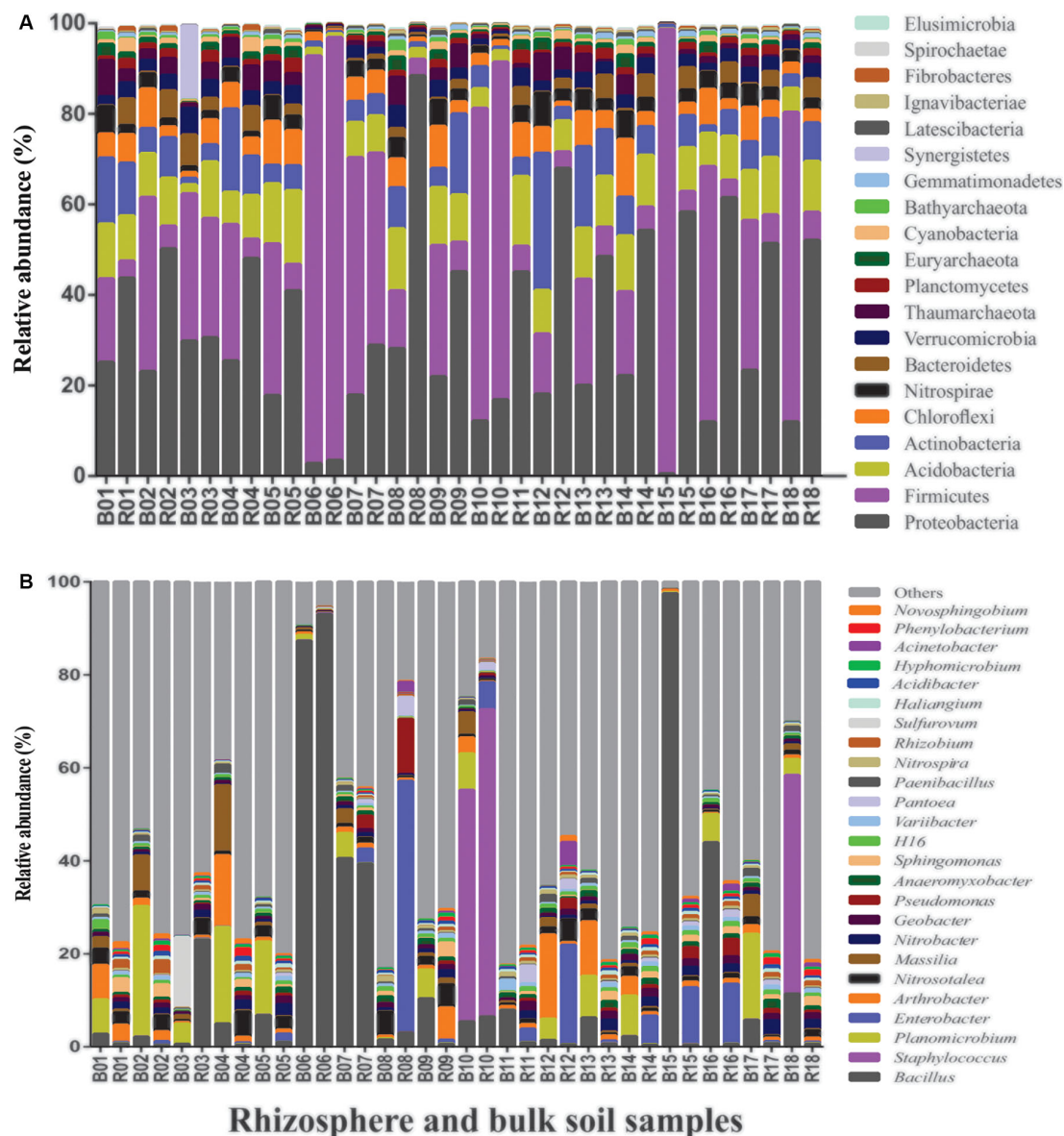


FIGURE 2 | Relative abundance of bacteria in the bulk soil and pea rhizosphere samples at the phylum (A) and genus (B) levels.

correlated. Significant negative correlations were also observed for the relative abundance of *Nitrosotalea* in bulk soil with pH, N, Ca, SMBC, and SMBP. However, the relative abundance *Bacillus* in bulk soil was positively correlated with Fe, whereas a negative correlation between Fe and the relative abundance *Arthrobacter* and *Massilia* was remarkably evident.

Moreover, significant negative correlations were also recorded between the relative abundance of Proteobacteria in rhizosphere and soil properties such as pH, N, P, K, Cu, and Mg, whereas the relative abundance of Thaumarchaeota in the rhizosphere was positively correlated with Mg and negatively correlated with Zn (Supplementary Table S4). The relative abundance of *Nitrosotalea* showed a significant positive correlation with Mn and a negative correlation with Zn in the rhizosphere.

A significant negative correlation was recorded between the relative abundance of *Pseudomonas* and P, whereas a positive correlation was observed for *Bacillus* with Cu.

Predictive Functional Analysis of the Microbial Community

The functional contributions of the bacteria were predicted based on OTUs, and the results revealed the presence of 398 different functional classes. Among these classes, transporters, two-component system, secretion system, ABC transporters, transcription factors, peptidases, ribosome, methane metabolism, quorum sensing, and bacterial motility proteins were the top 10 highly represented classes. The presence of 6596 KEGG orthologs

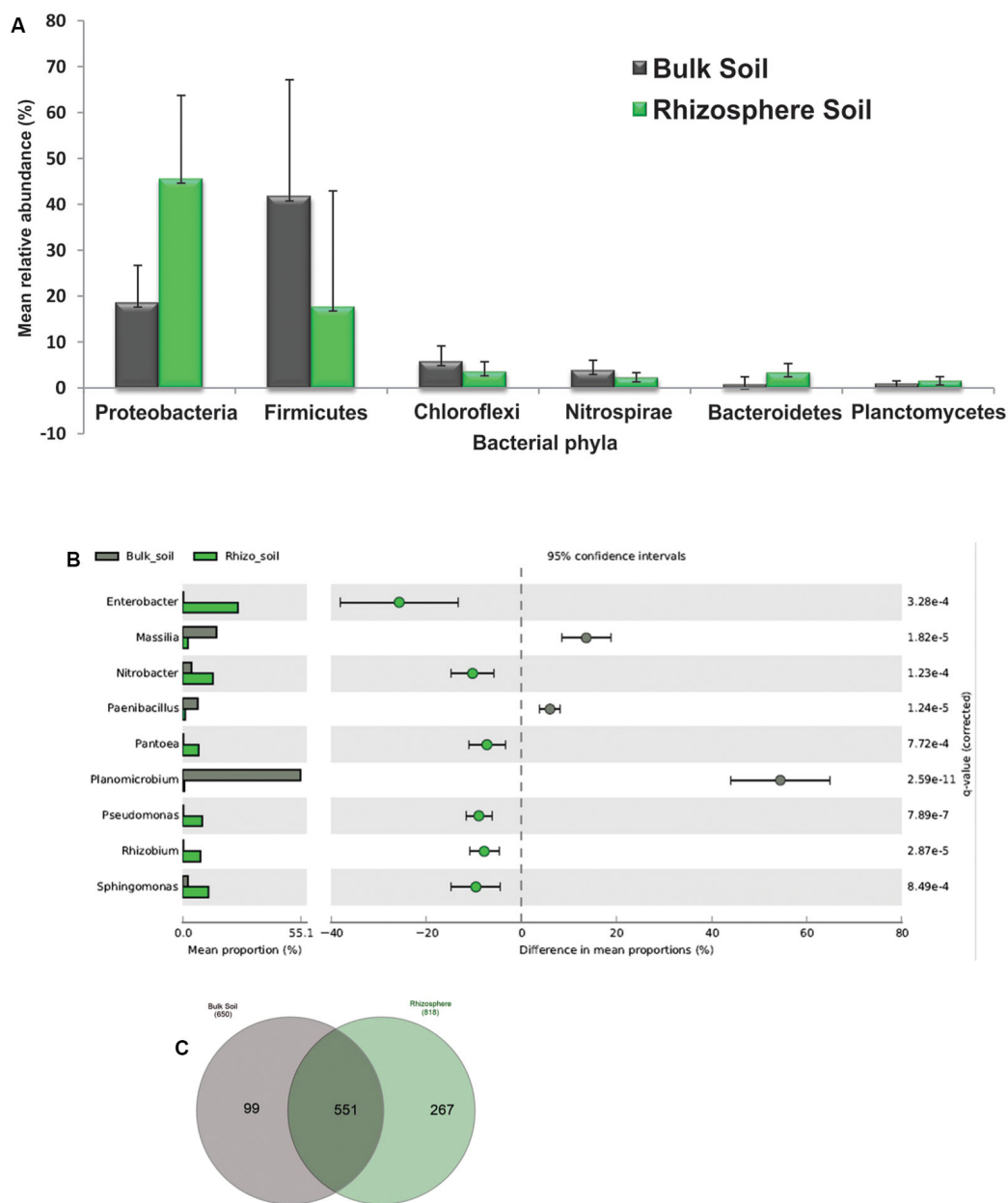
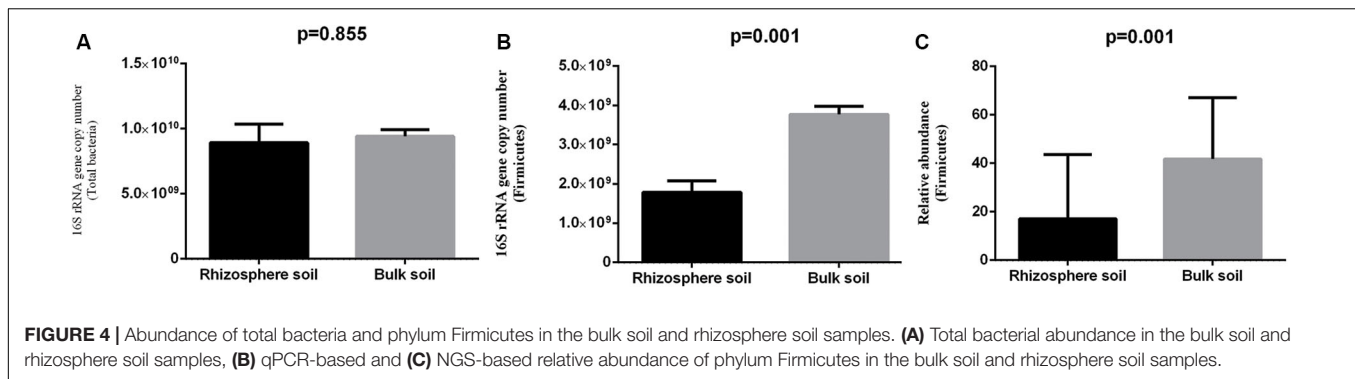


FIGURE 3 | Differentially abundant bacterial taxa across the bulk soil and pea rhizosphere samples. **(A)** At the phylum level. **(B)** At the genera level. **(C)** Venn diagram representing the number of shared and unique OTUs in the bulk soil and rhizosphere soil samples.

was predicted across all samples, belonging to metabolism, environmental information processing, cellular processes, human diseases, genetic information processing, and organismal systems. PCA based on the highly abundant functions clustered the bulk soil and rhizosphere samples into separate groups (**Supplementary Figure S3**). The majority of the rhizosphere samples is placed on the negative side of PC1 and the positive side of PC2, whereas most of the bulk samples were on the positive side of PC1 and the negative side of PC2 (**Supplementary Figure S3**). Bacterial genes associated with iron complex outer membrane receptor protein, cobalt–zinc–cadmium resistance

(CzcA) protein, RNA polymerase sigma-70 factor, ribonuclease E, translation initiation factor IF-2, serine/threonine-protein kinase, hydrophobic/amphiphilic exporter-1, beta-glucosidase, and multiple sugar transport system permease proteins showed high negative values for PC1 and positive for PC2 (**Supplementary Table S5**). Rhizosphere samples revealed high abundance of genes involved in nitrogen fixation (*NifQ*) by 64%, enterobactin (siderophore) production by 39%, plant hormone IAA production (tryptophan 2-monooxygenase) by 10%, and phosphate solubilization (pyrroloquinoline quinone C) by 09% in comparison to bulk soil samples (**Supplementary Table S6**).



DISCUSSION

Conservation agriculture offers a framework to improve soil structure, save water, enhance soil nutrient supply and cycling, increase yield, and maintain soil biodiversity. This study was designed to investigate the effect of long-term exposure to various tillage and residue management practices on the bacterial community structures of the bulk soils and how pea plant (a rotation crop) shapes the rhizosphere communities. Our results showed the dominance of Proteobacteria, Firmicutes, Acidobacteria, and Actinobacteria in both bulk and rhizosphere soils (**Figure 3A**) and are in agreement to the previous report on the dominance of copiotrophic microorganisms such as Proteobacteria, Firmicutes, and Actinobacteria in rich organic environments, and soils with low pH harbor more Acidobacteria (Fierer et al., 2007).

Significant differences were observed in the overall alpha diversity in the rhizosphere and bulk soil samples (**Supplementary Figure S1**), demonstrating higher species abundance and evenness (based on Shannon and Simpson indices) in rhizosphere samples. On the contrary, there was no significant difference in bacterial richness and evenness among the different tillage and residue management treatments ($P > 0.05$) in both rhizosphere and bulk soils (**Supplementary Figure S1**). This indicated that, in this study, the plant rhizosphere effect is the key driver for alpha diversity. Plants can alter the microbial communities by secreting a variety of nutrients and bioactive molecules into the rhizosphere (Hu et al., 2018; Huang et al., 2019). The enrichment of specific OTUs in the pea rhizosphere leading to the increased diversity was further confirmed, as all the rhizosphere samples were grouped in a small cluster, in comparison to a loose clustering of bulk soil samples in the PCoA biplot (**Figure 1**). The buildup of homogeneous bacterial communities in most of the rhizosphere samples can be attributed to the selection pressure of the pea roots, which continually release a large number of border cells and mucilage (Ropitiaux et al., 2019), and pose a strong rhizosphere effect on the bacteria. In addition to this, the pea is a nitrogen-fixing crop, and an increase in the diversity of rhizosphere bacteria with soil mineral nitrogen levels has been observed in a study on frequency cropping of pulses (Hamel et al., 2018). Impact of crop plant on the diversity of rhizosphere microbes has also been observed in different crops including barley, cotton, maize,

pulses, and wheat (Hamel et al., 2018; Yurgel et al., 2018; Babin et al., 2019; Kerdraon et al., 2019).

The majority of members of microbial communities in the host plant are horizontally acquired from the surrounding environment, and the soil is the main reservoir of a plant rhizosphere microbiome (Wagner et al., 2016; Sánchez-Cañizares et al., 2017). Our results on pea rhizosphere and bulk soils are consistent with this, as 551 (60%) genera of 917 were common in bulk and rhizosphere soil samples (**Figure 3C**). The dominance of Proteobacteria recorded in pea rhizosphere samples, with a significant increase in the abundance of genera including *Pseudomonas*, *Rhizobium*, *Pantoea*, *Enterobacter*, and *Sphingomonas* known for plant growth-promoting attributes, was in agreement with the previous studies on the rhizosphere microbiome analysis (Weinert et al., 2011; Mendes et al., 2013; Yurgel et al., 2018; Goss-Souza et al., 2019). Pea-*Rhizobium* symbiosis is well documented in Indian soils (Rahi et al., 2012); hence, an increase in the abundance of *Rhizobium* in the pea rhizosphere was expected. A higher abundance of *Pseudomonas* and *Sphingomonas* was also reported in the rhizospheres of crop plants such as lettuce, pea, wheat, and maize (Schreiter et al., 2014; Kerdraon et al., 2019). In addition to Proteobacteria, a significantly higher abundance of Bacteroidetes and Planctomycetes was also recorded in the rhizosphere soil in comparison to bulk soil samples (**Figures 2, 3**); positive correlations with increased abundance of Bacteroidetes and Planctomycetes have been observed to increase in organic carbon and phosphorus concentrations, respectively, in the rhizosphere of soybean (Goss-Souza et al., 2019). The genus *Nitrobacter* was at higher abundance in pea rhizosphere samples than bulk soils (**Figure 3B**), suggesting its enrichment by host plant as *Nitrobacter* converts nitrite to nitrate, making nitrogen more readily available to the host plant (Richardson et al., 2009; Hubbard et al., 2019).

Amplicon sequencing-based analysis revealed a higher abundance of phylum Firmicutes in the bulk soil samples in comparison to the rhizosphere samples, which was further substantiated with the similar results obtained by qPCR (**Figure 4**). The abundance of Firmicutes represented by members of genera, such as *Bacillus*, *Staphylococcus*, and *Planomicrobium*, proves a significant decrease in pea rhizosphere in comparison to bulk soil samples (**Figures 2, 3**); previous studies have also indicated a negative correlation of plant growth

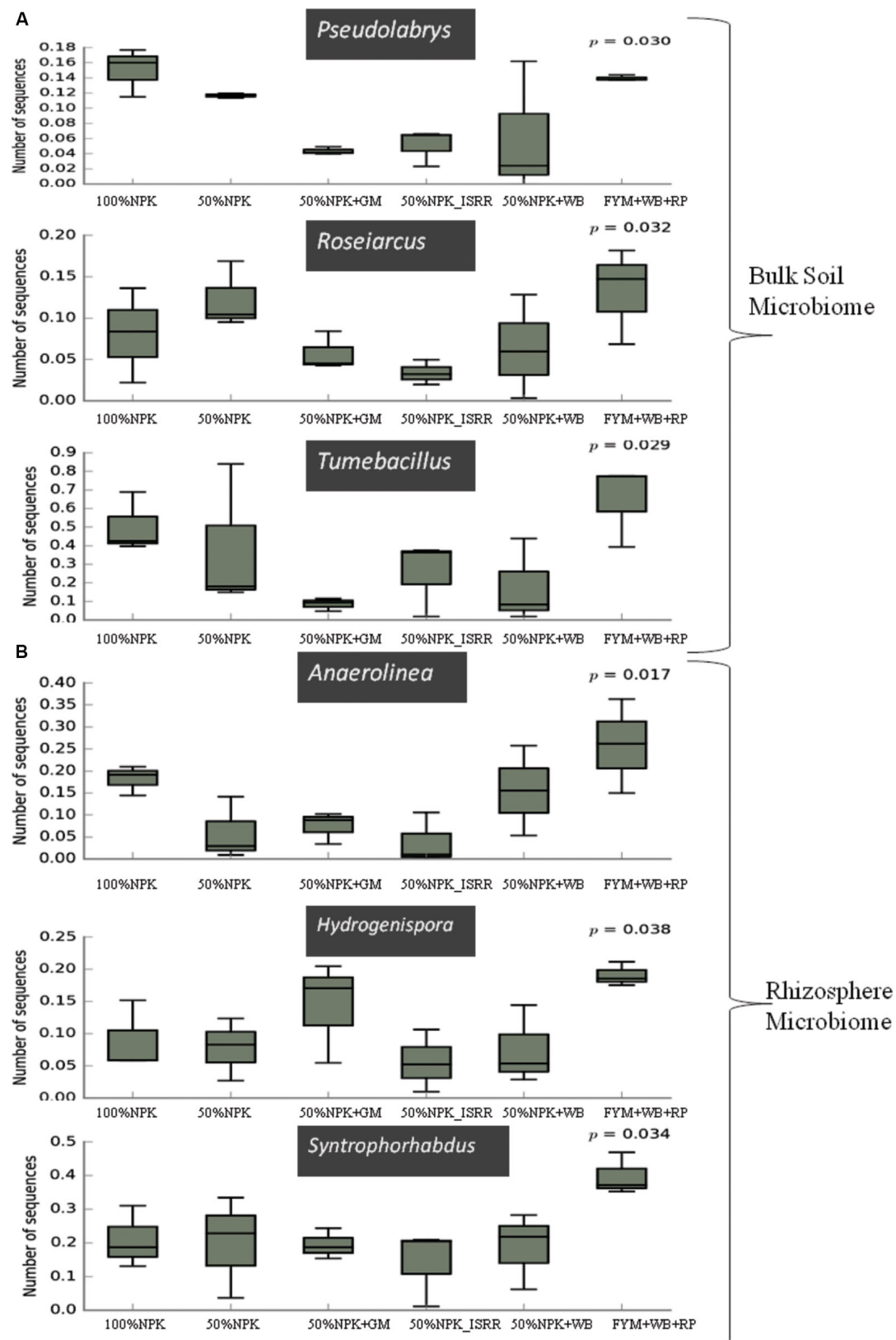


FIGURE 5 | Differentially abundant bacterial taxa detected across the different residue management practice groups in the **(A)** bulk soil and **(B)** pea rhizosphere samples.

TABLE 2 | Differentially abundant bacterial genera across the three different tillage treatments practices.

Genera	No-till (Mean \pm SD)	Conventional tillage (Mean \pm SD)	Minimum tillage (Mean \pm SD)	p-values (ANOVA)
<i>Flavobacterium</i>	0.989 \pm 0.684	2.197 \pm 0.428	0.523 \pm 0.54	0.001
<i>Lysobacter</i>	0.639 \pm 0.426	1.194 \pm 0.354	0.269 \pm 0.174	0.002
<i>Pseudolabrys</i>	1.071 \pm 0.533	1.298 \pm 0.158	0.345 \pm 0.36	0.003
<i>Rhodomicrobium</i>	0.437 \pm 0.253	0.888 \pm 0.18	0.299 \pm 0.264	0.003
<i>Pedomicrobium</i>	0.297 \pm 0.164	0.578 \pm 0.197	0.194 \pm 0.15	0.008
<i>Acidothermus</i>	0.421 \pm 0.233	0.836 \pm 0.418	0.2 \pm 0.127	0.01
<i>Bryobacter</i>	1.285 \pm 0.663	1.54 \pm 0.32	0.473 \pm 0.463	0.011
<i>Anaeromyxobacter</i>	1.763 \pm 1.184	3.445 \pm 1.398	1.107 \pm 0.885	0.018
<i>Rhizomicrobium</i>	0.72 \pm 0.645	1.179 \pm 0.423	0.274 \pm 0.224	0.025
<i>Nitrobacter</i>	4.096 \pm 2.36	5.912 \pm 1.874	2.104 \pm 1.812	0.031
<i>Sideroxydans</i>	0.401 \pm 0.366	0.629 \pm 0.121	0.195 \pm 0.196	0.047

TABLE 3 | Pearson's correlation coefficient (R) between the relative abundance of bulk soil bacterial taxa and measured soil characteristics.

Bacterial taxa	Measured soil characteristics																
	pH	N	P	K	TOC	SOC	Fe	Ca	Cu	DHA	Mg	Mn	S	SMBC	SMBN	SMBP	Zn
Phylum																	
Proteobacteria	-0.46	-0.32	-0.12	-0.14	-0.25	-0.02	-0.49*	-0.25	-0.18	-0.14	-0.03	-0.49*	0.02	-0.24	-0.14	-0.28	-0.24
Firmicutes	0.35	0.45	0.27	0.15	0.39	0.20	0.48	0.39	0.28	0.35	0.12	0.49*	0.07	0.31	0.24	0.37	0.27
Acidobacteria	-0.42	-0.59*	-0.4	-0.22	-0.45	-0.3	-0.3	-0.6	-0.27	-0.5*	-0.3	-0.24	-0.1	-0.33	-0.26	-0.47	-0.02
Actinobacteria	-0.05	-0.2	-0.1	0.01	-0.11	-0.15	-0.53*	-0.18	-0.4	-0.3	-0.03	-0.43	-0.07	-0.11	-0.15	-0.15	-0.42
Chloroflexi	-0.28	-0.56*	-0.47	-0.3	-0.65*	-0.4	-0.27	-0.52*	-0.32	-0.41	-0.25	-0.24	-0.2	-0.39	-0.36	-0.46	-0.05
Nitrospirae	-0.18	-0.42	-0.28	-0.07	-0.36	-0.24	-0.34	-0.44	-0.31	-0.47	-0.2	-0.26	-0.03	-0.16	-0.17	-0.3	0.01
Verrucomicrobia	-0.26	-0.21	-0.17	-0.12	-0.21	-0.07	-0.14	-0.23	-0.04	-0.21	-0.04	-0.29	0.05	-0.2	-0.07	-0.23	-0.06
Thaumarchaeota	-0.32	-0.46	-0.22	-0.09	-0.23	-0.17	-0.39	-0.38	-0.15	-0.29	-0.15	-0.4	-0.1	-0.26	-0.21	-0.33	-0.31
Bacteroidetes	0.03	0.17	0.14	0.07	0.07	0.16	0.08	0.25	0.27	0.23	0.21	-0.22	0.06	-0.03	0.08	0.10	-0.19
Planctomycetes	-0.45	-0.59*	-0.5*	-0.35	-0.61*	-0.44	-0.23	-0.62*	-0.36	-0.57*	-0.38	-0.09	-0.24	-0.41	-0.39	-0.53*	0.05
Genera																	
<i>Bacillus</i>	0.45	0.31	0.14	0.09	0.24	0.04	0.66*	0.21	0.37	0.21	0.02	0.43	-0.05	0.12	0.09	0.22	0.40
<i>Staphylococcus</i>	-0.18	0.16	0.04	0.00	0.21	0.09	-0.21	0.23	-0.18	0.06	-0.01	0.28	-0.02	0.21	0.09	0.14	-0.19
<i>Planomicrobium</i>	-0.27	-0.2	0.13	0.02	-0.2	0.08	-0.43	-0.05	-0.24	0.13	0.12	-0.41	0.12	-0.05	0.00	-0.04	-0.36
<i>Enterobacter</i>	0.14	0.20	0.23	0.19	0.21	0.21	-0.27	0.00	-0.24	0.02	0.19	-0.04	0.5*	0.35	0.37	0.22	0.34
<i>Arthrobacter</i>	0.07	-0.01	0.02	0.07	-0.02	-0.04	-0.51*	0.02	-0.35	-0.1	0.14	-0.38	0.03	0.03	-0.04	0.01	-0.4
<i>Nitrosotalea</i>	-0.58*	-0.68*	-0.44	-0.3	-0.39	-0.38	-0.16	-0.59*	-0.11	-0.48	-0.42	-0.23	-0.39	-0.57*	-0.44	-0.59*	-0.31
<i>Massilia</i>	-0.23	0.11	0.28	0.10	0.13	0.27	-0.56*	0.21	-0.23	0.31	0.30	-0.34	0.23	0.14	0.14	0.15	-0.33
<i>Nitrobacter</i>	-0.47	-0.58*	-0.44	-0.31	-0.58*	-0.53*	-0.06	-0.57*	-0.32	-0.58	-0.41	-0.09	-0.4	-0.53*	-0.48*	-0.54*	-0.11
<i>Geobacter</i>	-0.51*	-0.62*	-0.59*	-0.42	-0.63*	-0.5*	-0.35	-0.58*	-0.32	-0.53	-0.39	-0.27	-0.38	-0.53*	-0.52*	-0.61*	-0.11
<i>Pseudomonas</i>	-0.14	-0.2	-0.38	-0.43	-0.13	-0.17	0.06	-0.19	-0.3	-0.11	-0.36	0.25	-0.35	-0.27	-0.2	-0.29	0.32

N, Nitrogen; P, Phosphorous; K, Potassium; TOC, Total organic carbon; SOC, Soil organic carbon; Fe, Iron; Ca, Calcium; Cu, Copper; DHA, Dehydrogenase activity; Mg, Magnesium; Mn, Manganese; S, sulfur; SMBC, Soil microbial biomass carbon; SMBN, Soil microbial biomass nitrogen; SMBP, Soil microbial biomass Phosphorous; Zn, Zinc. *Correlation significant at 0.05 level (two-tailed).

and high abundance of Firmicutes in the soil (Zhang et al., 2014; Kumar et al., 2018). The consistently higher relative proportion of genus *Planomicrobium* in the majority of bulk soil samples (Figure 2) also confirms its dominance in soils (Hui et al., 2019). The abundance of bacterial phyla Chloroflexi and Nitrospirae was significantly higher in bulk soil in comparison to rhizosphere samples (Figure 3A), which is in agreement with the previous study on the impact of land-use intensity and plant functional identity on microbial communities (Schöps et al., 2018). Both Chloroflexi and Nitrospirae are slow-growers adapted to low substrate concentrations (Daims et al., 2015) and

could not cope up with other fast-growing bacterial communities in the nutrient-rich pea rhizosphere environment. Members of Nitrospirae are involved in nitrification process (oxidation of nitrite to nitrate) and have been reported to be dominant in N-fertilized treatment in paddy soil (Kumar et al., 2018). The significantly high abundance of *Massilia* and *Paenibacillus* was observed in bulk soil than rhizosphere samples (Figure 3B), which can be attributed to the presence of high cellulosic biomass in most of the treatment leading to the selection of the members of genera with potential to degrade cellulose (Ofek et al., 2012; Grady et al., 2016).

Minor differences were observed in the bacterial community composition in response to residue management treatments in both bulk and rhizosphere soils (**Figure 5**), exhibiting the complex responses of microbial communities to fertilizer applications (Hartmann et al., 2015). The higher proportion of *Pseudolabrys* in 100% NPK and organic treatments indicate its specific enrichment in these treatments to higher levels of nitrogen (**Figure 5**); previous studies based on the increased abundance of genera *Pseudolabrys*, *Elstera*, and *Ramlibacter* suggested them to be used as bacterial biomarkers for different nitrogen levels (Yan et al., 2017). *Roseiarcus* was abundant in the organic treatment (**Figure 5**). The member of this genus was isolated from acidic peat soil (Kulichevskaya et al., 2014). The function of this genus is not well understood. Higher abundance of *Tumebacillus* was recorded in organic treatment (**Figure 5**). *Tumebacillus* is a highly abundant member of Firmicutes in different soils (Lian et al., 2019). Among the rhizosphere sample, the significantly higher relative abundance of *Anaerolineae*, *Hydrogenispora*, and *Syntrophorhabdus* was observed for the organic treatment (**Figure 5**), all of these genera are capable of decomposing diverse carbon sources under anoxic environments and have been identified as the unique core taxa for rice soils (Jiao et al., 2019).

Although pea was cultivated under zero tillage, the influence of previous tillage treatments during rice cultivation was observed on the prevalence of few specific OTUs (**Supplementary Figure S2**), suggesting the plausible effect of tillage treatments on the bacterial community structure. This can be attributed to the fact that tillage practices alter soil bulk density, pore structure, water availability, soil organic carbon, and so on (Zhang et al., 2018). However, the impact to tillage treatments was not pronounced, and only 0.56% OTUs in the bulk soil and 2.60% OTUs in the rhizosphere soil were enriched across various tillage treatments (**Supplementary Figure S2**). Enrichment of OTUs assigned to different genera was recorded in the rhizosphere soil samples in the CT in comparison to ZT and MT (**Table 2**), which can be attributed to the fact that intense soil disturbance in conventional tillage accelerates soil organic matter oxidation, hence expected to enrich diverse member of bacteria (Ding et al., 2011).

The levels of N, P, K, and few micronutrients showed a significant negative correlation with the relative abundance of Proteobacteria in pea rhizosphere (**Supplementary Table S4**); a strong negative correlation was recorded between the relative abundance of Proteobacteria and available phosphorus in *Pinus tabuliformis* forest soils (Deng et al., 2018). Among the members of Proteobacteria, with the relative abundance of genus *Pseudomonas*, a significant negative correlation was recorded with values of P (**Supplementary Table S4**), exhibiting the enrichment of phosphate-solubilizing *Pseudomonas* in the pea rhizosphere in the soils with lower *P*-values (Gulati et al., 2008). The results of our study showed a significant negative correlation of multiple soil nutrients values with the relative abundance of Chloroflexi, and the level of N with Acidobacteria in the bulk soil (**Table 3**) is in agreement to the reports that Chloroflexi and Acidobacteria are generally oligotrophic and had slower growth rates (Fierer et al., 2012).

Planctomycetes in soils has been reported to be sensitive to soil history (Buckley and Schmidt, 2003); in our study, we found a significant negative correlation between the abundance of Planctomycetes in bulk soil and multiple parameters of soil nutrients (**Table 3**). Members of Chloroflexi and Planctomycetes have been reported for negative responses to fertilization (Eo and Park, 2016). Similar negative correlation to various soil properties, including available N, has been observed for *Nitrobacter* and *Nitrosotalea* (**Table 3**), as the members of these genera are known as nitrite and ammonium oxidizers, and contributes to nitrification in soils (Pajares and Bohannan, 2016).

Higher abundance of genes related to nitrogen fixation, phytohormone and siderophore production, and phosphate solubilization in the rhizosphere soil (**Supplementary Table S6**) substantiate our earlier conclusions on the selection of bacterial communities with plant growth-promoting potential in the rhizosphere (**Figure 3**). The selection of specific bacterial communities in the rhizosphere based on their putative functions was further confirmed by specific clustering of the majority of rhizosphere and a bulk sample in the PCA biplot constructed based on the highly abundant functions (**Supplementary Figure S3**). In addition to the high abundance of plant growth promotion genes, bacterial genes associated with iron complex outer membrane receptor protein, CzcA; RNA polymerase sigma-70 factor; and ribonuclease E were also abundant in rhizosphere soil as compared to the bulk soil (**Supplementary File S1**), revealing the possible role of bacterial communities in abiotic stress (low pH and high aluminum and iron toxicity) amelioration. The higher abundance of iron complex outer membrane receptor protein in the rhizosphere indicates the enrichment of Gram-negative bacteria, which outer membrane receptor system for siderophore-mediated Fe transportation (Ahmed and Holmström, 2014). The CzcA protein has been reported to be involved in the efflux of heavy metals ions (Nies, 2000) and was also present in metal resistance *Pseudomonas putida* S13.1.2 isolated from a vineyard soil (Chong et al., 2016). Rhizosphere offers a variety of abiotic stress, including temperature fluctuations, salinity, osmolarity, oxygen concentration, and nutrient concentration, to the bacteria; RNA polymerase sigma-70 factors play relevant roles in adapting to different kinds of stresses (Martínez-Salazar et al., 2009; Mishra et al., 2011).

CONCLUSION

Our work showed that pea plant is the most dominating selection factor shaping the microbial communities under diverse residue management and tillage treatments. Soil management and residue management practices also affect the bacterial community structure in both bulk soil and pea rhizosphere. Enrichment of bacterial taxa known for plant growth promotion attributes and removal of toxic elements from soil was recorded in the rhizosphere, indicating selection of rhizosphere communities by the plant to meet its requirements of nutrient uptake and combating stress. Predictive functional analysis also

revealed the plausible enrichment of plant growth-promoting and stress tolerance genes in the pea rhizosphere.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the sequence data is made available at NCBI SRA submission with accession number SUB5624752 (Bioproject ID: PRJNA544901).

AUTHOR CONTRIBUTIONS

PR, KR, AD, YS, and JL conceived the study. KR, SB, and BK provided the samples. KR, AD, JL, SB, and BK performed the soil physio-chemical analysis. DC performed the bioinformatics and statistical analyses. PR participated and provided guidance with the data analysis. PR and DC drafted the manuscript. KR contributed to manuscript revisions. All authors have read and approved the final 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/fmicb.2020.00968/full#supplementary-material>

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Linking Short-Chain N-Acyl Homoserine Lactone-Mediated Quorum Sensing and Replant Disease: A Case Study of *Rehmannia glutinosa*

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Rehmannia glutinosa, a perennial medicinal plant, suffers from severe replant disease under consecutive monoculture. The rhizosphere microbiome is vital for soil suppressiveness to diseases and for plant health. Moreover, N-acyl homoserine lactone (AHL)-mediated quorum sensing (QS) regulates diverse behavior in rhizosphere-inhabiting and plant pathogenic bacteria. The dynamics of short-chain AHL-mediated QS bacteria driven by consecutive monoculture and its relationships with *R. glutinosa* replant disease were explored in this study. The screening of QS bacteria showed that 65 out of 200 strains (32.5%) randomly selected from newly planted soil of *R. glutinosa* were detected as QS bacteria, mainly consisting of *Pseudomonas* spp. (55.4%). By contrast, 34 out of 200 (17%) strains from the diseased replant soil were detected as QS bacteria, mainly consisting of Enterobacteriaceae (73.5%). Functional analysis showed most of the QS bacteria belonging to the *Pseudomonas* genus showed strong antagonistic activities against *Fusarium oxysporum* or *Aspergillus flavus*, two main causal agents of *R. glutinosa* root rot disease. However, the QS strains dominant in the replant soil caused severe wilt disease in the tissue culture seedlings of *R. glutinosa*. Microbial growth assays demonstrated a concentration-dependent inhibitory effect on the growth of beneficial QS bacteria (i.e., *Pseudomonas brassicacearum*) by a phenolic acid mixture identified in the root exudates of *R. glutinosa*, but the opposite was true for harmful QS bacteria (i.e., *Enterobacter* spp.). Furthermore, it was found that the population of quorum quenching (QQ) bacteria that could disrupt the beneficial *P. brassicacearum* SZ50 QS system was significantly higher in the replant soil than in the newly planted soil. Most of these QQ bacteria in the replant soil were detected as *Acinetobacter* spp. The growth of specific QQ bacteria could be promoted by a phenolic acid mixture at a ratio similar to that found in the *R. glutinosa* rhizosphere. Moreover, these quorum-quenching bacteria showed strong pathogenicity toward the tissue culture seedlings of

R. glutinosa. In conclusion, consecutive monoculture of *R. glutinosa* contributed to the imbalance between beneficial and harmful short-chain AHL-mediated QS bacteria in the rhizosphere, which was mediated not only by specific root exudates but also by the QQ bacterial community.

Keywords: *Rehmannia glutinosa*, replant disease, quorum sensing, root exudate, quorum quenching

INTRODUCTION

To ensure food security and meet market needs, the practice of consecutive monoculture is becoming popular in intensive agriculture. However, large-scale crop monoculture results in many problems including a loss of crop genetic diversity, an increase in disease incidence, a decline in crop quality and even the fragility of ecosystem functioning (Jacques and Jacques, 2012; Mariotte et al., 2018; Li et al., 2019). Consecutive monoculture problems, also known as replant problems or replant disease, are especially severe in the cultivation of medicinal herbs, such as *Rehmannia glutinosa*, *Panax notoginseng*, *Pseudostellaria heterophylla*, and *Panax ginseng* (Dong et al., 2018a; Luo et al., 2019; Wu et al., 2019; Zhang et al., 2019). It was reported that approximately 70% of medicinal herbs using tuberous roots were attacked by replant disease (Wu et al., 2016c). *R. glutinosa*, a member of the Scrophulariaceae family, is a traditional and famous Chinese medicinal herb with various pharmacological effects. However, 2-year consecutive monoculture of this plant on the same land led to a substantial increase in root rot disease and a serious reduction in tuberous root yield in the field trial conducted at our long-term orientation station. The consecutively-monocultured plants had unexpanded tuberous roots and large numbers of adventitious fibrous roots, which are of no commercial value (Wu et al., 2015, 2018b). In addition, consecutive monoculture of this plant resulted in a significant increase in the abundance of several soil-borne fungal pathogens (i.e., *Aspergillus flavus* and *Fusarium oxysporum*), which were frequently isolated from the consecutively monocultured soil and diseased plants and are pathogenic to *R. glutinosa* seedlings (Wu et al., 2015, 2016b, 2018a). Fields used for *R. glutinosa* production need to be planted with other crops for at least 15 years before they can be replanted again (Yang et al., 2011). Each year, replant disease causes a dramatic decrease in the area of the geo-authentic production zone (34° 48' N to 35° 30' N, 112° 02' E to 113° 38' E) (Zhang et al., 2019), an optimal production area with the most suitable soil and climate conditions for *R. glutinosa* cultivation. The quality of *R. glutinosa* tuberous roots cannot be assured when grown outside the geo-authentic production zone. Therefore, it is urgent to gain insight into the mechanisms underlying replant disease.

The plant microbiome is a key determinant of plant growth, development and health. The interactions between plants and soil microorganisms play key roles in maintaining soil quality and ecosystem sustainability (Kwak et al., 2018; Wang and Li, 2019). Plant roots influence soil microbial community assembly and alter the relative abundance of beneficial, harmful and neutral microorganisms, which in turn exert positive or negative effects on plant growth and resistance (Bakker et al., 2018;

Stringlis et al., 2018; Zhalnina et al., 2018; Rolfe et al., 2019). Moreover, previous studies demonstrated that previous plants could affect the immunity and resistance of subsequent plant populations growing in the same soil through soil-borne microbial legacy (Bakker et al., 2018; Yuan et al., 2018; Kong et al., 2019). Therefore, recent research in the field of replant disease has increasingly focused on soil microbiome composition and function. Liu et al. (2019) indicated that consecutive soybean monoculture significantly decreased the fungal community diversity but increased the abundances of plant pathogens. Gao et al. (2019) found that consecutive sweet potato monoculture reduced the abundance of beneficial fungi such as *Chaetomium* but increased harmful fungi such as *Verticillium*, *Fusarium*, and *Colletotrichum*. Our previous study using barcoded pyrosequencing of 16S rDNA gene amplicons demonstrated that consecutive *R. glutinosa* monoculture modulated the rhizosphere microbiome with a reduction in the abundances of specific beneficial microorganisms and an increase in the harmful microorganisms (Wu et al., 2018b). Similar examples were also found for numerous medicinal plants including *Panax quinquefolius*, *P. notoginseng*, *P. heterophylla* and *P. ginseng* under a monoculture regime (Wu et al., 2016a; Zhao et al., 2017; Dong et al., 2018b; Jiang et al., 2019). Besides, a growing body of research has indicated that replant disease can be attributed to changes in the soil microbiome induced by phenolic allelochemicals, rather than their direct autotoxicity (Li et al., 2014; Wu et al., 2016a; Chen et al., 2017, 2018). Li et al. (2014) found that peanut root exudates could selectively stimulate or inhibit different microbial taxa, and the modifications in the soil microbiome mediated by phenolic acids led to the poor performance of the peanut plants. Our previous study found that the abundance of phenolic acids in *R. glutinosa* root exudates increased with the growth time of seedlings under sterile conditions but did not increase with the increasing years of monoculture under natural field conditions, suggesting that soil microbes might be involved in the degradation, utilization and conversion of root exudates (Wu et al., 2015).

The co-evolution between plants and associated microbial communities is common but complex in natural ecosystems (Zhang et al., 2017; Chagas et al., 2018). During coevolution with their host plant, microorganisms have evolved numerous strategies to talk with the hosts, intraspecific populations and other organisms for growth and survival (Badri et al., 2009; Venturi and Keel, 2016; Kan et al., 2017). Quorum sensing (QS) is a widespread phenomenon by which bacteria use intercellular communication to coordinate their behavior in response to environmental changes. Likewise, plant-associated bacteria utilize QS systems to sense the ecological niche,

adapt to environmental stress, distribute their population under the existing conditions, and thereby influence the growth and health of host plants (Loh et al., 2002; Khan et al., 2019). N-acyl homoserine lactone (AHL)-mediated QS plays important roles in root-microbe interactions and the motility and colonization of rhizobacteria (Loh et al., 2002; von Bodman et al., 2003; Zhang et al., 2017). Plant root exudates not only provide nutrients and energy sources for root-associated microorganisms, but also select, attract or repel specific QS rhizobacteria (Bauer and Mathesius, 2004; Venturi and Keel, 2016; Chagas et al., 2018). Neal et al. (2012) found that 2,4-dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA) released by maize roots could induce positive chemotaxis by *Pseudomonas putida* and attract these bacteria to the rhizosphere. Rosmarinic acid, a plant-derived compound that acts as a QS regulator agonist, activated the quorum sensing responses in *Pseudomonas aeruginosa* (Corral-Lugo et al., 2016). More interestingly, Schaefer et al. (2008) demonstrated that photosynthetic bacterium *Rhodospseudomonas palustris* had the ability to produce a novel QS signaling molecule, *p*-coumaroyl-homoserine lactone, by using a plant-derived aromatic acid, *p*-coumarate. In contrast, quorum quenching (QQ) refers to the process of interference in microbial QS (Dong et al., 2001; Uroz et al., 2009; Grandclement et al., 2016). Plant roots can also produce QS signal mimics or QS-interfering molecules to interfere with microbial QS systems (Teplitski et al., 2000; Zhang et al., 2017). Methyl gallate, a phenolic compound, was reported to inhibit both AHL synthesis and activity in *Chromobacterium violaceum* and to suppress biofilm formation and other QS-associated virulence factors in *P. aeruginosa*. In addition, microorganisms can utilize QQ strategies including the enzymatic degradation of QS molecules to prevent QS signaling and QS-regulated functions in plant-associated microorganisms.

Even though the rhizosphere microbiome plays crucial roles in maintaining soil health, the relationships between QS bacterial populations and replant disease are still unclear. A previous study on *P. heterophylla* replant disease indicated that the number of QS bacteria, all identified as *Serratia marcescens* that can rapidly cause wilt disease, significantly increased with the increasing years of monoculture. Moreover, it was found that *P. heterophylla* root exudates and root tuber extracts could significantly promote the growth of *S. marcescens* (Zhang et al., 2016). Our previous studies have demonstrated that consecutive *R. glutinosa* monoculture led to soil microbiome dysbiosis, and phenolic acids in root exudates could significantly promote the mycelial growth and toxin production of pathogenic *F. oxysporum* (Wu et al., 2015, 2018a,b). However, little is known about the shifts in QS bacterial populations in the *R. glutinosa* rhizosphere under consecutive monoculture, as well as the effects of phenolic acids in root exudates on the growth of specific QS bacteria. We hypothesized that consecutive *R. glutinosa* monoculture could restructure the short-chain AHL-mediated QS bacterial populations in the rhizosphere through the modulation of root exudates, with an increase in the abundance of harmful QS bacteria but a reduction in beneficial QS bacteria.

MATERIALS AND METHODS

Field Experiment and Soil Sampling

The field experiment was conducted in Jiaozuo city, Henan Province (34°56'N, 112°58'E), the geo-authentic production zone. The mean annual precipitation in this region is 552 mm, and the mean annual temperature is 14.3°C. *R. glutinosa* cultivar “Wen 85-5” was used as the experimental material in this study. To ensure uniform soil and climate conditions among different treatments, a single field previously cultivated with wheat was divided into two parts for two cropping patterns: the newly planted (NP) part and the 2-year consecutively monocultured (CM) part. The soil pH of the tested field was 7.43, and the soil organic matter content was 12.52 g·kg⁻¹. The contents of available nitrogen, phosphorus and potassium were 23.41, 51.36, and 223.89 mg·kg⁻¹, respectively. The total nitrogen, phosphorus and potassium were 0.51, 1.46, and 6.98 g·kg⁻¹, respectively. In brief, *R. glutinosa* in the NP plots was planted on April 15 in 2016 and harvested on October 30 in 2016. The CM plots were established in 2015; the plots were consecutively monocultured for 2 years (Wu et al., 2018b). Each treatment had three experimental repetitions (12 m²). All study plots had the same fertilization and water management during the whole experimental period.

The rhizosphere soil samples were collected from 5 random locations within each plot on July 15 in 2016. *R. glutinosa* tuberous roots were carefully excavated with a shovel, shaken to remove loosely attached soil, and then tightly attached soil on the tuberous roots was collected as rhizosphere soils. For bacterial isolation, the soil samples from three replicates of each treatment were combined into a composite sample. All soil samples were passed through a 2-mm sieve to remove plant residues, macrofauna and stones and then used immediately for bacterial isolation and soil total DNA extraction.

The Isolation and Identification of QS Bacteria From Rhizosphere Soil

The biosensor strain *Chromobacterium violaceum* CV026 (CV026), a mini-Tn5 mutant of *C. violaceum* ATCC31532 with kanamycin resistance (Sakr et al., 2013), was used to identify short-chain AHL-mediated QS bacteria (C4-C8-AHLs) (Remuzgo-Martínez et al., 2015). This strain cannot synthesize QS signal molecules but can sensitively respond to exogenous signal molecules (i.e., N-hexanoyl-L-homoserine lactone, C6-HSL) and produce purple violacein pigment (Hossain et al., 2017). The rhizosphere soils collected from healthy NP plants (denoted as NP soil) and the diseased CM plants with root rot disease symptoms (denoted as diseased soil, BT soil) were used to isolate short-chain AHL-mediated QS bacteria. Briefly, a serial dilution of soil suspension (10⁻¹, 10⁻², and 10⁻³) was prepared by using freshly collected soil. The soil suspension was spread on a plate containing Luria-Bertani (LB) medium and incubated at 37°C for 12 h. Then, 200 recognizable single colonies were randomly selected from an appropriate dilution (10⁻³ dilution) for each treatment because there were

200~250 single colonies in this dilution on the LB agar plates. Each single colony was streaked and co-cultured with the biosensor CV026 on the LB plates at 30°C. The inoculation of a known non-QS bacterium and C6-HSL standard solution was used as a negative and positive control, respectively. The production of purple pigmentation by CV026 indicated that the co-cultured bacteria could produce QS signal molecules; these were identified as QS bacteria. The identified QS bacteria were stored at -80°C and were cultured overnight in LB medium for further use. The DNA of QS bacteria was extracted and then used for 16s rDNA amplification with the primer pair 357F (5'-CTCCTAGGGAGGCAGCAG-3') and 1492r (5'-GGTTACCTTGTTACGACTT-3') for Sanger sequencing. The sequence data have been submitted to GenBank (accession number MT355703~MT355756).

Quantitative PCR (qPCR) of *Pseudomonas* sp. in Rhizosphere Soil

The abundance of *Pseudomonas* in NP and BT soils was determined via qPCR with the specific primer pairs Ps-for (5'-GGTCTGAGAGGATGATCAGT-3') and Ps-rev (5'-TTAGCTCCACCTCGC GGC-3') (Tan and Ji, 2010). The PCR reaction mixture and amplification conditions were set as described by Wu et al. (2015). Each treatment had three biological replicates.

Antagonistic Activity Assessment and Pathogenicity Test of QS Bacteria

The isolated QS bacteria were cultured overnight in LB medium at 37°C. The bacterial suspension was inoculated at the periphery of potato-dextrose-agar (PDA) plates and incubated for 2 days at 37°C. Then, the mycelium of *A. flavus* or *F. oxysporum*, two fungal causal agents of *R. glutinosa* root rot diseases (Wu et al., 2016b), was transferred to the center of the PDA plates and incubated again for several days to observe antagonistic activities of QS bacteria.

Simultaneously, QS bacteria were inoculated on Murashige-Skoog (MS) medium that was planted with tissue culture seedlings of *R. glutinosa* to assess the pathogenicity of QS bacteria. Firstly, seedlings shoots without roots were transferred to MS medium supplemented with 0.2 mg/L 6-benzyladenine, 0.2 mg/L indole-3-butyric acid and grown for 40 days. Then, the tissue culture seedlings inoculated with QS bacteria were cultured in a growth chamber at 25°C with a photoperiod of 16:8 h light/dark to observe the symptoms. Each treatment had three biological replicates.

The Effects of Phenolic Acid Mixture on the Growth of QS Bacteria

Based on the detection of the composition and total abundance of phenolic acids in the *R. glutinosa* rhizosphere (Wu et al., 2015), a phenolic acid mixture at the same ratio (molar ratio, protocatechuic acid : phthalic acid : *p*-hydroxybenzoic acid : vanillic acid : syringic acid : vanillin : ferulic acid : benzoic acid = 10 : 10 : 36 : 100 : 12 : 12 : 30 : 30) was applied to

assess the effects of phenolic acids on the chemotactic response and growth of QS bacteria. The chemotaxis of QS bacteria was performed using the drop assay described by Li et al. (2012) with some modification. In detail, 500 µL of QS bacterial suspension was added into 20 mL of minimal medium (Rani et al., 1996) and poured into a petri plate. Subsequently, 0.1 g of phenolic acid powder mixture at the above-mentioned ratio was placed in the center of a petri plate and chemotactic response was observed via migrating rings after 24 h of incubation at 37°C. The growth response of QS bacteria to phenolic acids was assessed through the optic density (OD) value assay. Briefly, the stock solution of phenolic acids was filtered through 0.22 µm filters and then added to an 8-fold dilution of LB broth (1/8 LB) medium to prepare a series of solutions with different final concentrations (0, 30, 60, 120, 240, 480 µmol·L⁻¹). Each treatment had three replicates. Thirty µL of QS bacteria was inoculated into 1/8 LB medium. After incubation at 200 rpm and 37°C for 8 h, the OD value at 600 nm (OD₆₀₀) was detected using a plate reader (Thermo Scientific Multiskan MK3, Shanghai, China).

The Isolation of QQ Bacteria That Could Disrupt the *Pseudomonas brassicacearum* SZ50 QS System

The QS bacteria belonging to the *Pseudomonas* genus were widespread in NP soil in this study and showed a declining trend under consecutive *R. glutinosa* monoculture. Therefore, *P. brassicacearum*, a *Pseudomonas* bacterium showing strong antagonistic activity against the fungal pathogen *A. flavus*, was selected as a target to isolate the corresponding QQ bacteria. In addition to NP and BT soils, another soil (denoted as consecutively cropped soil, CC soil) collected from 2-year CM plants, which had high numbers of adventitious fibrous roots but no root rot disease symptoms, was used for QQ bacterial isolation.

The biosensor strain CV026 was used to assess QQ activity against *P. brassicacearum* SZ50 and to isolate QQ bacteria from NP, CC and BT soils. Firstly, the *P. brassicacearum* SZ50 strain was cultured in LB medium for 16 h, and the QS signal molecules (AHL) were extracted using acidified ethyl acetate (supplemented with 1% acetic acid) according to the method of Anbazhagan et al. (2012) with modifications. AHL extracts were dissolved in HPLC-grade methanol and qualitatively detected by the well-diffusion assay using biosensor CV026 (Anbazhagan et al., 2012). Subsequently, the resulting AHL extracts were used for QQ bacterial isolation. In detail, the isolated strains were grown in sterile 96-well plates (plate No. 1) containing 300 µL of 1/2 TY broth (0.5% tryptone, 0.3% yeast extract, 15 mM KH₂PO₄ and 6 mM CaCl₂) at 200 rpm and 37°C for 16 h. Then, 50 µL culture broths of the isolated strains were transferred into each well of sterile 96-well plates (plate No. 2) containing 50 µL of 1/2 TY broth supplemented with 1 µL of *P. brassicacearum* AHL extracts and incubated at 300 rpm and 30°C for 24 h. Wells containing 50 µL culture broth of a known QQ bacterium, 50 µL of 1/2 TY broth and 1 µL of C6-HSL were used as a positive control. Wells containing only 100 µL of 1/2 TY broth supplemented with

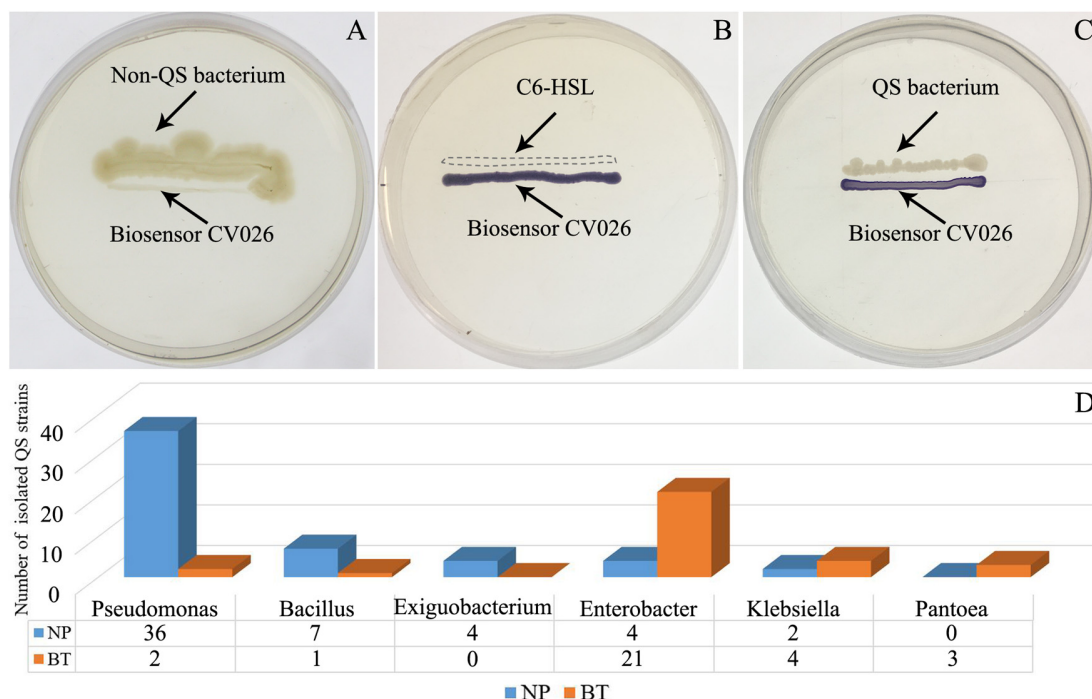


FIGURE 1 | Screening of short-chain AHL-mediated QS bacteria by biosensor strain CV026 (A–C) and the dynamic changes of QS bacteria in the *R. glutinosa* rhizosphere under consecutive monoculture (D). (A) The biosensor strain CV026 did not produce the purple pigmentation when co-cultured with a known non-QS bacterium. (B) CV026 produced the purple pigmentation when inoculated with C6-HSL standard, as indicated by a dashed box. (C) The CV026 colony developed a purple color when co-cultured with a QS bacterium randomly isolated from the *R. glutinosa* rhizosphere.

1 μ l of AHL extracts or C6-HSL were both used as a negative control. After ultraviolet (UV) sterilization for 30 min, 50 μ l of the sterilized culture broths were transferred into each well in new 96-well plates (plate No. 3) containing 100 μ l of 1/2 TY broth, 50 μ l of overnight-cultured CV026 and 0.18 μ l kanamycin (50 μ g·mL⁻¹) and incubated at 300 rpm and 30°C for 24 h to observe the QQ activity of the isolated strains. No purple pigmentation produced by CV026 in plates No. 3 indicated that the corresponding bacteria in plate No. 1 could degrade *P. brassicacearum* AHL signal molecules and could be identified as QQ bacteria (Supplementary Figure S1). The QQ candidates were checked again through the above-mentioned method. Similarly, the molecular identification of QQ bacteria was performed by using 357F and 1492r as mentioned above. The sequence data have been submitted to GenBank (accession numbers MT355757~MT355785).

The Functional Characterization of Specific QQ Bacteria

The effects of phenolic acid mixture on the growth of specific QQ bacteria and their pathogenicity test were carried out using the same method as described above for QS bacteria.

Statistical Analyses

Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) test ($P < 0.05$, $n = 3$) via Data Processing System (DPS)

software (version 7.05, Zhejiang University, Hangzhou, China). Phylogenetic tree construction was performed with Molecular Evolutionary Genetics Analysis (MEGA) version 4.1.

RESULTS

Changes in the Species Composition and Population Dynamics of Short-Chain AHL-Mediated QS Bacteria in the Rhizosphere Under Consecutive Monoculture

Compared with NP, CM plants had serious rot root disease and unexpanded tuberous roots. Moreover, 62% of consecutively-monocultured plants withered and died during sampling. The soil samples collected from the NP and diseased CM plants were used for QS bacterial isolation. Co-cultured with a non-QS bacterium, the biosensor strain CV026 did not produce purple pigmentation (Figure 1A). However, the positive control inoculated with the C6-HSL standard, the CV026 colony developed a deep purple color (Figure 1B). Based on this principle, the short-chain AHL-mediated QS bacteria were randomly isolated by biosensor CV026 from the newly planted (NP) soil and the diseased (BT) soil (Figure 1C). The results showed that 65 out of 200 strains (32.5%) randomly selected from the newly planted (NP) soil of *R. glutinosa* were

detected as QS bacteria, mainly consisting of *Pseudomonas* spp. (55.4%). By contrast, 34 out of 200 (17%) strains from the diseased (BT) soil were detected as QS bacteria, mainly consisting of Enterobacteriaceae (73.5%), including *Enterobacter* spp. (61.8%) and *Klebsiella* spp. (11.8%). The phylogenetic trees of 16S rDNA genes of QS bacteria isolated from NP and BT soils are shown in **Supplementary Figures S2, S3**, respectively. Moreover, the number of QS bacteria belonging to *Pseudomonas*, *Bacillus* and *Exiguobacterium* was markedly higher in the NP soil than in the BT soil. However, the number of QS bacteria belonging to *Enterobacter*, *Klebsiella* and *Pantoea* increased under consecutive *R. glutinosa* monoculture (**Figure 1D**). Quantitative PCR confirmed that the abundance of the *Pseudomonas* genus was significantly higher in the NP soil (3.7×10^7 copies \cdot g soil $^{-1}$) than in the BT soil (1.1×10^7 copies \cdot g soil $^{-1}$) (**Figure 2**).

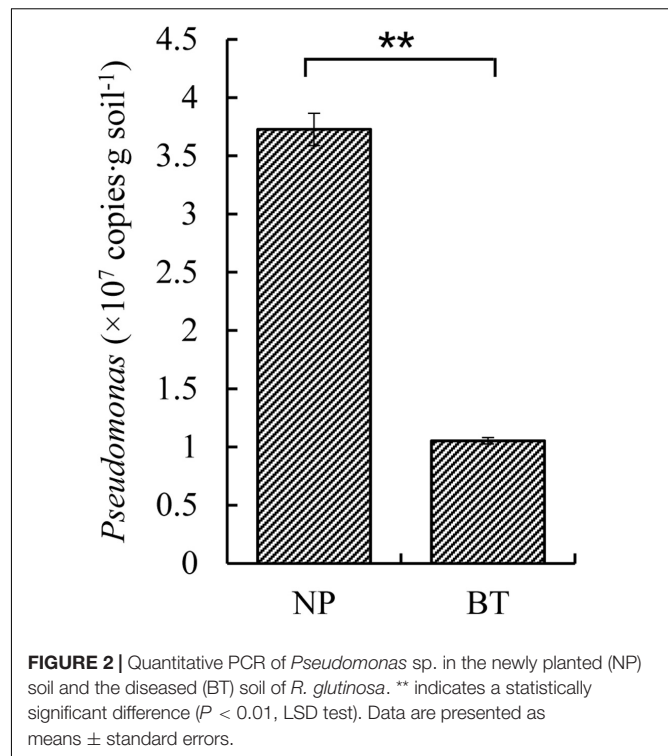
Antagonistic Activity Assessment and Pathogenicity Test of QS Bacteria

The antagonistic activity assessment showed that 33 out of 65 strains of QS bacteria (50.8%) in the NP soil had antagonistic activities against *A. flavus* or *F. oxysporum*, two fungal causal agents of *R. glutinosa* root rot diseases. In particular, most of the QS bacteria belonging to *Pseudomonas* (72.2%) had inhibitory effects on the growth of *A. flavus*. Further, several strains such as *Pseudomonas* spp. SZ88, SZ92, SZ95, SZ16, SZ90, and *Bacillus* sp. SZ93 possessed antagonistic activities against both fungal pathogens (**Supplementary Table S1** and **Figure 3**). By contrast, 14 out of 34 strains of QS bacteria (41.2%) in the BT soil showed antagonistic activity against one of the two fungal pathogens. Except for BT7 with antagonistic activity against *A. flavus*, the others only showed antagonistic activity against *F. oxysporum* (**Supplementary Table S1** and **Figure 3**). Moreover, the dominant QS bacteria in the BT soil, such as *Pantoea* sp. BT3, *Enterobacter* sp. BT22, *Enterobacter* sp. BT23, and *Enterobacter* sp. BT56, were highly pathogenic to the tissue culture seedlings of *R. glutinosa* (**Supplementary Figure S4**).

Different Responses of Beneficial and Harmful QS Bacteria to *R. glutinosa* Root Exudates

Based on the phylogenetic trees of isolated QS bacteria (**Supplementary Figures S2, S3**), most of the dominant taxa were selected to test their chemotactic responses toward phenolic acids identified in root exudates of *R. glutinosa* through the drop assay. The results showed that most of the *Enterobacter* spp. and *Klebsiella* spp., frequently isolated from the BT soil, exhibited distinct chemotaxis toward the phenolic acid mixture. However, several QS strains including *Pseudomonas* sp. SZ90, *Bacillus* sp. 31, *Bacillus* sp.77, *Exiguobacterium* sp. SZ17 and *Achromobacter* sp. SZ99 showed no appreciable chemotaxis (**Supplementary Figure S5**).

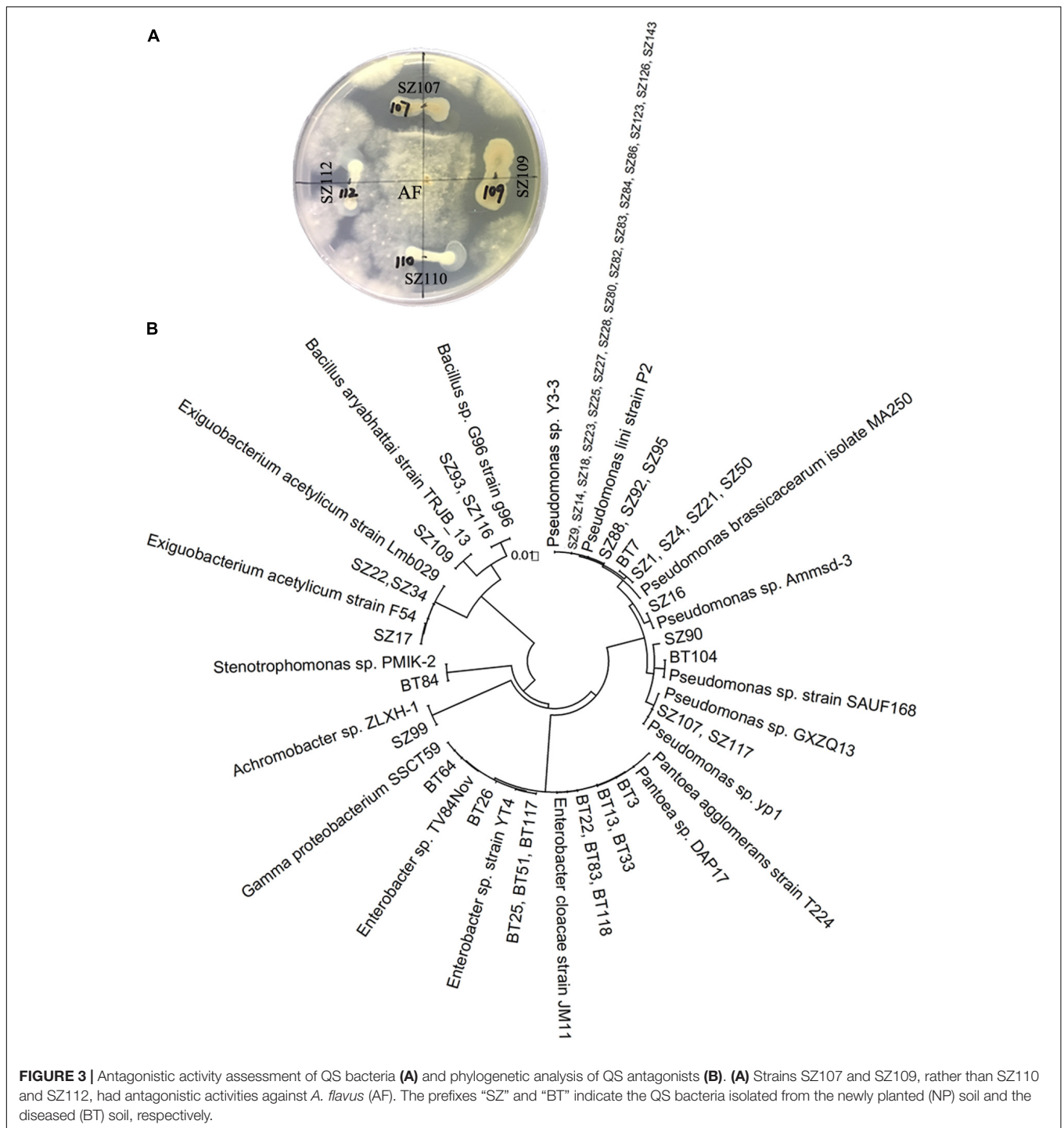
Furthermore, the different responses of specific beneficial and harmful QS bacteria to the phenolic acid mixture were



assessed through the OD value assay. The results showed that the phenolic acid mixture at the same ratio as detected in *R. glutinosa* rhizosphere soil had concentration-dependent inhibitory effects on the growth of beneficial QS bacteria including *Pseudomonas* spp. S9, SZ50, and SZ63, but not SZ8, that were dominant in the NP soil. However, the phenolic acid mixture significantly promoted the growth of harmful QS bacteria that were dominant in the BT soil including *Pantoea* sp. BT3, *Enterobacter* spp. BT22 and BT23, and *Klebsiella* sp. BT56, and the promotion effect increased as the concentration increased (**Figure 4**).

Dynamic Change in Bacteria With Quorum Quenching Activity Against Beneficial QS Bacteria Under Consecutive Monoculture

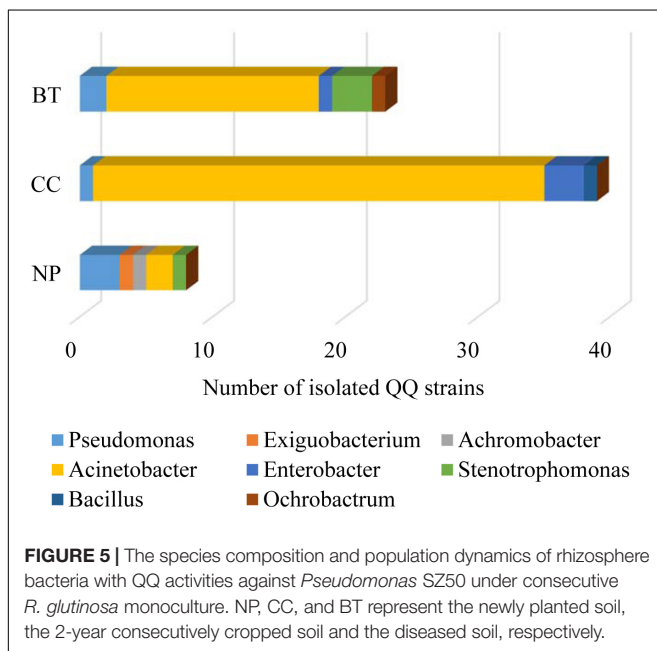
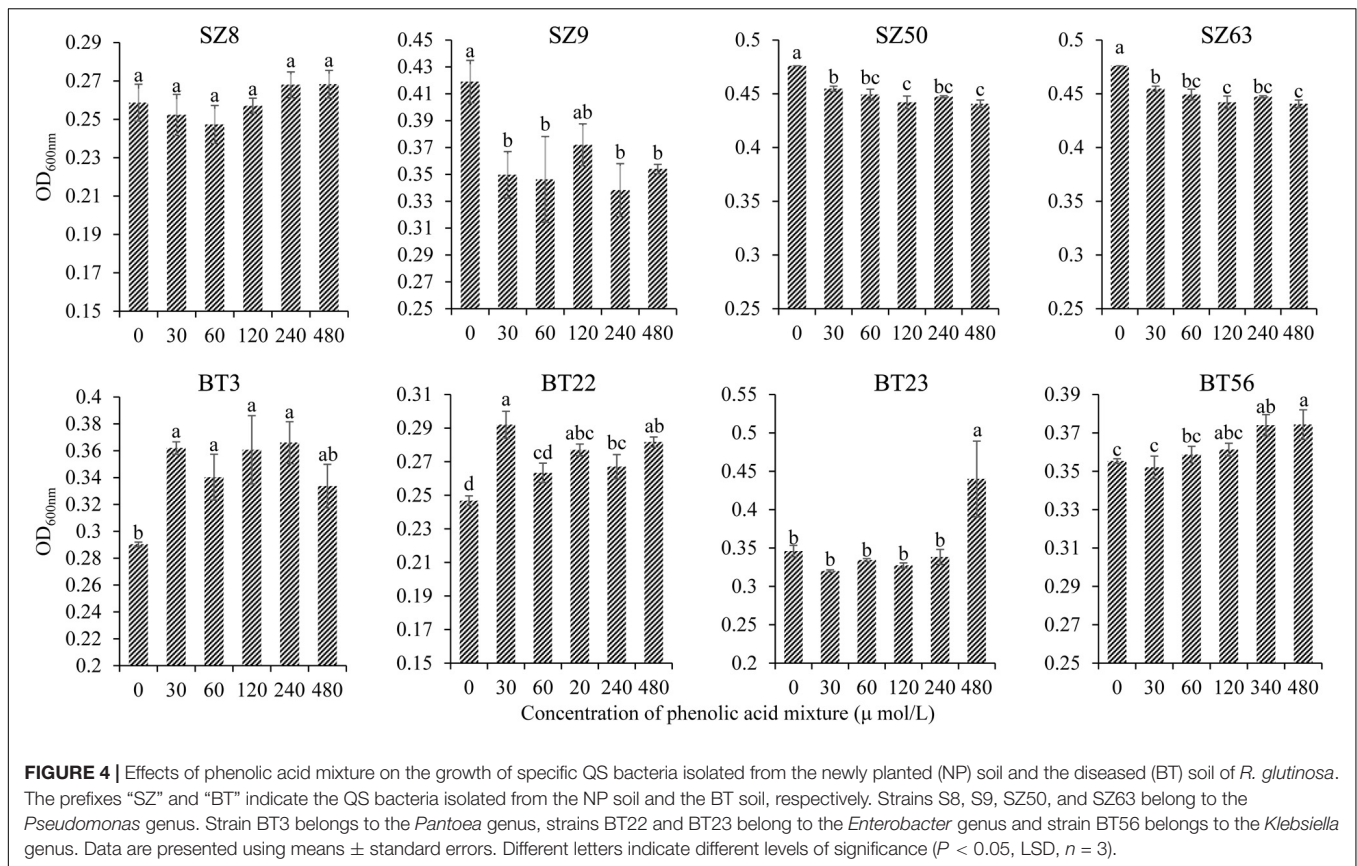
The well-diffusion assay using biosensor CV026 demonstrated that QS signal molecules (AHLs) were successfully extracted from the culture broth of *P. brassicacearum* SZ50 strain (**Supplementary Figure S6**). Screening of QQ bacteria with enzymatic degradation activity against SZ50 AHLs showed that 8 out of 200 strains (4.0%) randomly selected from the NP soil were detected as QQ bacteria, consisting of *Pseudomonas* (37.5%), *Exiguobacterium* (12.5%), *Achromobacter* (12.5%), *Acinetobacter* (25.0%), and *Stenotrophomonas* (12.5%). However, 39 out of 200 strains (19.5%) and 23 out of 200 strains (11.5%) were identified as QQ bacteria in the CC soil and BT soil, respectively. Moreover, QQ bacteria in the consecutively monocultured soils mainly consisted of *Acinetobacter* spp., accounting for 87.2% (34 strains) in CC soil and 69.6% (16 strains) in BT soil (**Figure 5**). Further,



several QQ bacteria belonging to *Enterobacter* spp. were detected in the consecutively monocultured soils, including *Enterobacter* sp. YQC23, *Enterobacter* sp. YQC24, and *Enterobacter* sp. YQC119. The phylogenetic trees of the 16S rDNA genes of QQ bacteria isolated from NP, CC, and BT soils are shown in **Supplementary Figure S7**.

Furthermore, the effects of phenolic acids on the growth of specific QQ bacteria isolated from the consecutively

monocultured soil were tested by OD value assay. The results showed that the phenolic acid mixture at the same ratio as that detected in *R. glutinosa* rhizosphere soil could significantly increase the growth of specific QQ bacteria belonging to *Acinetobacter* spp. (i.e., YQC11, YQC12, and YQB98) and *Enterobacter* spp. (i.e., YQC23, YQC24, and YQC119) (**Supplementary Figure S8**), which were isolated from the consecutively monocultured soils. Furthermore, a pathogenicity



test showed that these specific QQ bacteria isolated from the CC and BT soils could cause severe wilt disease in the tissue culture seedlings of *R. glutinosa* (Supplementary Figure S9).

DISCUSSION

The collective genome of the rhizosphere microbial community is referred to as the second genome of the plant and plays crucial roles in plant growth and health (Berendsen et al., 2012). A growing body of research has demonstrated that replant disease is closely associated with changes in the rhizosphere microbiome (Franke-Whittle et al., 2015; Chen et al., 2017; Yu et al., 2019). Plant roots release variable but substantial amounts of compounds and can shape the rhizosphere microbial community structure (Hu et al., 2018; Stringlis et al., 2018; Voges et al., 2019). Soil microorganisms can actively respond to environmental changes, alter their physiological behavior including motility and chemotaxis, and even modulate the plant root exudation profile (Matilla et al., 2010; Gu et al., 2016; Zhelnina et al., 2018). Quorum sensing, a cell density-dependent type of intercellular communication, allows bacteria to monitor the surrounding environment, coordinate the behavior of populations, and mediate microbe-microbe and host-microbe interactions (Loh et al., 2002; Taga and Bassler, 2003; Chagas et al., 2018). AHL-mediated QS regulates diverse behavior involving both intra- and interspecies interactions in rhizosphere-inhabiting and plant pathogenic bacteria (von Bodman et al., 2003; Zaytseva et al., 2019).

In this study, the species composition and population dynamics of QS bacteria driven by consecutive monoculture of

R. glutinosa were explored. The results showed that numerous bacteria in soil were detected as QS bacteria, accounting for 32.5% of all randomly selected strains in the NP soil and 17% in the diseased soil. This observation is consistent with the findings in previous studies that QS systems are widespread among the bacterial populations in the phytosphere (Veselova et al., 2003; Zhang et al., 2016). Molecular identification found that the QS bacteria isolated from the healthy NP soil mainly consisted of *Pseudomonas* spp. (accounting for 55.4%) and *Bacillus* spp. (accounting for 10.8%) (Figure 1D). Quantitative PCR confirmed the significant decline in *Pseudomonas* spp. in the *R. glutinosa* rhizosphere under consecutive monoculture (Figure 2). Moreover, more than half of the QS bacteria in the NP soil showed antagonistic activities against *A. flavus* or *F. oxysporum*, two fungal causal agents of *R. glutinosa* root rot diseases (Figure 3). Similar findings were reported in our previous study on the rhizosphere microbiome of *R. glutinosa* based on barcoded pyrosequencing (Wu et al., 2018b). Luo et al. (2019) indicated that *P. notoginseng* planting resulted in negative plant-soil feedback due to a decline in the beneficial rhizobacteria including the genera *Pseudomonas* and *Bacillus* in the rhizosphere. *Pseudomonas* spp. and *Bacillus* spp. have been widely proposed to play important roles in specific soil suppressiveness to plant pathogens (Mendes et al., 2011; Kyselková and Moënné-Loccoz, 2012; Shen et al., 2015; Zhou et al., 2019). By contrast, the QS bacteria isolated from the unhealthy BT soil mainly consisted of *Enterobacter* spp. (61.8%) and *Klebsiella* spp. (11.8%) (Figure 1D), and some of them were found to be pathogenic to *R. glutinosa* seedlings. Similarly, Lin et al. (2015) found that the abundance of *Enterobacter* spp. significantly increased in *P. heterophylla* rhizosphere soil with increasing years of monoculture. Köberl et al. (2017) indicated that *F. oxysporum*-infected banana plants harbored a higher abundance of Enterobacteriaceae known for their plant-degrading capacity. However, it should be noted that the culture-independent approaches in our previous study found that the relative abundance of Enterobacteriaceae was significantly higher in newly planted soil than in consecutively monocultured soil (Wu et al., 2018b), which might be due to the difference between culture-dependent and culture-independent assessments. Moreover, the CV026 reporter strain used in this study mainly detects short-chain AHLs (Remuzgo-Martínez et al., 2015). These results suggested that the imbalance between beneficial and harmful QS bacteria in the rhizosphere under consecutive monoculture might be an important factor for *R. glutinosa* replant disease. Further studies are needed to assess the effects of the synthetic multispecies community created using the isolated bacteria on the growth and development of tuberous roots of *R. glutinosa* in natural field sites.

Root exudates are the key determinant of rhizosphere microbiome assembly, acting as chemical attractants, repellants or antagonists of specific microorganisms in soil (el Zahar Haichar et al., 2014; Huang et al., 2014; Chagas et al., 2018; Stringlis et al., 2018). Hu et al. (2018) demonstrated that plant root exudates could modulate the rhizosphere microbiota and thereby affect the growth and defense of the next plant

generation. Here, it was found that the phenolic acid mixture at the same ratio as that detected in *R. glutinosa* rhizosphere soil showed concentration-dependent inhibitory effects on the growth of several beneficial QS bacteria (i.e., *Pseudomonas* spp.) but promoted the growth of harmful QS bacteria (i.e., *Enterobacter* spp.) (Figure 4). This result is in line with previous findings that phenolic acids in *P. heterophylla* root exudates could selectively inhibit beneficial microorganisms (i.e., *Pseudomonas* spp.) and stimulate certain pathogenic bacteria and fungi (Wu et al., 2016c; Chen et al., 2017). Zhang et al. (2016) also demonstrated that both root exudates and tuberous root extracts of *P. heterophylla* could significantly promote the growth of *S. marcescens*, a QS bacterium that rapidly causes wilt disease of *P. heterophylla*. Numerous studies indicated that indirect allelopathy through modifications in soil microbiome induced by root exudates contributes to replant disease in agriculture and horticulture (Li et al., 2014; Chen et al., 2018). Besides, our findings indicated that the QS system of *Pseudomonas* sp. was disrupted by specific QQ bacteria in the soil via the enzymatic destruction of signal molecules. Moreover, the number of bacteria with QQ activity against *P. brassicacearum* SZ50 was considerably higher in the CC soil and BT soil than in the NP soil, and the number of QQ bacteria belonging to *Acinetobacter* spp. greatly increased in the rhizosphere soil under consecutive *R. glutinosa* monoculture (Figure 5). The AHL-degrading activity of *Acinetobacter* spp. has been widely documented in previous studies (Kang et al., 2004; Chan et al., 2011; Ochiai et al., 2013). The QS system is known to play a pivotal role in regulating diverse behavior and functions, such as rhizosphere colonization and competence and biocontrol activities (Loh et al., 2002; Venturi and Keel, 2016). Therefore, QS disruption might be another factor contributing to a decline in antagonistic *Pseudomonas* spp. in the *R. glutinosa* rhizosphere under consecutive monoculture.

In conclusion, consecutive monoculture of *R. glutinosa* resulted in negative soil-borne legacy effects including the build-up of potentially harmful short-chain AHL-mediated QS bacteria in the rhizosphere and the reduction of beneficial short-chain AHL-mediated QS bacteria, which was not only mediated by specific root exudates but also by QQ bacteria. The findings in this work indicated the importance of the rhizosphere microbiome in maintaining soil health and highlighted a link between short-chain AHL-mediated quorum sensing and *R. glutinosa* replant disease. However, further studies will be performed to investigate the roles of rhizosphere bacteria producing the long-chain AHLs in replant disease and the functions of QQ bacteria that target QS signaling of other strains. In addition, further work is needed to restore soil health and overcome the replant disease of *R. glutinosa* by quorum quenching.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

LW and WL conceived the study. LW and QL wrote the manuscript. LW, YW, QL, JW, BY, JC, and HW performed the experiments. LW, BY, and QL performed the statistical analyses. JC, ZZ, and CL were involved in the field management and soil sampling. All authors discussed the results and commented on 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/fpls.2020.00787/full#supplementary-material>

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Multiple Genes of Symbiotic Plasmid and Chromosome in Type II Peanut *Bradyrhizobium* Strains Corresponding to the Incompatible Symbiosis With *Vigna radiata*

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Rhizobia are capable of establishing compatible symbiosis with their hosts of origin and plants in the cross-nodulation group that the hosts of origin belonged to. However, different from the normal peanut *Bradyrhizobium* (Type I strains), the Type II strains showed incompatible symbiosis with *Vigna radiata*. Here, we employed transposon mutagenesis to identify the genetic loci related to this incompatibility in Type II strain CCBAU 53363. As results, seven Tn5 transposon insertion mutants resulted in an increase in nodule number on *V. radiata*. By sequencing analysis of the sequence flanking Tn5 insertion, six mutants were located in the chromosome of CCBAU 53363, respectively encoding acyltransferase (L265) and hypothetical protein (L615)—unique to CCBAU 53363, two hypothetical proteins (L4 and L82), tripartite tricarboxylate transporter substrate binding protein (L373), and sulfur oxidation c-type cytochrome SoxA (L646), while one mutant was in symbiotic plasmid encoding alanine dehydrogenase (L147). Significant differences were observed in L147 gene sequences and the deduced protein 3D structures between the Type II (in symbiotic plasmid) and Type I strains (in chromosome). Conversely, strains in both types shared high homologies in the chromosome genes L373 and L646 and in their protein 3D structures. These data indicated that the symbiotic plasmid gene in Type II strains might have directly affected their symbiosis incompatibility, whereas the chromosome genes might be indirectly involved in this process by regulating the plasmid symbiosis genes. The seven genes may initially explain the complication associated with symbiotic incompatibility.

Keywords: *Bradyrhizobium*, *Vigna radiata*, incompatible symbiosis, interactions, genetic differences, protein 3D structure

INTRODUCTION

Symbiotic relationships between legume plants and soil bacteria, collectively termed rhizobia, are characterized by the formation of root nodules, a specialized plant organ, in which rhizobia differentiate into nitrogen-fixing bacteroids and reduce nitrogen to ammonia as nutrient for plant. In exchange, plants provide specialized environment and carbohydrates to rhizobia

(Krishnan et al., 2003; Nguyen et al., 2017). The association between legumes and rhizobia is highly specific, meaning that each rhizobial species establishes symbiosis with only a limited set of host plants and vice versa; this specificity led to the definition of cross-nodulation groups, which is used for description of symbiotic diversity and rhizobial species (Yang et al., 2010). The symbiotic specificity is determined by a fine-tuned exchange of molecular signals between host plant and its bacterial symbiont (Perret et al., 2000). Rhizobial specificity-related factors, such as NodD, exopolysaccharides, lipopolysaccharides, secreted proteins, Nod-factors, and so on, have been reported to affect the nodulation and host specificity (Radutoiu et al., 2007; Okazaki et al., 2013). Mutations in these related genes can cause incompatible symbiosis between rhizobia and legumes with the phenomenon that a rhizobium is unable to nodulate a particular host plant or forms nodules that are incapable of fixing nitrogen (Faruque et al., 2015; Wang et al., 2018). This incompatible relationship takes place at the early stages of the interaction and is demonstrated to result from signal changing between the host plants and bacteria, which is the molecular basis for the recognition mechanisms evolved in the process of coadaptation (Tang et al., 2016; Fan et al., 2017). This phenomenon also frequently happens at the later stages of nodule development with causing nitrogen-fixing efficiency difference between various plant–bacterium combinations (Wang et al., 2017; Yang et al., 2017). Studies have shown that genes with different functions participate in the control of incompatible symbiosis between rhizobia and plants. In *Bradyrhizobium elkanii* USDA 61, T3 secretion system (T3SS) participates in its incompatible symbiosis with *Vigna radiata* plant (Nguyen et al., 2017). In *Bradyrhizobium diazoefficiens* USDA 110, the metabolic pathways, transporters, chemotaxis, and mobility negatively influence the nodulation with *Glycine max* (host of origin) and *Sophora flavescens* (incompatible host) (Liu et al., 2018a). Cell surface exopolysaccharides (EPS) in *Sinorhizobium meliloti* and lipopolysaccharide (LPS) in *Mesorhizobium loti* 2231 were reported to affect the incompatible symbiosis with *Medicago sativa* (Barnett and Long, 2018) and *Lotus corniculatus* (Turska-Szenczuk et al., 2008), respectively.

It is generally believed that peanut (*Arachis hypogaea* L.) and mung bean (*V. radiata*) belong to the same cross-nodulation group; therefore, the peanut bradyrhizobia have the ability of establishing effective symbiosis with *V. radiata* (Zhang et al., 2011; Li et al., 2019). However, our previous study revealed that the majority of peanut bradyrhizobia (Type I) could establish normal symbiosis with *V. radiata* and the minority of strains (Type II) showed incompatible symbiosis with the same plant, and all the Type II strains contained a symbiotic plasmid (Li, 2019). In detail, Type I strains formed efficient and numerous nodules, and Type II strains formed ineffective and less nodules with *V. radiata* (Li, 2019). Genotype-specific symbiotic compatibility in interactions between legumes and rhizobia is an important trait for the use of root nodule bacteria to improve the crop yield (Triplett and Sadowsky, 1992). The incompatible symbiosis between the peanut rhizobia and *V. radiata* offered a valuable model for investigation of the mechanisms involved

in the symbiotic efficiency of rhizobia, which is not clearly described up to date.

In order to understand the causes for the incompatible interaction between Type II strains and *V. radiata*, we performed the present study. A genetic approach of Tn5 transposon mutagenesis was taken with Type II representative strain *B. guangxiense* CCBAU 53363 to construct a mutant library for screening the potential genes that regulate its effective nodulation on *V. radiata* plant. The mutants with compatible symbiotic phenotype with *V. radiata* were selected by nodulation experiments. Mutational analysis identified seven genes associated with the symbiotic incompatibility, and subsequently, the 3D structures of their predicted proteins were compared between the Type I and II strains. The results in this study would improve our understanding about the symbiotic incompatible mechanisms in legume–rhizobium interactions.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

Bacterial strains, plasmids, and primers used in this study are listed in **Table 1** and **Supplementary Table S1**. Rhizobia and *Escherichia coli* strains were cultured with tryptone yeast (TY) or yeast mannitol agar (YMA) medium at 28°C (Beringer, 1974) and Luria–Bertani (LB) medium at 37°C (Sambrook et al., 1989), respectively. When required, the media were supplemented with sucrose (7%, wt/vol) and/or antibiotics at the final concentrations of kanamycin (Km), 50 µg/ml; gentamicin (Gen), 30 µg/ml; and trimethoprim (Tnp), 10 µg/ml.

Tn5 Mutant Library and Positive Clones Screening

A Tn5 insertion mutant library of CCBAU 53363 was built by triparental conjugation method reported by Liu et al. (2018b) with some modifications. Tn5 transposon was introduced into CCBAU 53363 (recipient) by conjugative transfer of the plasmid pRL1063a-2 (donor) with the help of plasmid pRK2013 (helper). Due to the low Tn5 transposition efficiency (1.25%), using pRL1063a plasmid as donor in CCBAU 53363 chromosome, pRL1063a-2 was constructed in this study by inserting the *sacB* gene (sucrose sensitive gene) in the *EcoRI* site of pRL1063a by seamless cloning, which was located downstream of the Tn5 transposon gene of pRL1063a (see plasmid structure in Wolk et al., 1991). Then, the plasmid pRL1063a-2 was used as the donor in triparental conjugate test, and the transposition efficiency was significantly increased to 18%. After 4 days' triparental conjugating, transconjugants were selected on TY medium containing Tmp, Km, and sucrose. Colonies grown on plates were collected and washed with 0.8% of NaCl solution, resuspended to the concentration of OD₆₀₀ = 0.2, and inoculated to *V. radiata* seedlings at the dose of 1 ml/plant. A total of 400 plants were grown in Leonard jars filled with vermiculite moistened with low-N nutrient solution (Vincent, 1970) at 25°C in greenhouse with a daylight illumination period of 12 h. Nodules were harvested in 30 days postinoculation (dpi) and sterilized by three steps of washing orderly with ethanol (95%, v/v) for 30 s, NaClO (2%,

TABLE 1 | Bacterial strains and plasmids used in this study.

Strains or plasmids	Genotype or relevant characteristics	Source or references
Bacterial strains		
<i>Bradyrhizobium guangxiense</i>		
CCBAU 53363	Wild type of Type II strain; Tmp ^r	Li et al., 2015
L4-T	Tn5 inserted in gene encoding for hypothetical protein, Tmp ^r , Km ^r	This work
L4-P	pJQ200SK knockout in gene encoding for hypothetical protein, Tmp ^r	This work
L82-T	Tn5 inserted in gene encoding for hypothetical protein, Tmp ^r , Km ^r	This work
L82-P	pJQ200SK knockout in gene encoding for hypothetical protein, Tmp ^r	This work
L147-T	Tn5 inserted in <i>ald</i> gene encoding for alanine dehydrogenase, Tmp ^r , Km ^r	This work
L147-P	pJQ200SK knockout in <i>ald</i> gene encoding for alanine dehydrogenase, Tmp ^r	This work
L265-T	Tn5 inserted in gene encoding for acyltransferase, Tmp ^r , Km ^r	This work
L265-P	pJQ200SK knockout in gene encoding for acyltransferase, Tmp ^r	This work
L373-T	Tn5 inserted in gene encoding for tripartite tricarboxylate transporter substrate binding protein, Tmp ^r , Km ^r	This work
L373-P	pJQ200SK knockout in gene encoding for tripartite tricarboxylate transporter substrate binding protein, Tmp ^r	This work
L615-T	Tn5 inserted in gene encoding for hypothetical protein, Tmp ^r , Km ^r	This work
L615-P	pJQ200SK knockout in gene encoding for hypothetical protein, Tmp ^r	This work
L646-T	Tn5 inserted in <i>soxA</i> gene encoding for sulfur oxidation c-type cytochrome SoxA, Tmp ^r , Km ^r	This work
L646-P	pJQ200SK knockout in gene encoding for sulfur oxidation c-type cytochrome SoxA, Tmp ^r	This work
<i>B. guangzhouense</i>		
CCBAU 51670 ^T	Type II strain; Tmp ^r	Li et al., 2019
<i>B. guangdongense</i>		
CCBAU 51649 ^T	Type II strain; Tmp ^r	Li et al., 2019
<i>B. nanningense</i>		
CCBAU 53390 ^T	Type I strain; Tmp ^r	Li et al., 2019
CCBAU 51757	Type I strain; Tmp ^r	Li et al., 2019
<i>B. zhanjiangense</i>		
CCBAU 51787	Type I strain; Tmp ^r	Li et al., 2019
CCBAU 51778 ^T	Type I strain; Tmp ^r	Li et al., 2019
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Sambrook et al., 1989
Plasmids		
pRK2013	Helper plasmid that provides plasmid transfer functions; Km ^r	Figurski and Helinski, 1979
pRL1063a-2	Suicide plasmid for Tn5 mutagenesis of <i>Bradyrhizobium</i> , containing <i>sacB</i> gene (sucrose sensitive gene) from pJQ200SK; Km ^r	This work
pJQ200SK	<i>sacB</i> , suicide plasmid for mutagenesis of <i>Bradyrhizobium</i> ; Gen ^r	Quandt and Hynes, 1993
pJQ200SK-L4	Constructed plasmid for L4 gene knockout	This work
pJQ200SK-L82	Constructed plasmid for L82 gene knockout	This work
pJQ200SK-L147	Constructed plasmid for L147 gene knockout	This work
pJQ200SK-L265	Constructed plasmid for L265 gene knockout	This work
pJQ200SK-L373	Constructed plasmid for L373 gene knockout	This work
pJQ200SK-L615	Constructed plasmid for L615 gene knockout	This work
pJQ200SK-L646	Constructed plasmid for L646 gene knockout	This work
Primers		
Sequences		
415L	5'-CCATTGCGCACTCTCTTT-3' (7,247–7,265 bp)	Liu et al., 2018b
415R	5'-TACTGCCCGCTTGGTTAA-3' (7,644–7,661 bp)	Liu et al., 2018b
T5BF	5'-TTGCTCGTCGGTGATGTA-3' (10,549–10,566 bp)	This work
T5BR	5'-TGCCAAAGGTTTCGTGTA-3' (11,210–11,227 bp)	This work
PM	5'-TCATCTAATGCTAAGGCTGC-3' (199–218 bp)	Liu et al., 2018b

w/v) for 5 min, and sterile distilled water for eight times. Each sterilized nodule was crushed in a sterilized tube, and the crude extract was streaked onto YMA plates supplied with Tmp and Km. After being fostered for nearly 15 days in a 28°C incubator,

isolates were tested by PCR method with two primer pairs 415L/415R (inner primer of Tn5 transposon) and T5BF/T5BR (external primer of Tn5 transposon, designed on the base of the *sacB* gene located in downstream of Tn5 transposon) (Table 1).

The strains with the positive amplification reaction by primer pair 415L/415R and the negative reaction by T5BF/T5BR were identified as positive mutants. Screened positive mutants were verified using colony purification and nodulation validation for twice or thrice in order to confirm their symbiotic stability on nodulation and nodule numbers with *V. radiata*.

Mapping and Sequencing Analysis of Transposon Insertion Sites

For identifying the genes mutated by Tn5 insertion, the transposon insertion sites including the mutated genes were investigated with the following procedure. Total DNA for each Tn5-transposon-inserted mutant was extracted using Promega Wizard Genome DNA Purification Kit (Promega, Madison, WI, United States) and digested with *EcoRI*. The digested DNA fragments were precipitated using nucleic acid precipitation kit (Dr. Gen TLE precipitation carrier) from TaKaRa (Dalian, China), then self-ligated by T4 ligase (NEB) and transferred into DH5 α competent cells by heat shock. Positive clones with resistance to Km were verified by PCR with inner primers 415L/415R for Tn5 transposon, and the transposon gene junction region was amplified and sequenced using the specific primer PM (Table 1). To characterize the acquired genes, gene sequences were searched with BLASTX programs at the GenBank database of National Center for Biotechnology Information (NCBI, Bethesda, MD, United States¹).

Knockout of Tn5-Transposon-Inserted Genes With pJQ200SK Plasmid

In order to exclude false positive of compatible nodulation resulted by the polarity effect derived from Tn5 transposon insertion mutation, knockout of Tn5-transposon-inserted genes were conducted with the triparental conjugation method mentioned above (Liu et al., 2018b), with some modifications, in which the plasmid pRL1063a-2 was replaced with the reformed suicide plasmid pJQ200SK (donor) with the ability of homologous double-crossover recombination. For example, in order to knockout Tn5-inserted L82 gene of CCBAU 53363, pJQ200SK-L82 was constructed using the described methods (Quandt and Hynes, 1993; Sha et al., 2001). First, L82 gene with its upstream and downstream sequences were searched and acquired from the complete genome database of CCBAU 53363 using BioEdit and IGV 2.3, respectively (Li et al., 2015). Based on the obtained gene sequences, the two primer pairs L82-1F/L82-1R and L82-2F/L82-2R (Supplementary Table S1) were designed and used to amplify the upstream and downstream DNA fragments of L82 gene, respectively, by PCR method. Second, the two fragments were connected to the *SmaI* restriction site of the suicide plasmid pJQ200SK by seamless cloning, and then, the constructed pJQ200SK-L82 was transformed into DH5 α -competent cells of *E. coli*. Third, this plasmid was verified by PCR amplification with primer pair M13F/L82-2R (Supplementary Table S1) to ensure that there was no point mutation in the inserted two fragments and then used as donor in the following triparental conjugation experiment.

During the triparental experiment, the constructed plasmid pJQ200SK-L82 (donor) was introduced into CCBAU 53363 (recipient) with the help of pRK2013 (helper). After triparental conjugating for 4 days, single-crossover transconjugants were selected on TY agar plates containing Gen and Tmp and verified by PCR amplification using the detection forward primer and M13R (L82-F/M13R). The succeeded single-crossover isolates were cultured in TY broth containing Tmp with agitation at 180 rpm for 5 days, and subsequently coated on TY agar supplied with Tmp and sucrose for double-crossover filtering. Double-crossover transconjugants were verified by PCR amplification with external (L82SF/L82SR, positive) and intra (L82NF/L82SR, negative) PCR primers. Isolates were purified three times on TY agar with Tmp and sucrose.

Symbiotic Phenotype Analysis

Symbiotic phenotypes on *V. radiata* were tested by inoculating separately with the wild-type strain CCBAU 53363, acquired Tn5-inserted mutants and gene knockout mutants, Type I strain CCBAU 51778 (as positive control), and 0.8% NaCl solution (as negative control). *V. radiata* seeds were dipped 1 min in 95% ethanol solution for surface dehydration and then sterilized in 2.5% (w/v) NaClO solution for 8 min. After being rinsed in sterile distilled water for eight times, seeds were transferred onto 0.6% agar–water plates and germinated for 2 days at 28°C. Seedlings in Leonard jars were inoculated with 1 ml of rhizobial suspension with the concentration of OD₆₀₀ = 0.2. Plant chlorophyll content, shoot dry weights, nodule numbers, and nodule fresh weights of all treatments were recorded 30 dpi (Jiao et al., 2015), and nitrogenase activity per plant of each treatment was also measured with exception for that of Tn5-inserted mutants' treatments (Liu et al., 2017). Each treatment consisted of 10 plants in triplicate. Data were processed with Duncan's *t* test ($P = 0.05$) by SPSS.

Phylogenetic Analysis and Modeling of Proteins

For understanding the mutated genes' function and phylogenetic correlations, the authorized mutated genes' protein sequences of CCBAU 53363, homologous protein sequences of the closely related strains, and the two representative strains for types I and II were acquired by searching corresponding genes through BLASTX in National Center for Biotechnology information (NCBI) website. Phylogenetic tree, based on each mutant' protein sequences of the strain CCBAU 53363 and the homologous sequence of the closely related strains, was built respectively by maximum likelihood (ML) method in MEGA 5.05 (Tamura et al., 2011), and the identity percentages were calculated by Poisson correction model. In the same way, phylogenetic trees based on each mutated genes and corresponding protein sequences of the strain CCBAU 53363 and the representative strains for types I and II were separately constructed as well. Bootstrap analyses were performed using 1,000 replicates, and only the bootstraps values > 60% were indicated in the corresponding nodes of the trees. Protein 3D models were predicted by SWISS-MODEL web server and Pymol software.

¹<http://blast.ncbi.nlm.nih.gov/Blast.cgi>

RESULTS

Characterization of the Seven Mutants of CCBAU 53363

To investigate molecular mechanisms underlying unstable nodulation of *B. guangxiense* CCBAU 53363 on *V. radiata* plants, a library containing about 4.5×10^7 Tn5-transposon-inserted mutants was created. From 400 *V. radiata* plants inoculated with Tn5 transposon mutant library, 647 Tn5-transposon-inserted mutants of CCBAU 53363 presented increase in nodule numbers comparing with that of the wild-type strain CCBAU 53363, and they were preliminarily isolated and purified. Then, 53 out of the 647 mutants were verified to have a better nodulation capability than the others through reinoculation to this plant twice three times, since they showed stable compatibility with *V. radiata*. The knockout mutants of the 53 genes were further constructed through triparental conjugation method, and 7 of the 53 genes were ultimately demonstrated to be responsible for the incompatible symbiosis with *V. radiata* by nodulation tests. By mapping and sequence analysis of the seven mutants of CCBAU 53363, the characteristics including seven mutation gene length and product, protein accession number, and amino acid sequence identities (%) with that of the closed related strains are shown in **Table 2**. The mutants L265 and L615 were tentatively considered as acyltransferase and hypothetical protein due to their low amino acid sequence identity of 27–35.9% and 10.4–27.8%, respectively with the known proteins of some strains of *Bradyrhizobium* spp. and *Phenylobacterium zucineum*. Another two protein products derived from mutated genes L4 and L82 shared 88.5–92.1% and 77.9–84.2% amino acid identities with some hypothetical proteins of *Bradyrhizobium* spp. Mutant L147, Tn5 insertion in the 1,113-bp open reading frame (ORF) encoding alanine dehydrogenase, shared the highest identity of 91.5% with AlaDH protein sequence of *Bradyrhizobium* sp. WSM4349 (WP_018459455.1). A product of gene L373, a Tn5 insertion in the 978-bp ORF encoding tripartite tricarboxylate transporter substrate binding protein (TTT SBP), had the greatest identity of 96.1% with the TTT SBP protein sequence of *Bradyrhizobium* sp. BK707 (WP_130362841.1). The predicted protein of L646 mutant, a Tn5 insertion in the 867-bp ORF encoding sulfur oxidation c-type cytochrome SoxA, shared 96.1% amino acid sequence identity with SoxA of *B. zhanjiangense* CCBAU 51787 (WP_164934866.1).

Symbiotic Phenotypes of Tn5-Transposon-Inserted Mutants on *V. radiata* Plant

In symbiotic test, the symbiosis between Type I strain CCBAU 51778 (positive control) and *V. radiata* was stable or effective, which formed deep red interior nodules and dark green leaves, and the plants showed that chlorophyll content, nodule numbers, and fresh weight and shoot dry weight were significantly higher than those of the other plants. On the other hand, wild-type strain CCBAU 53363 showed incompatible symbiosis, as expressed by the following: (1) no nodules appeared in ~40% of the inoculated plants, and the other 60% plants formed one to three pink nodules, which evidenced the incompatible nodulation and

(2) it showed significantly lower chlorophyll content and shoot dry weight than that of CCBAU 51778, which were similar to that of the non-inoculated controls. Significantly, the seven Tn5-transposon-inserted mutants increased nodule number and nodule fresh weight on the inoculated plants, indicating the stable or effective nodulation capacity when compared with the wild-type CCBAU 53363, but still a little bit lower than that of the Type I strain CCBAU 51778, except of the L373-T mutant. Generally, the rhizobial gene mutations did not influence plant chlorophyll content and shoot dry weight compared with the wild-type strain. Therefore, these mutated genes were preliminarily speculated to participate in negatively regulating nodulation of CCBAU 53363 with *V. radiata* (**Supplementary Figures S1, S2**).

Symbiotic Phenotypes of Gene Knockout Mutants on *V. radiata* Plant

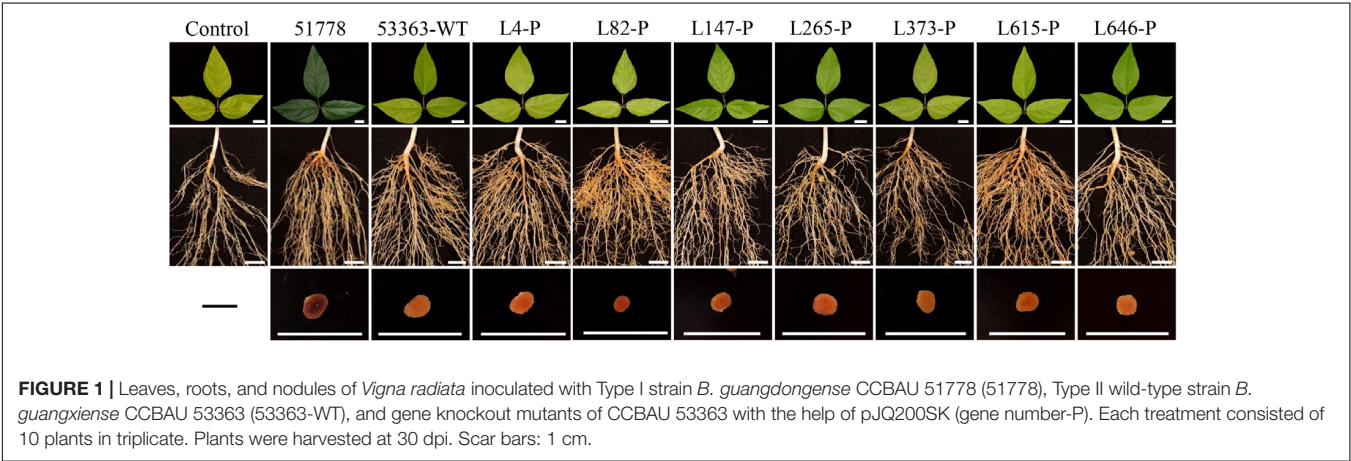
Seven mutated genes mentioned above were completely knockout by plasmid pJQ200SK, and symbiotic phenotype verification was performed with newly constructed mutants separately inoculated on *V. radiata*. Results showed that, with the exception of L373-P, symbiotic phenotypes of *V. radiata* inoculated with the other six gene knockout mutants (L4-P, L82-P, L147-P, L265-P, L615-P, L646-P) were the same as that of their Tn5-transposon-inserted mutants, demonstrating that they were responsible for the stable or effective nodulation of CCBAU 53363 with *V. radiata*. Nodule number and nodule fresh weight of *V. radiata* induced by L373-P mutant were remarkably lower than that of L373-T mutant but still more than that of wild-type strain CCBAU 53363, suggesting that L373-T mutant resulted in a polar effect to some extent but the knockout mutant L373-P were verified to negatively regulate nodulation of CCBAU 53363 with *V. radiata*. The result confirmed the association of the seven mutated genes with nodulation incompatibility on *V. radiata*; however, comparing with CCBAU 53363, the increased nodule number and nodule fresh weight induced by the seven mutants had no significant effects on plant chlorophyll content and shoot dry weight, implying that the problem of plant nitrogen deficiency had not been thoroughly solved (**Figures 1, 2A–D** and **Supplementary Figure S2**).

In order to determine relations between mutated genes and the nitrogen fixation efficiency of nodules, as well as the increased nodule number and plant nitrogen deficiency phenotypes, we tested nitrogenase activity of nodules induced by CCBAU 51778, CCBAU 53363, and mutant inoculated plants, respectively. The results (**Figure 2E**) showed that nitrogenase activity per plant inoculated with mutants were significantly higher than that of the wild-type CCBAU 53363, with exception of mutant L373-P, but lower than that of Type I strain CCBAU 51778. It might explain that nitrogen fixed by the mutant-induced nodules could not completely meet the necessity for plant growth. As to nitrogenase activity per nodule (**Figure 2F**), CCBAU 53363 and its seven mutants showed significantly lower level of activity than CCBAU 51778, and the four mutants L4-P, L82-P, L147-P, and L615-P presented similar nitrogen-fixing capacity with the original strain CCBAU 53363, implying that the four mutants were not associated with nitrogen-fixing efficiency. L265-P showed

TABLE 2 | Characteristics of the seven mutated genes in the study.

Gene	Length (bp)	Annotation	Protein accession no.	Strain (protein accession no., identity percentage ^a)
L4	1,305	Hypothetical protein	WP_164937829.1	<i>B. nanningense</i> CCBAU 51757 (WP_164936447.1, 92.1%) <i>Bradyrhizobium</i> sp. INPA54B (WP_100231454.1, 90.8%) <i>Bradyrhizobium</i> sp. WSM1743 (WP_156952278.1, 90.1%) <i>B. shewense</i> ERR11 (WP_165637841.1, 88.5%) <i>Bradyrhizobium</i> sp. TSA1 (PIT04988.1, 88.5%)
L82	291	Hypothetical protein	WP_128925684.1	<i>Bradyrhizobium</i> sp. MOS003 (WP_106950693.1, 84.2%) <i>Bradyrhizobium</i> sp. AC87j1 (WP_104462656.1, 83.0%) <i>Bradyrhizobium</i> sp. Rc3b (WP_092258140.1, 83.0%) <i>Bradyrhizobium</i> sp. WSM2793 (WP_018320481.1, 81.8%) <i>Bradyrhizobium</i> sp. TSA1 (WP_100174730.1, 77.9%)
L147	1113	Alanine dehydrogenase	WP_128929853.1 (<i>ald</i>)	<i>Bradyrhizobium</i> sp. WSM4349 (WP_018459455.1, 91.5%) <i>Bradyrhizobium</i> sp. DOA9 (WP_025038509.1, 91.2%) <i>Bradyrhizobium</i> sp. aSej3 (WP_148742750.1, 91.0%) <i>Bradyrhizobium</i> sp. LMTR 3 (WP_065746120.1, 91.0%) <i>Bradyrhizobium sacchari</i> BR10555 (WP_080137720.1, 89.2%)
L265	1113	Acyltransferase	WP_128928564.1	<i>Bradyrhizobium</i> sp. 63S1MB (QIO34022.1, 35.9%) <i>Bradyrhizobium</i> sp. BK707 (WP_130363856.1, 29.8%) <i>Bradyrhizobium</i> sp. cf659 (WP_092190185.1, 27.0%)
L373	978	Tripartite tricarboxylate transporter substrate binding protein	WP_128928985.1	<i>Bradyrhizobium</i> sp. BK707 (WP_130362841.1, 96.1%) <i>Bradyrhizobium sacchari</i> BR10555 (WP_080137426.1, 95.0%) <i>Bradyrhizobium</i> sp. WSM2254 (WP_084302701.1, 92.3%) <i>Bradyrhizobium</i> sp. MOS001 (WP_135216717.1, 91.7%) <i>Bradyrhizobium</i> sp. CNPSO 3448 (WP_135178836.1, 91.7%)
L615	852	Hypothetical protein	WP_128923995.1	<i>Phenyllobacterium zucineum</i> (PZQ60673.1, 27.8%) <i>B. lablabi</i> MT34 (SHK62758.1, 10.4%)
L646	867	Sulfur oxidation c-type cytochrome SoxA	WP_164938020.1 (<i>soxA</i>)	<i>B. zhanjiangense</i> CCBAU 51787 (WP_164934866.1, 96.1%) <i>Bradyrhizobium</i> sp. Rc3b (SFM39897.1, 95.7%) <i>Bradyrhizobium</i> sp. Rc3b (WP_092251643.1, 95.7%) <i>Bradyrhizobium</i> sp. WSM2254 (WP_084301459.1, 94.3%) <i>B. vignae</i> LMG 28791 (WP_122406152.1, 92.0%)

^aIdentity percentages were obtained by the phylogenetic analysis of Mega 5.05 ML Analysis.

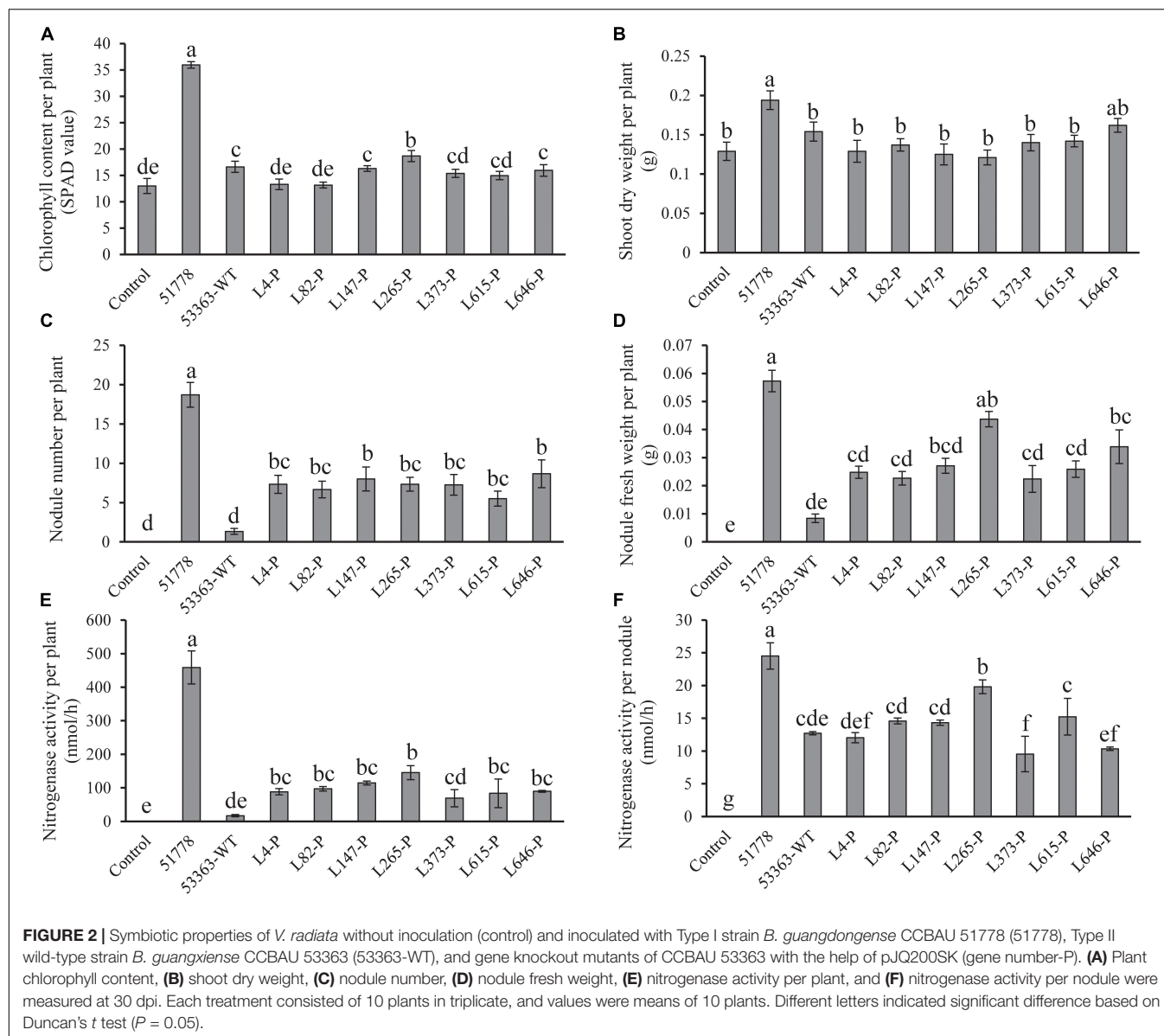


significantly higher nitrogenase activity, whereas L373-P was lower than CCBAU 53363, implying that L265-P or L373-P might have positive or negative correlations with nitrogen-fixation efficiency.

Nucleotide Sequence Analysis of Mutation Genes of Type I and II Strains

The seven symbiotic-related genes detected in CCBAU 53363 were identified to have negative regulatory effects on its nodulation with *V. radiata* in this study. Furthermore, we

collected and aligned these gene sequences of CCBAU 53363 with those of the other Type I and II strains to find the differences separately shared by the strains in each type, which might be the foremost reason for incompatible symbiosis of CCBAU 53363 and the other Type II strains with *V. radiata*. For this analysis, genomes of the Type I strains CCBAU 51757, CCBAU 51778, CCBAU 51787, and CCBAU 53390, and the Type II strains CCBAU 51649 and CCBAU 51670 were used. Results (Supplementary Table S2) displayed that L4, L82, L373, and L646 genes were located in the chromosomes of all the tested



strains with one copy, except for CCBAU 51649 in which the L373 gene was missing, while L265 and L615 were recognized as unique genes of CCBAU 53363 with the ability of causing restrictive nodulation on *V. radiata* plant. Through sequence comparison in this study, we found that Type II strains possessed two copies of L147 gene, which were located in the symbiotic plasmid (L147-p, identical to that of the inserted/knockout gene of CCBAU 53363) and chromosomal symbiotic gene cluster (L147-c, 66.3–66.9% identity with L147-p of CCBAU 53363), respectively. However, only one copy of its homolog (L147-c) was identified in the chromosomal symbiotic gene cluster of type I strains.

Further phylogenetic analyses based on the nucleotide sequences of these genes were performed to verify the evolutionary correlations between Type I and II strains. Results showed that genes L4, L82, L373, and L646 of CCBAU 53363

shared high level identities of 81.8–91.4%, 88.3–94.8%, 81.9–83.4%, and 87.7–92.7% ($\geq 80\%$) with homologous genes of the other tested strains (**Supplementary Table S2**). L147-p gene of CCBAU 53363 was identical to that in the symbiotic plasmid of Type II strains CCBAU 51649 and CCBAU 51670 and was more different from that (70.7–74.1% identity) in chromosomes of Type I strains and L147-c (66.8–69.3% identity) of Type II strains (**Supplementary Table S2**). Within strains in each of the Type I and II or between the Type I and II strains, L373 and L646 genes shared sequence identities of 82.8–100%, 81.9%, and 81.4–83.4% for L373 (**Supplementary Table S3**) and 92–100%, 87.7–91.6%, and 88.2–92.7% for L646 (**Table 3**), respectively, indicating that there was no major difference (identities $\geq 81.8\%$) of the chromosome genes within or between the Type I and II strains. Results for L4 and L82 were not shown due to similar properties of high-level

TABLE 3 | Identity percentages (%) of the L646 gene sequence among the tested *Bradyrhizobium* strains.

L646 gene	Type I				Type II		
	51757 ^a	51778	51787	53390	51649	51670	53363
Type I							
51757	100.0	92.0	92.0	100.0	90.2	88.2	92.7
51778	92.0	100.0	100.0	92.0	91.2	89.1	91.9
51787	92.0	100.0	100.0	92.0	91.2	89.1	91.9
53390	100.0	92.0	92.0	100.0	90.2	88.2	92.7
Type II							
51649	90.2	91.2	91.2	90.2	100.0	91.6	90.2
51670	88.2	89.1	89.1	88.2	91.6	100.0	87.7
53363	92.7	91.9	91.9	92.7	90.2	87.7	100.0

^aCCBAU strain number. Identities (%) were calculated by using MEGA 5.05.

TABLE 4 | Identity percentages (%) of the L147 gene sequences among the tested *Bradyrhizobium* strains.

L147 gene	Type I				Type II			
	51757-c ^a	51778-c	51787-c	53390-c	51649-c	51670-c	53363-c	53363-p
Type I								
51757-c	100.0	89.8	75.0	90.4	62.5	63.4	64.5	70.7
51778-c	89.8	100.0	78.5	93.1	65.9	66.5	67.2	74.1
51787-c	75.0	78.5	100.0	76.0	65.5	65.7	66.3	73.8
53390-c	90.4	93.1	76.0	100.0	63.2	64.9	66.6	73.5
Type II								
51649-c	62.5	65.9	65.5	63.2	100.0	85.8	85.7	66.9
51670-c	63.4	66.5	65.7	64.9	85.8	100.0	86.4	66.8
53363-c	64.5	67.2	66.3	66.6	85.7	86.4	100.0	69.3
^b 53363-p	70.7	74.1	73.8	73.5	66.9	66.8	69.3	100.0

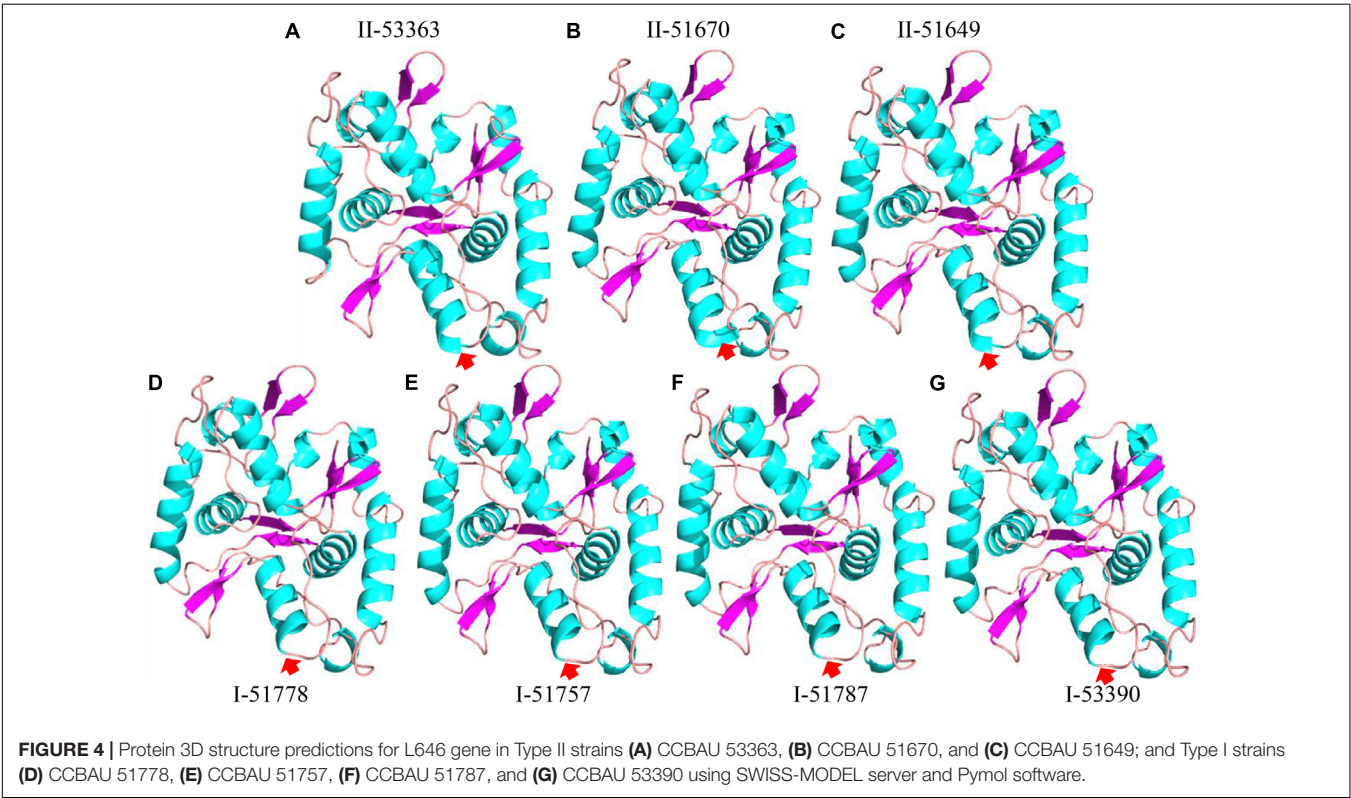
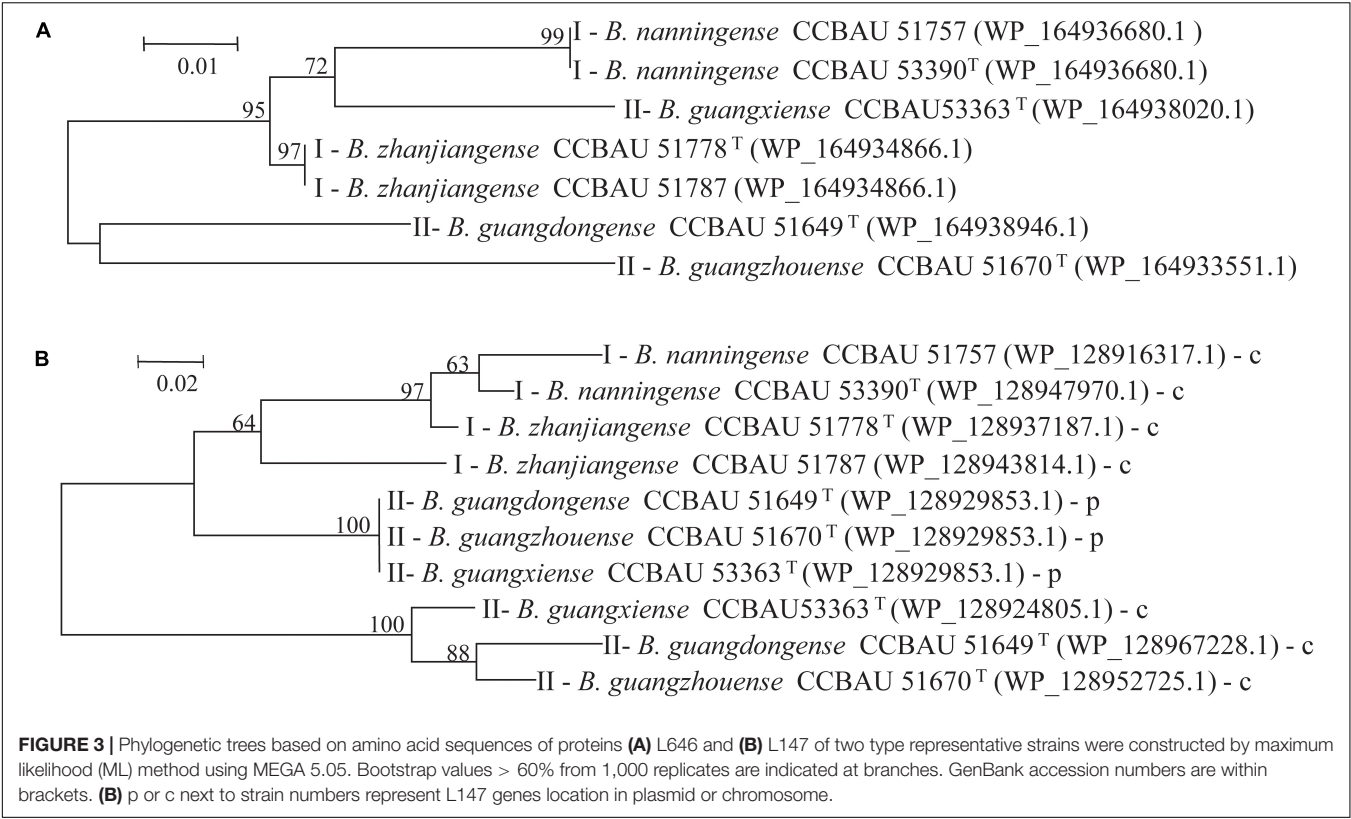
^aAll strains number are CCBAU strain number. Strain number with c indicates the gene L147 locating in chromosome, with p locating in symbiotic plasmid. Due to similarities of L147 gene were total identical in Type II strains symbiotic plasmid, we only used CCBAU 53363-p as representative strain of Type II strains symbiotic plasmid. Identities (%) were calculated using MEGA 5.05.

gene nucleotide identities ($\geq 81.8\%$) and one copy gene in the chromosome among two types of strains compared with that of the genes L373 and L646. In the same analysis for L147-c or L147-p of the Type II strains and L147-c of the Type I strains, the situation was complicated to some extent (Table 4). L147-p gene in the symbiosis plasmid of CCBAU 53363 was identical to that in the symbiosis plasmid of Type II strains CCBAU 51649 and CCBAU 51670 and was different from L147-c in the chromosomes of Type I and II strains with identities of 70.7–74.1% and 66.8–69.3%, respectively. Sequence identities were 75–93.1% for chromosome gene L147-c among the Type I strains, 85.7–86.4% for L147-c among the Type II strains, and 62.5–67.2% for L147-c between the Type I and II strains. Interestingly, L147-p was lightly closely related to L147-c in the chromosome of Type I than L147-c of Type II strains, implying that the two copies of L147 gene in Type II strains might have different evolutionary histories. In general, the mutated genes in the chromosome between Type I and II strains had minor differences (identities $\geq 81.8\%$) compared with that between the homologous genes in the Type II unique symbiotic

plasmid and in the chromosome (identities $\leq 74.1\%$) of both Type I and II strains.

Phylogenetic Tree and 3D Structure Prediction of Symbiotic-Related Proteins

To further analyze the effects of genetic differences between Type I and II strains on protein phylogenetic relationships, 3D structures, and functions, we performed amino acid sequence alignments and constructed phylogenetic trees, as well as predicted 3D structures for L147, L373, and L646 proteins. It was shown that the phylogenies for L373 and L646 amino acid sequences were very similar, and all strains were divided into two branches: one consisted of the Type II strain CCBAU 53363 and all the Type I strains with the identities of 87.9–100% for L373 proteins and 94.6–100% for L646 proteins; another one included the remaining Type II strains CCBAU 51670 and CCBAU 51649 (only for L646) with identities of 92.4% for L646 proteins (Figure 3A, Supplementary Figure S3, and Supplementary Tables S4, S5). However, protein 3D structures in the two types



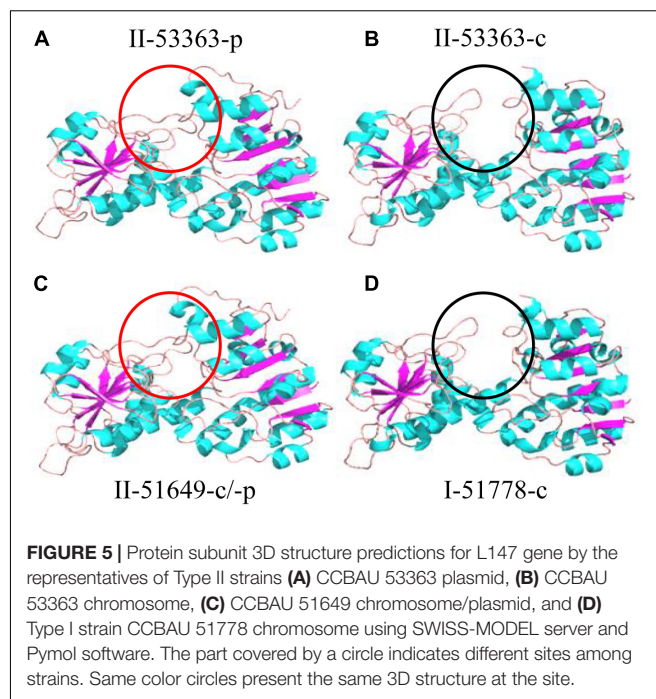
were not significantly affected, and only minor difference in one α -helix (red arrow) was found (Figure 4 and Supplementary Figure S4). These results indicated that the differences in amino acid sequences deduced from the mentioned chromosome genes in the Type I and II strains had no great effects on the 3D structure of the proteins.

Phylogeny of amino acid sequences deduced from the L147 genes classified the tested strains into three clades represented respectively by the Type I strains, symbiotic plasmid copy of the Type II strains, and chromosome symbiotic gene copy of the Type II strains, with inner-clade identities of 87.6–96.7%, 100%, and 93.3–94.7% amino acid sequences (Figure 3B and Supplementary Table S6). However, analysis of protein 3D structures (subunit and hexamer) identified them into two categories: category 1 covered the Type II strains CCBAU 53363-p (gene in plasmid), CCBAU 51649-c (gene in chromosome) and CCBAU 51649-p (the same structure), and CCBAU 51670-c and CCBAU 51670-p (the same structure); category 2 consisted of all the Type I representative strains (CCBAU 51778-c, CCBAU 51757-c, CCBAU 51787-c, CCBAU 53390-c) and the Type II strain CCBAU 53363-c (Figure 5 and Supplementary Figures S5, S6). These results indicated that protein 3D structures were not identical with their amino acid sequences phylogeny, which might be caused by amino acid residues, polarities, and hydrophobicity, which affected the folding of amino acid sequence into the 3D structure. The main distinctions between the two categories of 3D subunit structures were located on the spatial conformation of catalytic binding groove (red circle for category one and black circle for category two) (Figure 5 and Supplementary Figure S5). Similar to the subunits, the 3D structures of the L147 hexamers also indicated two different spatial structures (red or black rectangle gave an indication of a different site) (Supplementary Figure S6).

In addition, CCBAU 53363 simultaneously possessed both categories of L147 proteins, representing that it synthesized both the chromosome (Type I strain, L147-c) and symbiotic plasmid (Type II strain, L147-p) encoded proteins. However, the other Type II strains only translated L147-p protein. Considering the same symbiotic phenotypes of CCBAU 53363 as Type II strains on *V. radiata* (Li, 2019), it could be concluded that, for strain CCBAU 53363, L147-p protein functioned greater than that of L147-c, meaning that L147 in plasmid played a critical role in negatively regulating the symbiotic compatibility on *V. radiata*.

DISCUSSION

Contrary to conventional cognition, a previous study demonstrated that Type II peanut bradyrhizobia strains possessed incompatible symbiotic phenotypes with *V. radiata*, a plant belonging to the *A. hypogaea* cross-nodulation group (Li, 2019). Due to the differences between the genomes of strains in types I and II (Li, 2019), reasons for the incompatibility of Type II strains with *V. radiata* plants appear to be a genetic barrier. It is that the inactivation of genes associated with the rhizobial negative factor allowed the mutants to overcome the nodulation restriction conferred by plant and successfully achieve symbiosis, similar to the incompatible symbiosis between



soybean plants carrying Rj4 and USDA 61 strain (Faruque et al., 2015). This study used Tn5 mutagenesis to screen for mutants of Type II strain CCBAU 53363 compatible with *V. radiata* to investigate the genetic mechanisms of its incompatibility with *V. radiata*. Successful isolation of seven mutants with the ability of stable nodulation with *V. radiata* (Figures 1, 2 and Supplementary Figures S1, S2), comparative analysis results of the mutation genes' sequence (L373, L646, and L147) (Tables 3, 4 and Supplementary Tables S2, S3) and corresponding amino acid sequences (Supplementary Tables S4–S6), amino acid sequence phylogenetic trees (Figure 3 and Supplementary Figure S3), and protein 3D structure predictions (Figures 4, 5 and Supplementary Figures S4, S5) in the present study initially supported our speculation that genetic barrier, caused by the presence of seven genes in the Type II strains, is a crucial cause of the incompatible symbiosis. However, generally, mutants' lower efficient nitrogen fixation ability in nodules showed that other barriers also play roles in the incompatible symbiosis between the Type II strains and *V. radiata*, which needed to be further studied.

The mutation of L147 was located in the gene of encoding L-alanine dehydrogenase (AlaDH, EC 1.4.1.1) that participates in producing alanine from pyruvate and $\text{NH}_3/\text{NH}_4^+$ in *B. japonicum* strain 110 bacteroids inside the soybean nodules, but it is not essential for symbiosis (Lodwig et al., 2004). This enzyme influences amino acid cycle and pyruvate metabolism level in *Rhizobium leguminosarum* cells through the alanine synthesis, which in turn affects the plant nitrogen content and the rhizobial system of tricarboxylic acid cycle and ultimately affects the plant biomass accumulation in soybean plant and bacteroid metabolism in pea nodules (Smith and Emerich, 1993; Lodwig et al., 2004; Dave and Kadeppagari, 2019). Thus, we can conclude that AlaDH participates in the complex metabolic

regulation network of rhizobia. However, it remains unclear how AlaDH regulates nodulation or host infection of rhizobia. In this research, comparison analysis demonstrated that L147-p functioned stronger than L147-c in CCBAU 53363. Symbiotic test found that L147-p gene knockout mutant of CCBAU 53363 did not affect chlorophyll content, shoot dry weight, and nodule's nitrogenase activity, indicating that both L147-p and L147-c did not promote plant biomass accumulation or enhance nodule N₂-fixation efficiency in the case of plant nitrogen deficiency. However, this mutant enhanced nodule number and nodule fresh weight, illustrating that L147-p negatively regulated rhizobial nodulation with *V. radiata*. The role of AlaDH in regulating rhizobial nodulation has not been reported before; therefore, we first found that AlaDH plays a significant role in regulating rhizobial stable nodulation with legume under the premise of plant nitrogen deficiency, which may be affected by the cell regulatory network, and detailed mechanisms needed to be further detected. Furthermore, the mutation of L147-c or double mutation of L147-c-p gene of CCBAU 53363 will help us better understand the function of L147 gene and its regulation mechanism.

The L373 mutant was located in the gene of translating TTT SBP. TTT family is a poorly characterized group of prokaryotic secondary solute transport systems, which employ a periplasmic SBP for initial ligand recognition and present in many bacteria (Winnen et al., 2003; Rosa et al., 2017). SBPs bind with high affinity to diverse classes of substrates, such as tricarboxylates, amino acids, nicotinic acid, nicotinamide, and benzoate (Herrou et al., 2007); terephthalate and other aromatics (Hosaka et al., 2013); 3-sulfolactate (Denger and Cook, 2010); and C₄-dicarboxylic acids (plant secretions, such as succinate, fumarate, and malate, etc.) (Rosa et al., 2019), which deliver substrates to the trans-membrane domains to be imported into the cell. C₄-Dicarboxylic acids have been shown to play important roles as substrates and signal compounds for rhizobia and are considered to be the major carbon sources utilized by free-living *Rhizobium* species during the colonization on root surface (Robinson and Bauer, 1993). However, there is no research on the relationship between TTT SBP and rhizobial symbiosis with legumes. In this study, with the knockout of L373 gene, mutant significantly stabilized the plant nodulation by enhancing nodule number and nodule fresh weight, but it had no influences on chlorophyll content and shoot dry weight, with decreased nodule nitrogenase activity, when compared with the wild-type strain CCBAU 53363. We speculated that the substrate recognition and absorption system involved in the TTT SBP protein might have no preference for *V. radiata* plant root secretion substrates. With the mutation of TTT SBP protein, rhizobial affinity substrate recognition and absorption system to the plant root secretions were enhanced by some unknown reasons and followed with affected rhizobial colonization and nodulation efficiency on root surface. It is the first finding that TTT SBP substrate absorption system of CCBAU 53363 is involved in regulating compatible symbiosis with *V. radiata* and nodule nitrogen fixation efficiency.

In the mutant L646, a gene encoding sulfur oxidation c-type cytochrome SoxA protein was found to be mutated,

which was a subunit of SoxAX cytochromes, a part of c-type cytochromes that catalyzes the transformation of inorganic sulfur compounds (Bamford et al., 2002; Ogawa et al., 2008). Inorganic sulfur compounds' oxidation capability is frequently found in phylogenetically and physiologically diverse bacteria, including the members of *Bradyrhizobiaceae* with *sox* homologs of *B. japonicum* USDA110 (Masuda et al., 2010). However, the relationship between inorganic sulfur metabolism and symbiosis of rhizobia with plants has not been studied. In this research, *soxA* knockout mutant significantly increased the nodule number and nodule fresh weight, but *V. radiata* plant's chlorophyll content, shoot dry weight, and nodule nitrogen fixation efficiency were not influenced, indicating that *soxA* negatively regulated CCBAU 53363 nodulation on *V. radiata*, and had no significant effects on the extremely inefficient nitrogen fixation ability. Therefore, a certain regulatory relationship might exist between the inorganic sulfur metabolism of rhizobia and their stable nodulation with legumes.

Studies have demonstrated that a successful symbiosis depends on the interaction of rhizobia and plant with complex chemical signaling communication (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003; Arrighi et al., 2006), recruitment and attachment of rhizobia to growing root hair tips (Murray, 2011), activation and inhibition of plant defense systems during the rhizobial infection (Bright and Bulgheresi, 2010; Tóth and Stacey, 2015), and rhizobial differentiation and bacteroids metabolism in plant cells (Oldroyd and Downie, 2008). In this research, the Type II strains occasionally nodulated with *V. radiata*, illustrating the incompatible and unsuccessful interactions between these strains and *V. radiata*. According to the results of mutants' symbiosis with *V. radiata*, we found that factors with multiple functions in Type II strains participated in the incompatible symbiosis with *V. radiata*, such as substrate recognition and absorption before infection (L373), inorganic sulfur metabolism (L646), and proteins with unknown functions (L4, L82, L265, L615). At the same time, with the exception of new genes, we also discovered new functions for some well-known genes. For example, during the incompatible symbiosis of CCBAU 53363 and *V. radiata* (in the case of plant nitrogen deficiency), L147 gene did not function as mentioned in other studies (Lodwig et al., 2004) but participated in the negative regulation of stable nodulation. Our mutant screening analysis identified several genetic factors of CCBAU 53363 involved in the incompatibility with *V. radiata* and implied that diverse and multiple mechanisms might cause these host-specific interactions.

Further protein prediction revealed that the 3D structure (category 1) deduced by symbiosis-related gene L147-p of CCBAU 53363 and L147-c/-p of other Type II strains was different from that of the corresponding protein (category 2) coded by chromosome genes in Type I and II strains. The difference might be the crucial site affecting L147 protease catalytic activity or efficiency and would be directly related to the symbiotic phenotype divergence between Type II and Type I strains. It could be supposed that the gene L147-p in CCBAU 53363 functioned stronger than the L147-c counterpart, although it needs to be further confirmed through experiments.

According to L373 and L646 genes and the other symbiosis-related genes, the chromosome genes of Type II strains possessed high genetic homology and similar 3D protein structures with the corresponding genes in chromosome of Type I strains, which illustrated that they might indirectly regulate the symbiosis by an unknown way and eventually led to this incompatible symbiosis on *V. radiata*. It is therefore likely that the genetic barrier exists between Type II strains and *V. radiata*: the mutated genes associated with rhizobial negative factor directly or indirectly allowed mutants to overcome the condition of unstable nodulation, a part of incompatible symbiotic barrier.

In brief, the present study initially demonstrated seven genes of Type II strain CCBAU 53363 responsible for compatible nodulation with *V. radiata*, in which the regulation mechanism is needed to be further researched. These results partially explained the restrictive nodulation of CCBAU 53363 with *V. radiata*. Simultaneously, we also found that the L265 gene negatively regulated nodule nitrogenase activity. However, L265-T/P mutant inoculated plants were still in nitrogen deficiency state due to the low number of nodules, indicating that trials on enhancing nodules number will be needed. Besides, with the comparison of original strain CCBAU 53363, L4, L82, L147, L615, and L646 gene knockout mutants did not influence the nodule nitrogen fixation efficiency, which implied that the experiments on improving nodule nitrogenase activity need to be implemented. That is, Tn5 transposon randomly inserted in mutants' genome and selected by *V. radiata* with the different standards mentioned above will help us to explore the determinants and regulatory networks for incompatible symbiosis between Type II strains and *V. radiata*; Songwattana et al. (2019) demonstrated that the photosynthetic bradyrhizobial strain ORS278 acquired a broader host range with the ability to form nodules on *Crotalaria juncea* and *Macroptilium atropurpureum* through acquiring a symbiotic mega-plasmid from the non-photosynthetic *Bradyrhizobium* strain DOA9. Otherwise, the "experimental evolution" approaches used by Marchetti et al. (2010) evolved a plant pathogen into legume symbiont after transferring a symbiotic plasmid. All these data verified that the symbiotic plasmid played a great role in rhizobial host range and symbiotic compatibility with plants. Coincidentally, we found that gene in plasmid functioned stronger than the copy in chromosome in CCBAU 53363, and only Type II strains contain symbiotic plasmid with identical nucleotide sequences (Li, 2019). Therefore, symbiotic plasmid might also play a role in Type II strains' symbiotic compatibility

with *V. radiata*. It would be an interesting and positive certification for this supposes to transfer the symbiotic plasmid from Type II to Type I strains, as Type I strains obtain plasmid and show incompatible symbiosis similar to type II strains.

DATA AVAILABILITY STATEMENT

Publicly available datasets were analyzed in this study. This data can be found here: CP022219.1, CP030053.1, CP030051.1, CP022221.1, LBJQ00000000, LBJM00000000, LBJC00000000, WP_128916317.1, WP_128937187.1, WP_128943814.1, WP_128947970.1, WP_128967228.1, WP_128952725.1, WP_128924805.1, WP_128929853.1, WP_128918822.1, WP_128930686.1, WP_128947785.1, RXH23619.1, WP_128949221.1, WP_128928985.1, WP_164936680.1, WP_164934866.1, WP_164938946.1, WP_164933551.1, and WP_164938020.1.

AUTHOR CONTRIBUTIONS

YW, XS, and YL conceived the study. YW, JS, LC, and BH performed the experiments. YW and XS analyzed the data and wrote the manuscript along with the help of EW. CT, WFC, and WXC provided resources. 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/fmicb.2020.01175/full#supplementary-material>

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A Compost Treatment Acts as a Suppressive Agent in *Phytophthora capsici* – *Cucurbita pepo* Pathosystem by Modifying the Rhizosphere Microbiota

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Phytophthora capsici Leonian (PHC) is a filamentous pathogen oomycete that causes root, fruit, foliar and crown rot over a wide host range, including the economically and nutritionally important summer squash (*Cucurbita pepo* var. *cylindrica* L.) crop. PHC chemical control strategies are difficult to adopt, due to the limited number of registered chemicals that are permitted and the scalar harvest system. For these reasons, other strategies, such as the use of waste-based composts that can act as suppressive agents against several soilborne pathogens, have been studied intensively. It is well known that compost's microbiota plays an important role to confer its suppressive ability. In this study, four different composts were analyzed with both 16S rRNA gene and 18S rRNA gene real-time PCR amplification and with 26S gene amplicon-based sequencing; the total abundance of the bacterial and fungal communities was found to be higher compared to literature, thus confirming that the four composts were a good inoculum source for agricultural applications. The core mycobiota was mainly composed of 31 genera; nevertheless, it was possible to observe a clear predominance of the same few taxa in all the composts. The four composts were then tested, at different concentrations (1–10–20% v/v), to establish their ability to confer suppressiveness to the *Phytophthora capsici* (PHC) – *Cucurbita pepo* pathosystem in controlled greenhouse pot trials. A total of 12 compost mixtures were considered, and of these, one (*Trichoderma*-enriched compost at 10% v/v) was able to statistically reduce the disease incidence caused by PHC (by 50% compared to the untreated control). Hence, the microbiota composition of the most effective compost treatment was investigated and compared with untreated and chemical (metalaxyl) controls. Mycobiota sequencing showed genera differences between the three treatments, with relative abundances of several fungal genera that were significantly different among the samples. Moreover, PCA analyses clustered the

compost treatment differently from the chemical and the untreated controls. These findings suggest that suppressive activity of a compost is strictly influenced by its microbiota and the applied dosage, but the ability to induce a shaping in the rhizosphere microbial composition is also required.

Keywords: *Phytophthora capsici*, *Cucurbita pepo*, compost, *Trichoderma*, soil metatranscriptomics, mycobiota

INTRODUCTION

Phytophthora capsici Leonian (PHC) is a filamentous pathogen oomycete that causes root, fruit, foliar, and crown rot over a wide host range, including some economically and nutritionally important horticultural crops (cucurbits, tomatoes, pepper, eggplant) (Lamour et al., 2012). In Italy, and throughout the world, summer squash (*Cucurbita pepo* var. *cylindrica* L.) cultivation is strongly affected by the natural and human-derived presence of PHC inoculum (Erwin and Ribeiro, 1996). Chemical control strategies are difficult to adopt, due to the limited number of registered chemicals that are permitted and the scalar harvest system (Gilardi et al., 2015). However, many efforts have been made to find a source of genetic resistance in squash accessions to PHC strains (Michael et al., 2019; Siddique et al., 2019), but this approach is still at its beginning. For these reasons, alternative PHC control agents are being studied and adopted in agriculture. Moreover, one of the most promising and most studied technique to prevent the infection of soil-borne pathogens is the application of organic amendments (Meghvansi and Varma, 2015; Gilardi et al., 2016; De Corato et al., 2018a,b).

In the last few decades, interest in using organic amendments has grown throughout the world, due to the possibility of reintroducing recycled biowastes and organic matter into the primary production industry, which is connected to the concept of circular economy. Among the various organic amendments, compost has been studied the most and has been used because of its suppressiveness activity (Pugliese et al., 2015; Bonanomi et al., 2018). The phenomenon of suppressiveness is the ability to limit or avoid the spread of a disease where both susceptible crop variety and pathogen are present in the field; this is connected with the available amount or the addition of organic matter and the ability of the seeds and roots to establish a connection with diverse microorganisms present in the soil that uptake exudates and, consequently, limit the outbreak of pathogens and parasites (Topalović et al., 2020).

Moreover, plant root exudates have been shown to have the ability to select a specific microbiota and influence the colonization of root areas by microorganisms; this means that rhizosphere microbiota is closely connected not only to the soil but also to the plant genotype since different cultivars were shown to be able to select different microbiota (Weller et al., 2002; Haas and Défago, 2005; Bulgarelli et al., 2012; Doornbos et al., 2012; Badri et al., 2013; Mazzola et al., 2015).

Nevertheless, since each compost can be different, suppressive action is not always guaranteed, and, sometimes, a compost can even play a conducive role. Bonanomi et al. (2007) redacted a list of 2,423 articles in this field and showed that only 54% of the studied composts were able to induce suppressiveness. It is also

known that a mature compost can play both a suppressive and a conducive role as a function of the pathosystem (Bonanomi et al., 2010). Compost microbiota plays a major role in the suppressive activity of composts, and in an attempt to prove this, many studies have compared sterilized composts with their non-sterilized counterparts and have found that the inactivation of microbiota is connected to the loss of suppressive activity (Reuveni et al., 2002; Tilston et al., 2002; Papasotiriou et al., 2013; De Corato et al., 2019). The microorganisms present in a compost are also selected by root exudates, according to the need and the genotype of the plant, resulting in the shaping of the rhizosphere composition and accomplishing the suppressive activity against several biotic stresses such as fungi and nematodes (Antonioni et al., 2017; Mwaheb et al., 2017; Cucu et al., 2019; Zhang et al., 2020). Thus, in order to prevent soil-borne diseases when pathogens are present in field, it is very important to know which microorganisms confer compost suppressiveness according to the type of pathosystem. Many studies have investigated the microbial communities present in several composts, but most of them were based on *in vitro* isolation, which has the drawback of excluding non-cultivable microorganisms and therefore of not giving a full picture of the entire complexity. Moreover, recent studies have pointed out the importance of using molecular culture independent methods (Šišić et al., 2018; Cao et al., 2019; Carrasco et al., 2019; Fernández-Bayo et al., 2019; Zhao et al., 2019; Zhou G. et al., 2019).

The objectives of this work were to investigate the microbiota populations of four different composts using targeting (real-time PCR) and non-targeting (amplicon-based Illumina sequencing) molecular approaches; to test their ability to confer suppressiveness in a squash – PHC pathosystem in controlled greenhouse pot trials; and to investigate, on the basis of the greenhouse trial results, the microbiota composition of the best compost treatment, in comparison with an untreated and a chemical treatment, by means of the same molecular tools used for the compost characterization.

MATERIALS AND METHODS

Composts Used in This Study

Four different commercial composts were used in this study: (i) a green waste compost produced in a dynamic composting system for 6 months and sifted with a 10 mm sieve (ANT's Compost V – CV; AgriNewTech s.r.l., Italy), (ii) the same green compost enriched with experimental BCA “*Trichoderma* sp. TW2” (ANT's compost M – CM; AgriNewTech s.r.l., Italy), (iii) a municipal biowaste compost produced using green and urban organic

fraction biowastes in a dynamic composting system for 4 months (ANT's Compost B – CB; AgriNewTech s.r.l., Italy), and (iv) a green compost produced in a dynamic composting system for 6 months and sifted with a 20 mm sieve (ANT's compost V2 – CV2; AgriNewTech s.r.l., Italy). At the end of the maturation process, CV, CM, CV2, and CB were analyzed by an external laboratory to establish their chemical compositions (**Table 1**).

Greenhouse Trials

Summer squash (*Cucurbita pepo* var. *cylindrica* L. cv Genovese) seeds were sown in seed cells in a peat substrate (Tecno 2, 70% white peat and 30% clay, pH 5.5–6, N 110–190 mg/L, P₂O₅ 140–230 mg/L, K₂O 170–280 mg/L, Turco Silvestro terricci, Bastia d'Albenga, SV, Italy) and kept in a nursery for 2 weeks at 26 ± 1°C. In the meantime, substrates were prepared for potted plants, by adding different percentage of each compost (1–10–20% v/v) to the same peat used for sowing. After 1 week, each substrate mixture was infested with 2 g/l of fresh biomass of one strain of PHC (AGROINNOVA collection), grown for 2 weeks in grain-hemp (60:40) flasks, according to the method described in Gilardi et al. (2015). A chemical treatment, in which a suspension of metalaxyl (Ridomil gold, 480 g/l, Syngenta Crop Protection) and water was used in order to reach a final concentration of 50 µl/l of substrate, was carried out at the same time as the inoculation. One week after the infestation, the seedlings were transplanted into 21 pots, with 3 plants per pot, and placed in a greenhouse kept at 24 ± 1°C. Each treatment was replicated in three different pots per trial, with a randomized experimental design. The experiment was carried out twice independently.

Disease Assessment

Disease incidence (DI) was evaluated by counting the number of diseased plants in each pot twice during the trials, according to the formula: $\frac{\text{number of diseased plants}}{\text{number of total plants}} \times 100$; an intermediate disease assessment was performed 1 week after transplantation; the final evaluation was performed 1 week later. The fresh biomass of the plants was also weighed. The area under the disease progress curve (AUDPC) was calculated according to Pandey et al. (1989).

TABLE 1 | Chemical composition of the tested composts.

	CV/CM	CV2	CB
pH	7.92	8.08	8.08
Humidity (%)	42.00	43.10	40.90
Organic C (g/kg dry matter)	210.00	258.00	220.00
Organic N (g/kg dry matter)	15.70	23.60	21.30
C/N ratio	13.00	9.44	9.16
Total N (g/kg dry matter)	16.30	15.50	24.00
Organic N/total N ratio	96.00	88.45	88.75
Hg (mg/kg dry matter)	0.16	<0.01	<1.50
Ni (mg/kg dry matter)	93.10	11.6	84.00
Pb (mg/kg dry matter)	47.90	32.1	39.00
Zn (mg/kg dry matter)	143.80	140.10	206.00
Cu (mg/kg dry matter)	52.50	56.60	148.00

Sampling and DNA Extraction

Each compost was collected individually from different and random parts of big bags (total volume 50 ml). Two separate DNA extractions were carried out for each compost using 100 mg of fresh compost. The rhizosphere was collected at the end of the pot trials. Three biological replicates were collected from three different pots per treatment and per trial, the plant roots were shaken to remove any excess peat, and the particles that were still adhered to the root system were collected in 50 ml vials. Total microbial DNA extraction was carried out with EZNA soil DNA kit using 100 mg of soil (Omega Bio-Tek, Norcross, GA, United States), following manufacturer's instructions. DNA quantity was assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States), while DNA integrity was verified by running 5 µl of each sample in a 1% agarose electrophoretic gel.

Real-Time PCR Assays

Real-time PCR assays were performed using a StepOne-Plus™ Real-Time System (Applied Biosystems, Foster City, CA, United States). The abundance of the total fungal (18S rRNA gene) and bacterial (16S rRNA gene) communities in the compost samples was determined. The abundance in the rhizosphere samples was instead assessed, for the total fungi, total bacteria, and *Phytophthora capsici*, with the primers by Lan et al. (2013) and under the conditions described by Cucu et al. (2020). Real-time PCR was performed for each extraction in triplicate, and the average values were then transformed into Log of gene copies per gram of dry compost; these data were mediated between two extractions of each sample. **Table 2** summarizes the primers and real-time PCR conditions.

Amplicon-Based Sequencing

The mycobiota were evaluated by amplifying the D1 domain of the 26S gene using the primers and condition described by Mota-Gutierrez et al. (2019). A library preparation was performed according to the Illumina metagenomic procedure. Sequencing was performed using a MiSeq instrument (Illumina) with V3 chemistry and 250-bp generated paired-end reads, following the manufacturer's instructions. After sequencing, reads were assembled, quality filtered and processed using QIIME 1.9.0

TABLE 2 | Description of the primer sets and amplification conditions of the quantitative real-time PCR assays.

Gene	Primers	Real-time PCR conditions
18S rRNA gene, total fungal abundance	FR1 (Vainio and Hantula, 2000) 390FF (Vainio and Hantula, 2000)	45 cycles 95°C 30", 50°C 30", 70°C 60"
16S rRNA gene, total bacterial abundance	Eub338 (Muyzer et al., 1993) Eub51 (Muyzer et al., 1993)	40 cycles 95°C 30", 55°C 35", 72°C 45"
<i>Phytophthora capsici</i> , pathogen abundance	Pc1F (Lan et al., 2013) Pc1R (Lan et al., 2013)	40 cycles 95°C 30", 60°C 35", 72°C 45"

software (Caporaso), and the pipeline described by Mota-Gutierrez et al. (2019). Centroids sequences of each cluster were manually checked by Blast tool to confirm the taxonomic assignment. QIIME was used to rarefy the OTU table at the lowest number of sequences per sample and to build the OTU table. The OTU table displays the highest taxonomy resolution that was reached; when the taxonomy assignment was not able to reach the genus level, family name was displayed. Relative abundance of OTUs was used to build a principal component analysis (PCA) as a function of the treatment. Anosim statistical test was used, through the *vegan* function of R, to identify any significant differences as a function of the treatments. α -diversity was assessed by Chao1 index, estimating the number of different taxa, and by Shannon diversity index, evaluating the taxa richness calculated using the diversity function of the *vegan* package in R environment. The Wilcoxon matched pairs test was used to establish the difference in OTUs abundance as a function of the treatment. *P*-values were adjusted for multiple testing using the Benjamini–Hochberg procedure, which assesses the false discovery rate (FDR).

Statistical Analyses

Statistical analyses were performed, with SPSS software (IBM SPSS Statistics, Westland, MI, United States), for the disease incidence, fresh biomass, AUDPC, and real-time PCR data. ANOVA and Tukey's *post hoc* tests were performed to establish the statistical values of the differences ($P < 0.05$). The DI, fresh biomass, and AUDPC data were unified for the two separate trials.

Availability of the Sequence Data

The sequencing data were deposited at the Sequence Read Archive of the National Centre for Biotechnology Information under BioProject number PRJNA580394.

RESULTS

Abundance of the Total Microbial Community in Four Composts

Analyses were carried out, with real-time PCR, to describe the microbiological assessment in four composts, in terms of gene abundance expressed as Log of copy⁻¹ numbers per gram of dry matter (Figures 1A,B). The fungal 18S rRNA gene copies per 1 g of dry compost were between 10.29 and 10.56 for the four composts, while the bacterial 16S rRNA gene copies were between 9.48 and 9.84. No statistical differences were observed for the fungal or bacterial communities, since the four composts showed a similar abundance.

Mycobiota Composition of the Four Composts Used in This Study

A total of 244,298 raw reads (2×250 bp) were obtained after sequencing. After quality filtering, a total of 242,702 clean reads were used, with an average value of 60,675 reads/sample and an average sequence length of 386 bp. 26S rRNA gene sequencing showed differences between the four composts used in this study at a genus level. Thirty-one genera were

detected (Figure 2 and Supplementary Table S1), and it was possible to observe a clear predominance of a few taxa in all the composts. CV was mostly populated by *Phialophora* (5.5%), *Coniochaeta* (4.2%), and *Aureobasidium* (8.5%); CV2 showed *Penicillium* (21.1%), *Myceliophthora* (9.8%), *Coniochaeta* (2.5%), *Cladosporium* (10.7%), *Aspergillus* (8.5%), *Arthoderma* (13.3%), and *Pseudoeurotium* (5%); CB showed *Scopulariopsis* (2.6%), *Pseudoeurotium* (4%), and *Chaetomium* (2.9%). CM was mostly populated by three main genera: *Trichoderma* (6.4%), *Phialophora* (3.4%), and *Fusarium* (11.5%).

Disease Suppression by the Compost Mixtures

The negative control – non-inoculated (NC) and chemical control (CC) showed no disease symptoms at the end of both trials. The inoculated untreated control (UC) showed up to 90% of disease incidence at the end of the trials. All the treatments showed a numerical reduction in DI, compared to the UC, and all the CM mixtures had the lowest disease index, but only samples from CM – 10% showed a statistical reduction of DI, compared to the UC ($P < 0.05$), that ranged from 90 to 45%. As far as the fresh biomass is concerned, the untreated control showed the lowest value of all (5.8 ± 1.9 g), while the highest was for the non-inoculated treatment (34.0 ± 2.4 g). Of all the compost mixtures, CM – 10% was the only one that was significantly different from the UC, with an average fresh biomass of 26.3 g ($P < 0.05$).

The area under the disease progress curve (AUDPC) values highlighted a similar situation as the DI and fresh biomass: the inoculated untreated control (UC) had the highest AUDPC value (351) of all the treatments, but only CM – 10% was significantly different from UC, with a value of 137. All the data are shown in Table 3, where the results of Trial 1 and Trial 2 were averaged.

Abundance of Fungi, Bacteria, and PHC in the Rhizosphere of UC, CC, and CM – 10%

Since the only effective treatment was CM – 10%, molecular analyses were carried out using rhizosphere soils collected at the end of the trials for the untreated control, chemical control, and CM – 10% treatments. Data from two trials are shown in Figures 3A–C. No differences were evident for the abundance of fungi and bacteria between the three treatments. The specific PHC gene was found in both the untreated control and in the CM – 10% treatment at levels of 3 and 2.82, respectively [Log of copy⁻¹ numbers per gram of dry matter], while it was not found in the chemical treatment.

Mycobiota Composition of the Rhizosphere Soils

The total number of paired sequences obtained from samples reached 884,148 raw reads. A total of 784,797 reads were obtained after quality filtering, with an average value of $35,672 \pm 17,772$ reads/sample and a mean sequence length of 393 bp. The α -diversity index showed a satisfactory coverage for all the samples (>96%), but did not show a different level of complexity on the basis of the treatment. No significant difference in mycobiota composition or in α -diversity index

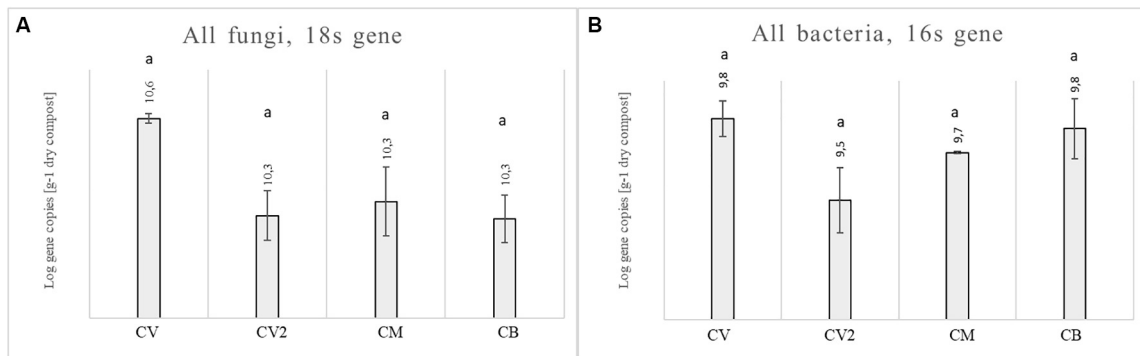


FIGURE 1 | Abundance of the fungal 18S rRNA gene **(A)** and bacterial 16S rRNA gene **(B)** for four composts: CV, CM, CB, and CV2. Different letters indicate statistical differences between the four composts, as obtained with the ANOVA test and Tukey's *post hoc* test.

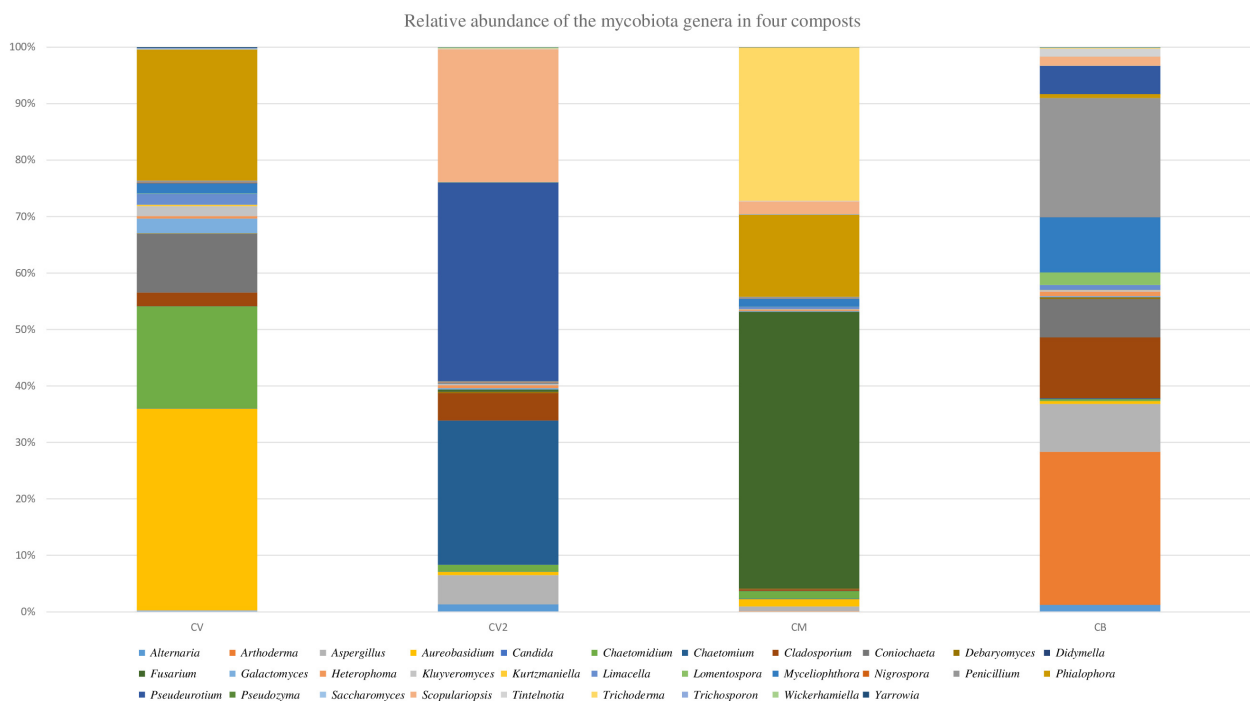


FIGURE 2 | Relative abundance of the fungal community in the four analyzed composts: CV green compost, CV2 green compost, CM green compost with the addition of *Trichoderma* sp. TW2 and CB mixed compost. The OTUs were selected by discarding the ones that were not present in the four composts under a threshold of 0.5%.

was observed (**Supplementary Figure S1**), and the data were therefore averaged.

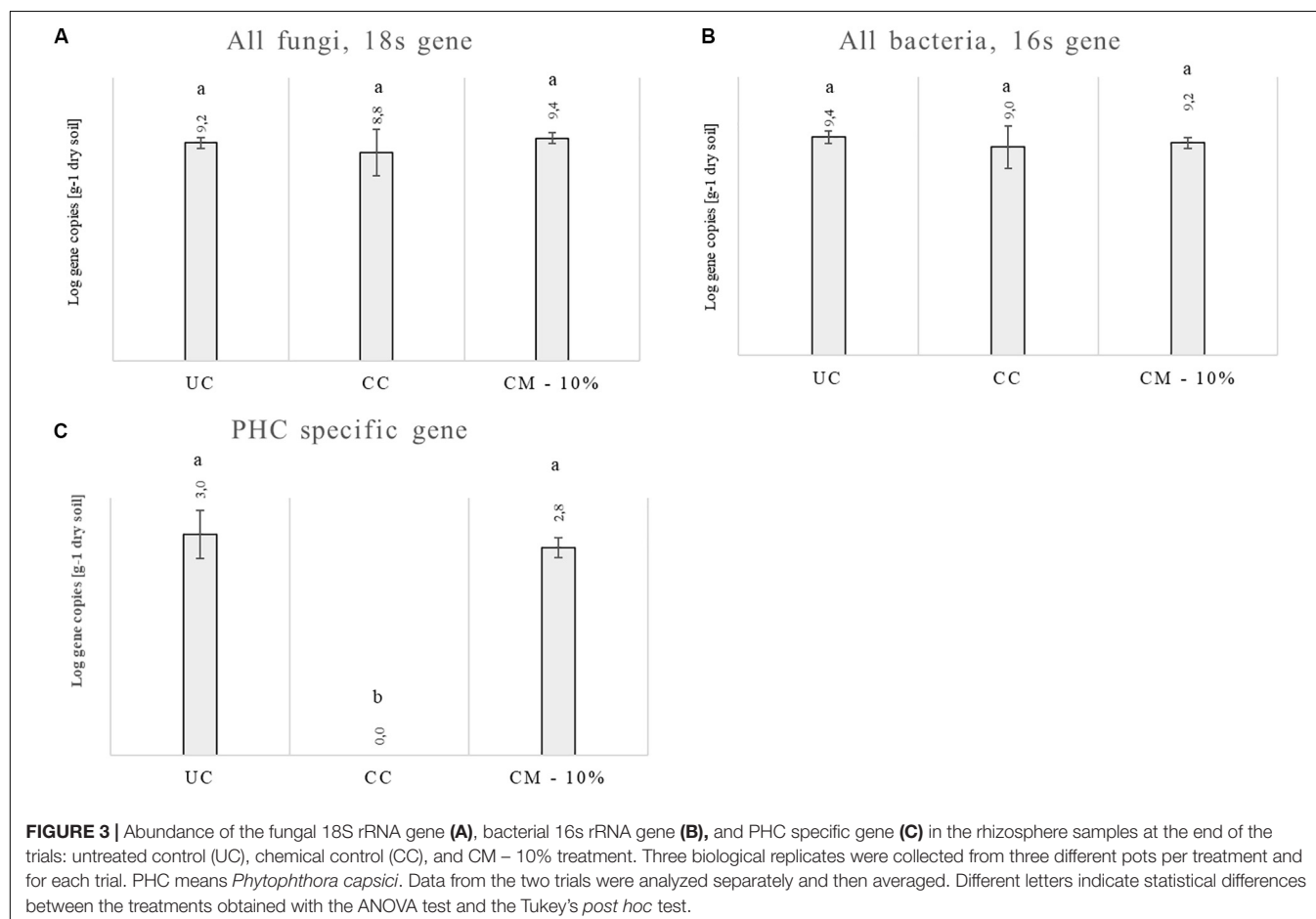
Taking into account the microbiota composition at the highest taxonomic level (**Figure 4** and **Supplementary Table S2**), it is possible to observe a core mycobiota, composed of *Fusarium*, which reaches about 3% of the relative abundance in the control samples (UC), 1% in the compost (CM – 10%) and 2% in the chemical treatment (CC); *Glomus*, which reaches 9, 5, and 15% in UC, CM – 10%, and CC, respectively; *Penicillium*, which reaches 6, 1, and 4% of the relative abundance in UC, CM – 10%, and CC, respectively;

Saccharomycetales, which reaches 4, 1, and 3%; *Torrubiella*, which reaches 2, 2, and 1%; *Trichoderma*, which reaches 10, 12, and 7%; and *Zygoascus*, which reaches 1, 1, and 3%, respectively (**Figure 4**). A further separation of the samples, based on the treatment, was also observed, through the principal component analysis (PCA, **Figure 5**), and the result was confirmed by means of the ANOSIM statistical test ($P = 0.003$). Moreover, it was possible to observe a clear separation of the samples treated with compost, while the chemical treatment and control ones clustered together (**Figure 5**). By taking into account the significant difference in the OTUs among

TABLE 3 | Efficacy of the compost mixtures to suppress *Phytophthora capsici* disease on summer squash plants expressed as disease incidence (%), fresh biomass (g), and AUDPC values.

Treatments	Disease incidence	Standard error	Tukey	AUDPC	Standard error	Tukey	Fresh biomass	Standard error	Tukey
Non-inoculated control	0.0	0.0	c	0.0	0.0	a	34.0	2.4	d
Untreated control	90.0	4.7	a	351.3	28.8	c	5.8	1.9	a
Chemical control	0.0	0.0	c	0.0	0.0	a	32.8	3.1	cd
CV – 1%	70.0	9.4	ab	268.8	47.3	bc	9.8	3.0	ab
CV – 10%	75.0	12.5	ab	301.3	49.1	bc	17.6	5.0	abc
CV – 20%	57.5	11.6	ab	212.9	48.6	abc	21.2	2.9	abc
CV2 – 1%	72.5	13.3	ab	287.5	58.2	bc	10.6	4.2	ab
CV2 – 10%	75.0	10.3	ab	286.3	44.9	bc	17.5	3.5	abc
CV2 – 20%	70.0	6.3	ab	257.5	30.6	bc	18.7	4.3	abc
CM – 1%	55.0	9.4	ab	245.4	44.0	bc	12.4	3.1	ab
CM – 10%	45.0	9.1	b	137.1	36.7	ab	26.3	3.3	bcd
CM – 20%	55.0	12.9	ab	181.3	49.1	abc	21.7	2.9	abc
CB – 1%	67.5	10.6	ab	251.7	41.2	bc	16.6	2.3	abc
CB – 10%	67.5	6.6	ab	226.7	30.7	bc	21.5	2.7	abc
CB – 20%	72.5	10.3	ab	286.3	42.8	bc	18.1	5.9	abc

The letters refer to the Tukey's post hoc test, which was performed after one-way ANOVA ($P < 0.05$).



treatment (Figure 6, FDR < 0.05), it was possible to observe that the compost treatment (CM – 10%) was characterized by the presence of minor fraction OTUs. In other words, a

higher presence of *Chaetomiaceae*, *Microascaceae*, *Arthrographis*, *Myceliophthora*, and *Phialophora* was observed (Figure 6), while *Penicillium* and *Pseudeurotium* were reduced in the

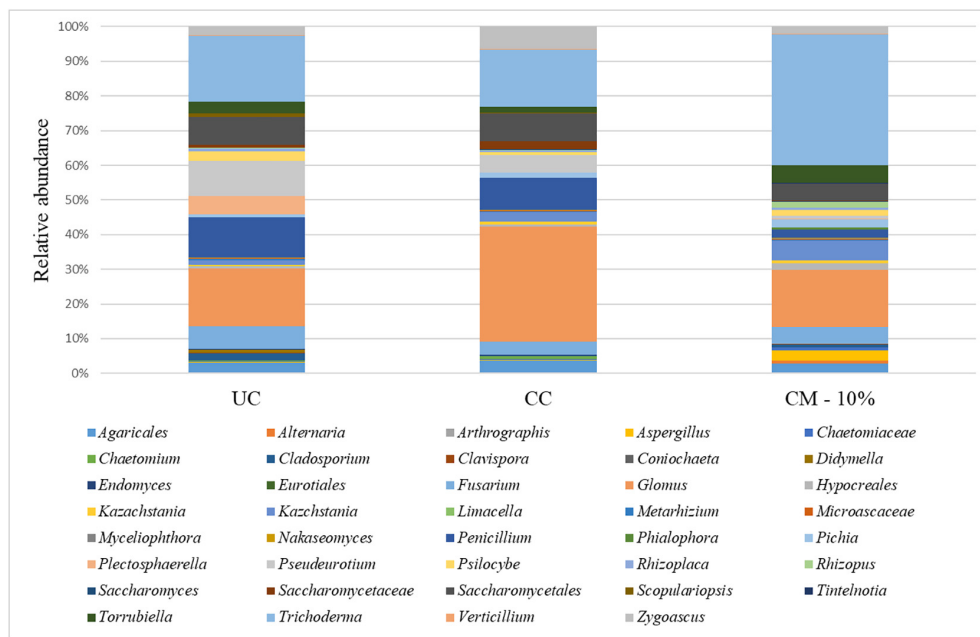


FIGURE 4 | Relative abundance of the mycobiota in the rhizosphere samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. Only OTUs which showed an incidence above 0.2% in at least two samples are shown. The data from replicates were averaged.

compost treated samples, compared with UC and with CC. It should be observed that *Didymella* was reduced by both CM-10% and chemical treatments, compared to the untreated control (Figure 6).

Co-occurrence Co-exclusion Analysis of the Mycobiota of the Rhizosphere Soils

The OTU co-occurrence/exclusion pattern of the rhizosphere soils is shown in Figure 7, where only significant correlations are reported (at a false discovery rate [FDR] of <0.01). As far as the main OTUs shared in the datasets are concerned, we observed that *Trichoderma* co-occurs with *Arthrographis*, *Myceliophthora*, *Phialophora*, and *Glomus*; *Verticillium* co-occurs with *Alternaria* and *Cladosporium*, while there is co-exclusion with *Rhizopus*; *Penicillium* shows co-exclusion with *Arthrographis*, *Limacella*, and *Endomyces*, while it co-occurs with *Chaetomium*; *Aspergillus* co-occurs with *Myceliophthora* and shows co-exclusion with *Endomyces*; *Alternaria* co-occurs with *Cladosporium*, *Limacella*, and *Verticillium*, while it co-excludes with *Zygoascus*; *Fusarium* co-occurs with *Hypocreales* and *Psilocybe*.

DISCUSSION

Four composts were analyzed to characterize their microbial community. Real-time PCR assays of the absolute abundance of genes have shown that all the four composts used in this study had a higher number of bacteria and fungi compared to literature (Epelde et al., 2018; Tortosa et al., 2020), confirming that all of them were potential sources of inoculum for agricultural

applications. The mycobiota composition of the four compost analyzed showed a highest biological complexity.

In details we observed that CB showed several fungal genera that were less present in the other composts (such as *Scopulariopsis*, *Pseudoeurotium*, and *Chaetomium*) highlighting the difference between green waste based and mixed waste based composts. A different mycobiota distribution was also observed among CV and CV2 probably due to the different procedures of composting. These aspects are in accordance with other studies that reported an implication of the composting procedures in the microbial modulation, especially when wastes were used (Anastasi et al., 2005; Neher et al., 2013). CV and CM were the same green composts, but CM was added with *Trichoderma* sp. TW2 strain; it is interesting to observe the difference, in terms of the relative abundance of the fungal genera, between these two composts which suggests that the composition of stable systems, such as a green commercial compost with a strong microbiome, can also be altered. The disease assessments showed that CM was the most effective; however, only CM – 10% was able to suppress the disease in the summer squash-PHC pathosystem and to reduce the disease incidence by 50% if compared to the untreated control. The fresh biomass and AUDPC were in accordance with the disease incidence in both experiments, and since no significative differences were found between the two trials, data were collapsed. CV, CV2, and CB were not effective in the protection of summer squash against PHC in any mixture concentration. This suggests that their microbial asset did not induce a modification of the rhizosphere microbial community able to establish the suppressive activity. As already reported by Bonanomi et al. (2007), composts suppressiveness is not a

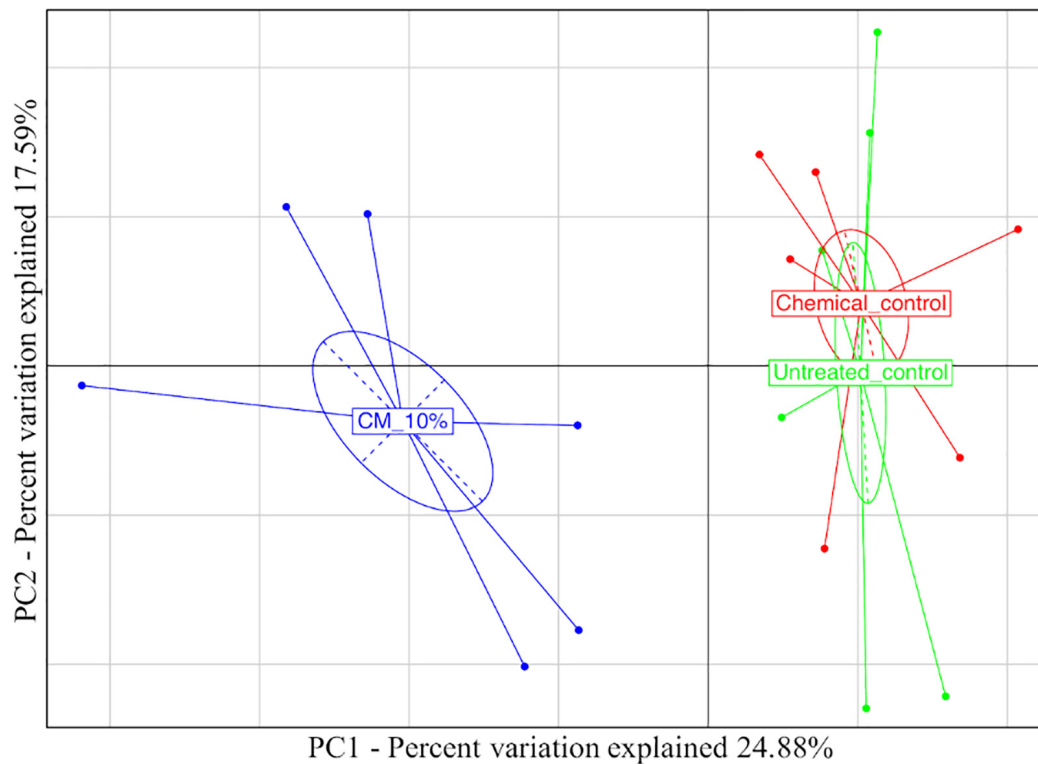


FIGURE 5 | Principal component analysis based on the mycobiota composition referred to the rhizosphere samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. The samples are color-coded according to the treatment.

constant event. CV is the same compost as CM, but without the addition of the *Trichoderma* sp. TW2 strain, suggesting that the enrichment of composts with biological control agents may be a good strategy for this pathosystem. In addition, *Trichoderma* was effective against PHC, as already reported by many authors (Ahmed et al., 2000; Ezziyyani et al., 2007; Lorito et al., 2010; Bae et al., 2011). Furthermore, in a previous study carried out in field conditions (Gilardi et al., 2019), the application of *Trichoderma* TW2 alone against *Fusarium* wilt in lettuce was less effective compared to the use of CM compost, in combination with *Trichoderma* TW2.

Bonanomi et al. (2018) reviewed several works on biocontrol agents (BCAs) compost enrichment and pointed out that this could be the most promising way to achieve a long-term suppressiveness against soil-borne pathogens. In this context, the study of different composts, with the addition of different BCAs for several pathosystems, helps to clarify and identify the best one to use. On the other hand, BCAs can be selected by composts that showed a high suppressive action and can be used as inoculum to enhance the ability of other systems to suppress soil-borne pathogens (Pugliese et al., 2008). It is also important to underline that in this study, an excessive application of CM (20%) did not significantly suppress PHC disease incidence compared with the CM – 10%. This is in agreement with other papers (Bonanomi et al., 2007; Noble, 2011; Pugliese et al., 2011). The level of suppression is not always affected by compost dosage

rate, and increased application rate only partially corresponds to increased disease suppression for composts (Bonanomi et al., 2007). Soil amended with $\geq 20\%$ v/v of compost in 6 out of 79 experiments even showed a disease promotion effect (Noble, 2011). Furthermore, according to Pugliese et al. (2011), in non-sterilized composts, the addition of *Trichoderma* is not providing a dose-dependent effect against *Phytophthora*, as observed here. This dose-related effect of composts can be related to complex interactions among BCAs, compost's initial microbiota and rhizosphere, which can explain why CM applied at the rate of 20% was not as effective as CM at 10%. Moreover, an occurrence of phytotoxicity due to the increasing rate of compost is also known (Bonanomi et al., 2007) and can explain the reduction in the efficacy of CM. Since only one compost mixture was suppressive against PHC, analyses of the rhizosphere of this treatment were carried out in order to establish whether the microbial communities were altered by the addition of CM – 10%. Bacteria and fungi had almost similar gene copy number. This is in agreement with previous studies (Cucu et al., 2019), where the fungal and bacterial abundance in rhizosphere were found to be at the same level compared to bulk soil.

Real-time PCR of the total gene abundance showed no differences in the rhizosphere soils at the end of the trial for the CM – 10% treatment, chemical treatment, and untreated control for bacterial and fungal communities. Interestingly, PHC gene was found in the CM – 10% treatment and untreated

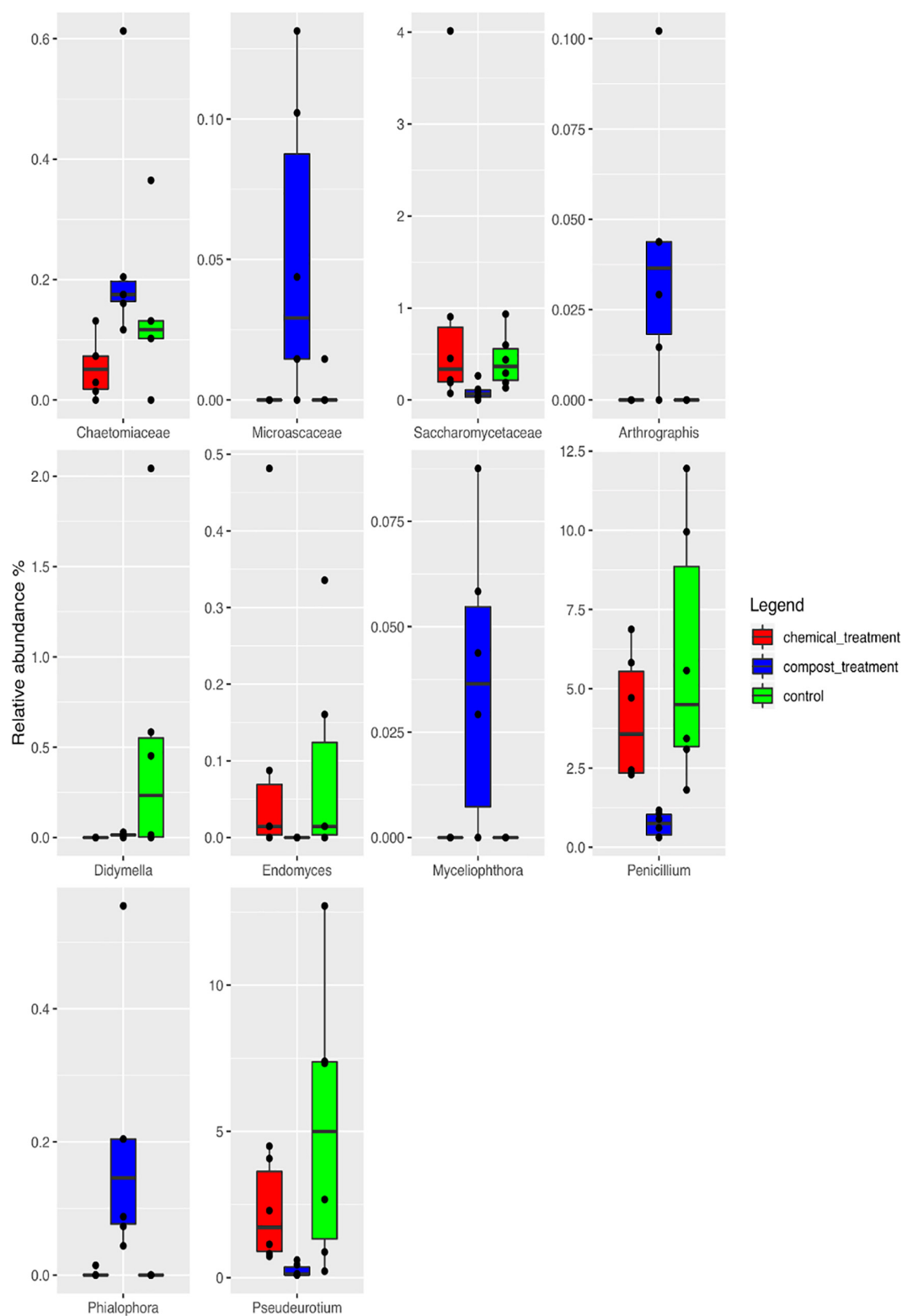


FIGURE 6 | Boxplots showing the relative abundance of the differentially abundant OTUs based on the Wilcoxon matched pairs test ($FDR \leq 0.05$) of the rhizosphere soil samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. The boxes represent the interquartile range (IQR) between the first and third quartiles, and the line inside represents the median (2ND quartile). The whiskers denote the lowest and the highest values within 1.56 IQR from the first and third quartiles, respectively. The circles represent outliers beyond the whiskers.

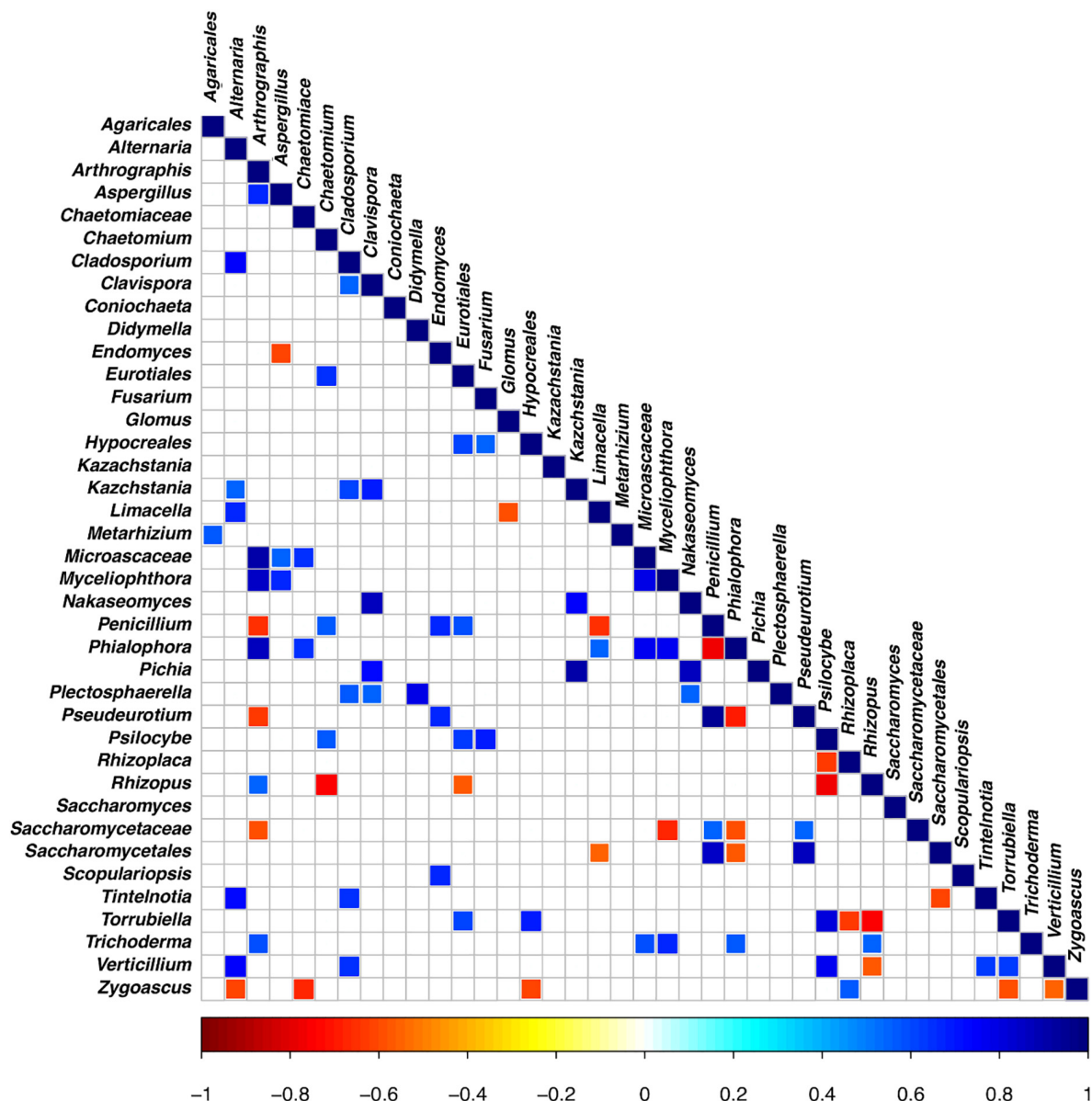


FIGURE 7 | Significant co-occurrence and co-exclusion relationships between the OTUs of the rhizosphere soil samples at the end of the trials: untreated control (UC), chemical control (CC), and CM – 10% treatment. Three biological replicates were collected from three different pots per treatment and for each trial. The figure presents a Spearman's rank correlation matrix (FDR < 0.01). The color of the scale bar denotes the nature of the correlation, with 1 indicating a perfect positive correlation (dark blue) and -1 indicating a perfect negative correlation (dark red).

control at similar levels, while it was not found in the chemical treatment, thus suggesting that the reduction in disease incidence in CM – 10% treatment was mediated by the complex interaction between *Trichoderma* and others microorganisms. The positive effect of CM – 10% treatment can also be due by the induction of resistance, which has already been reported to be stimulated by composts and *Trichoderma* spp. on many pathosystems (Vallad et al., 2003; Sang et al., 2010; Martínez-Medina et al., 2017; Savitha and Sriram, 2017). As for the mycobiota composition in the CM – 10% treatment, it was possible to observe that *Chaetomiaceae* and *Microascaceae* were higher than that in the untreated and

chemical controls. *Chaetomiaceae* is a family composed of several genera, commonly found in air or soil environment (Rodríguez et al., 2002), that has already been related to beneficial actions, such as suppression of *Lisianthus Fusarium* wilt (Zhou X. et al., 2019), antibacterial activity (Chovanová and Zámocký, 2016), and putative entomopathogenic activity against Khapra Beetle (Mohammed et al., 2019). The high presence of *Microascaceae* in compost is not surprising, since this family has already been reported to increase during compost maturation (Galitskaya et al., 2017). Interestingly, the levels of *Penicillium* were lower in the CM – 10% compost treatment than in the chemical and

untreated control, thus suggesting that this compost treatment could be able to reduce the presence of this genus, which is a well-known plant pathogen and mycotoxin producer (Olsen et al., 2019; Schmidt-Heydt et al., 2019; Vidal et al., 2019; Zinedine and El Akhdari, 2019). PCA analyses clearly showed a separation of CM – 10% treatment samples from chemical and untreated controls. The chemical control and untreated control clustered together in the PCA analyses, but the disease incidence was absent in the chemical control, thus suggesting that the fungicide used in this study was effective against *Phytophthora capsici* but did not alter the rhizosphere mycobiome, compared to the untreated control, while the CM – 10% treatment altered the equilibrium of the rhizosphere. This result is a further confirmation of the CM suppressive action conferred by the microbial community as a result of the interaction with *Trichoderma* sp. TW2. The co-occurrence and co-exclusion analyses highlighted that *Verticillium*, *Alternaria*, and *Cladosporium* occurred together, which is interesting because of the well-known pathogenic activity of these three genera (Rotem, 1994; Crous et al., 2007; Klosterman et al., 2009; Bensch et al., 2012). The fact that these three genera co-occurred in the rhizosphere samples suggests their potential cooperation in biotic stressed plants. In addition, we observed that *Trichoderma* co-occurred with *Glomus*, a genus considered the one that contains the highest number of arbuscular mycorrhizal species (Schwarzott et al., 2001). This suggests that a beneficial effect of *Trichoderma* is guaranteed in the rhizosphere environment, not only as a suppressive agent against *Phytophthora capsici* but also by improving the rhizosphere microbiome in terms of quality.

CONCLUSION

This study involved four different composts analyzed for their microbial community composition and then used in a greenhouse pot trial in order to test their suppressive activity to prevent *Phytophthora capsici* infection against summer squash. The fungal community of the four composts were different, highlighting the central role of wastes choices and composting procedures in the selection of the mycobiota. Above 12 compost-peat mixtures, only CM – 10% was able to suppress PHC, due to its microbial composition that can play a major role in its suppressiveness (Reuveni et al., 2002; Tilston et al., 2002; Papasotiriou et al., 2013; De Corato et al., 2019). The mycobiota composition of the CM – 10% treated pots clustered separately if compared to CC and UC, confirming that beneficial microorganisms present in CM – 10% treatment can protect the plant root system by microbiota modulation (Antonioni et al., 2017; Mwaheb et al., 2017; Cucu et al., 2019; Topalović et al., 2020; Zhang et al., 2020). Further investigations

should be necessary to obtain a deeper understanding of how this protection is conferred because the complex interaction between rhizosphere, microbiota and pathogens is less explored. Moreover, an activation of the systemic resistance of the host plants by CM – 10% treatment cannot be excluded. This study points out the importance of the exploration of microbial community of composts and their *in vivo* application to control soil borne diseases, in order to better understand how to predict the suppressive ability of a compost.

DATA AVAILABILITY STATEMENT

All sequencing data generated by this study can be found in the NCBI using accession number PRJNA580394.

AUTHOR CONTRIBUTIONS

AB, MP, and IF contributed to the conception and design of the study and writing of the original draft. AB and IF performed the statistical analysis. AB, MC, and IF contributed to the methodology and investigation. MP and MG contributed to reviewing. AB and MP contributed to editing. MP, MG, and AG supervised the study. MP contributed to the resources, project administration, and funding acquisition. 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/fpls.2020.00885/full#supplementary-material>

FIGURE S1 | Boxplots to describe α -diversity measures of the rhizosphere soil samples at the end of the trials: Untreated control (UC), chemical control (CC), and CM – 10% treatment. Samples are color coded according to the treatment.

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The remaining 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 Microbiomes: Do Different Preservation Approaches and Primer Sets Alter Our Capacity to Assess Microbial Diversity and Community Composition?

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The microbial communities associated with plants (the plant microbiome) play critical roles in regulating plant health and productivity. Because of this, in recent years, there have been significant increase in studies targeting the plant microbiome. Amplicon sequencing is widely used to investigate the plant microbiome and to develop sustainable microbial agricultural tools. However, performing large microbiome surveys at the regional and global scales pose several logistic challenges. One of these challenges is related with the preservation of plant materials for sequencing aiming to maintain the integrity of the original diversity and community composition of the plant microbiome. Another significant challenge involves the existence of multiple primer sets used in amplicon sequencing that, especially for bacterial communities, hampers the comparability of datasets across studies. Here, we aimed to examine the effect of different preservation approaches (snap freezing, fresh and kept on ice, and air drying) on the bacterial and fungal diversity and community composition on plant leaves, stems and roots from seven plant species from contrasting functional groups (e.g. C3, C4, N-Fixers, etc.). Another major challenge comes when comparing plant to soil microbiomes, as different primers sets are often used for plant vs. soil microbiomes. Thus, we also investigated if widely used 16S rRNA primer set (779F/1193R) for plant microbiome studies provides comparable data to those often used for soil microbiomes (341F/805R) using 86 soil samples. We found that the community composition and diversity of bacteria or fungi were robust to contrasting preservation methods. The primer sets often used for plants provided similar results to those often used for soil studies suggesting that simultaneous studies on plant and soil microbiomes are possible. Our findings provide novel evidence that preservation approaches do not significantly impact plant microbiome

data interpretation and primer differences do not impact the treatment effect, which has significant implication for future large-scale and global surveys of plant microbiomes.

Keywords: plant microbiome, sampling, preservation methods, amplicon sequencing, soil, sequencing primers

INTRODUCTION

The microbial communities associated with different plants compartments, from roots to leaves (the plant microbiome), play a crucial role in plant health and productivity (Köberl et al., 2013; Andreote et al., 2014; Berg et al., 2016; Rosier et al., 2016; Colla et al., 2017; Qiu et al., 2019). The plant microbiome can perform key functions in supplying nutrients and helping to control pathogens (Schmalenberger et al., 2008; Compant et al., 2010; Suárez-Moreno et al., 2012; Sessitsch and Mitter, 2015). Because of this, harnessing beneficial microbes associated with plants is considered a promising emerging tool to improve agricultural productivity and sustainability. Understanding the plant microbiome assembly mechanisms and how these microbiomes interact with their hosts is a fundamental first step to achieve this goal. Over the last few years, there has been an increasing number of studies using next generation sequencing to unveil the plant microbiome structure and dynamics (Berg et al., 2014; Agler et al., 2016; Hamonts et al., 2018). Particularly, amplicon sequencing has been widely used to discover the fundamental process of microbial assembly in plant germination, growth, metabolism and defence (Mayak et al., 2004; Weyens et al., 2009; Schmidt et al., 2014).

Despite the importance and potential opportunities offered by the plant microbiome, there are still multiple challenges that need to be addressed to advance our knowledge including lack of large-scale study to identify the processes that govern assembly and function of plant microbiomes. Large-scale studies in plant microbiomes is constrained by logistic issues including plant preservation after samples collection, and its important role in maintaining the original microbial community intact. For example, the snap freezing (in liquid nitrogen) preservation method for sampling and transporting to laboratory is considered the gold standard method for field surveys, as samples are immediately placed at -20°C or below after collection *in situ* to minimise the disruptions of the plant tissue and its microbiome (Agler et al., 2016; Timm et al., 2016; Hamonts et al., 2018). However, in some circumstances, snap freezing is impractical due to logistic and financial difficulties, especially when large number of samples are required from remote areas, or in global and regional studies. This has led to the development of alternative approaches for storing samples, which have also proven effective on non-plant samples, including the use of FTA cards (Song et al., 2016), ethanol (Estes et al., 2013; Koch et al., 2013), CTAB (Hammer et al., 2015) and RNAlater (Campbell et al., 2004; Sanders et al., 2014). However, most of these methods are not applicable for plant microbiome due to the requirement of the tissue integrity for downstream analysis. In plant microbiome studies, snap freezing is still the most common method for preserving plant materials (Agler et al., 2016; Deyett and Rolshausen, 2020), but in

suboptimal conditions, air-dry with silica gel (Bazzicalupo et al., 2013) ice incubation or fridging (-4°C to 4°C , Kaushal et al., 2020) have also been used for sample preservation, but the effect of this approach on microbiome integrity has not been fully tested compared to snap freezing method. With the increasing interests of harnessing plant microbiome to sustainably promote crop productivity, more initiatives and projects aimed to unfold the plant microbiome from regional to global scales have been launched recently. Therefore, finding practical and cost-effective preservation approaches is critical to accommodate the ever-increasing number of samples for sequencing, and ultimately harnessing microbial-based knowledge for the development of sustainable agricultural technologies.

Another major challenge is associated with the fact that plant bacterial microbiomes are often assessed with different primer sets (e.g. 799F-1193R) than those used for soils (e.g. 341F-805R, 515F-806R). This is not an issue for fungi as plant and soil studies can sequence the same fungal ITS region without getting huge variance. In the case of bacteria, plant microbiome studies often use 16S rRNA gene primers (799F-1193R) targeting the V5–V7 region of the gene (Bai et al., 2015; Liu et al., 2017). Unlike for the primer sets most used for soils (e.g. 341F-805R; region V3–V4, Delgado-Baquerizo et al., 2016; Feng et al., 2016; 515F-806R; region V4, Caporaso et al., 2011; Caporaso et al., 2012; Walters et al., 2016), these plant microbiome primer sets minimise the sequencing of chloroplast and mitochondrial 16S rRNA gene (Beckers et al., 2016). Although alternative approaches are available such as using PNA blockers (Fitzpatrick et al., 2018), the efficiency to reduce the amplification of plant material was still far from ideal (Hamonts et al., 2018). This poses an important challenge, as the lack of demonstration that the primer set (799F-1193R) is valid for soils, and yields similar results to those from 341F-805R, limiting any attempt to compare both soil and plant microbiomes. Because of this, it is critical that we investigate whether the typical primer set used for plant microbiomes is also valid for soil, and provides comparable data to commonly used soil primer sets.

Here, we aim to 1) examine the effect of different preservation approaches on plant microbiome analysis and to identify the best preservation method to maintain sample integrity, and 2) to evaluate whether the plant primer pair targeting V5–V7 regions is valid for soil microbiomes, and provide similar results in this environment than those primers that target the V3–V4 region. To assess the effect of plant preservation methods, we implemented three preservation approaches commonly used in plant microbiome studies: a) silica gel desiccation, with samples incubated at room temperature until fully dehydrated; b) incubation on ice for 24 h; and c) snap freezing in liquid nitrogen immediately after sample collection and then transfer to -80°C . To further evaluate the variability of microbiome and distinct difference of the leaf traits that could potentially affect plant microbiome across species, we

selected five plant species from contrasting functional groups, including C3 (wallaby grass *Austrodanthonia caespitosa*) and C4 (kangaroo grass *Themeda triandra* and rhodes *Chloris gayana*) grasses, the nitrogen fixing legume lucerne (*Medicago sativa*), as well as an economically important crop (the cotton plant *Gossypium hirsutum*) for amplicon sequencing targeting both bacterial 16S rRNA gene and fungal ITS region, in order to compare the microbial communities under different preservation methods. For primer pairs comparisons, we used the 341F-805R and 799F-1193R primers on the same soil samples.

MATERIAL AND METHODS

Plant Preservation Approaches

Plant leaves from *A. caespitosa*, *M. sativa*, *T. triandra* and *C. gayana*, were collected from Pastures And Climate Extremes (PACE) Facility, Western Sydney University, Richmond, Australia. Briefly, each plant species was collected from control monoculture blocks by cutting the leaves with a sterilised scissors before being aseptically transferred into a clear zip lock bag. For the snap freezing and ice incubation methods, plant leaves from each plant species were subsampled into a clean zip lock bag ($n = 6$ for each treatment) before immediately being stored in liquid nitrogen and on ice, respectively. For air dry method, plant leaf from each plant species were subsampled into a paper bag ($n = 6$) before being stored in a desiccator filled with silica gel at bottom. Samples were incubated for approximately two days at room temperature until complete dehydration.

Plant leaves, stems and roots from cotton (*G. hirsutum*, genotype Sicot 71BRF) were collected from two-week old cotton plants (10–15 cm tall) grown in a glasshouse with daytime temperature of 32°C and night-time temperature of 25°C. Cotton leaves (top two leaves) and stems (0–5 cm above soil surface) were cut with a sterilised scissors before being transferred into clear zip lock bags ($n = 6$) while cotton roots were cut and simply washed with distilled water before transferred into a clear zip lock bag. Preservation treatments were conducted as described above.

A total number of 126 frozen plant tissues (~15 mg dry weight, finely cut into ~2 mm × 2 mm pieces) were weighed and DNA was extracted using DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany), following the manufacturer's instructions. Extracted DNA was quality checked by NanoDrop 2000 (Thermo Fisher Scientific, Waltham, Massachusetts, US), quantity checked by Qubit Fluorometer (Thermo Fisher Scientific) and PCR checked to confirm the amplifiability.

Amplicons using 799F/1193R targeting 16S rRNA gene targeting V5-V7 region for bacterial communities to reduce chloroplast sequences from the plant tissue (Chelius and Triplett, 2001), and ITS2 region (FITS7-ITS4R, Ihrmark et al., 2012) for fungal communities were obtained *via* PCR.

Plant-Based Primer Set Applicability to Soil Samples

We used 86 soil samples from to compare how different primers affect the community determination. These samples were obtained

from a glass-house experiment that aimed to evaluate how different verities of rice, soil types, and salinity impact soil microbiomes (unpublished data). DNA for soil samples was extracted after plant harvest as explained above for plants (250 mg of soil was used in the extraction). Each soil sample was collected in a cryogenic tube from the greenhouse and kept under -80°C before DNA extraction. Primer pairs 341F-805R (Herlemann et al., 2011) and 799F-1193R (Chelius and Triplett, 2001) were used to amplify the 16S rRNA gene from all soil samples. Two PCR were performed as: initial denaturation at 95°C for 3 min, followed by 25 cycles consisting of denaturation (95°C for 30 s), annealing (95°C for 30 s) and extension (72°C for 30 s) and a final extension at 72°C for 5 min before merging the samples for downstream process. All sequencing (plant and soil) were performed at Western Sydney University Next Generation Sequencing (NGS) facility (Sydney, Australia) using Illumina MiSeq 2 × 300 bp paired end chemistry. A mock community consists strains belong to order Bacillales, Lactobacillales, Enterobacteriales Pseudomonadales in the following proportion: 47, 28.3, 20.5, and 4.2%, was sequenced with both primers to evaluate the validity of primer comparison. All raw sequence data related to this study are available in the European Nucleotide Archive (The European Bioinformatics Institute, EMBL-EBI) database (Accession No. PRJEB38041).

Microbial Community Analysis

Raw data obtained from NGS facility were processed using Mothur standard operating procedure (Schloss et al., 2009). Briefly, forward and reverse sequences were merged into contigs. Sequences that contained unidentified bases or had greater than eight homopolymers were filtered out. For bacterial sequences, an additional step aligning sequences against Silva 16S rRNA gene database version 132 (Pruesse et al., 2007) was applied, and unaligned sequences were removed. Refined sequences were pre-clustered (diffs = 1) and chimera checked using UCHIME (Edgar et al., 2011) and singleton was removed to reduce error (Reeder and Knight, 2009). Bacterial and fungal sequences were then taxonomically classified according to the Silva database version 132 and UNITE database version 8, respectively, with 60% cut-off confidence and sequences that match cotton mitochondria, chloroplast, archaea (bacteria) and host ITS regions (fungi) were removed. Remaining sequences were clustered into Operational Taxonomic Units (OTUs) at 100% identity where taxonomy was assigned to, generating 80,617 and 25,577 bacterial and fungal OTUs, respectively.

For plant preservation approaches, the OTU matrices were rarefied to 808 bacterial and 6736 fungal sequences per sample, respectively (Figures S2A, B). Rare OTUs (contributed less than 0.1% of total abundances) were removed from the OTU matrices, resulted in 8,218 bacterial OTUs and 3,255 fungal OTUs for downstream analyses. Datasets were analysed using permutational multivariate analysis of variance (Anderson, 2001a) in PRIMER v. 6 (PRIMER-E, UK) to compare bacterial and fungal communities under different preservation methods (snap frozen, ice incubation and air dry). Block effects driving microbial difference were not considered because we only compare difference between preservation treatments. Similarity matrices were calculated based

on Bray–Curtis distances on square-root transformed abundance data to compare the composition and abundances of community structure, and on Jaccard distances to compare the presence/absence of the community members in PRIMER. Analyses used 9,999 permutations of residuals under a reduced model (Anderson, 2001b). Pair-wise analyses were performed to compare the differences between preservation methods, and p-values were adjusted following Holm's method (Holm, 1979) to reduce the bias generated in statistical analysis. Permutational multivariate dispersion (PERMDISP) analysis was used to test for homogeneity of multivariate dispersion within groups in PRIMER (Anderson, 2006). Alpha and beta diversity were analysed using R package “phyloseq”. Data visualisation including Principal Coordinates Analysis (PCoA) plots were generated based on Bray–Curtis and Jaccard distance, and taxonomic analysis based on the Bray–Curtis dissimilarity matrix with heatmap were performed using R packages “phyloseq”, “dplyr” and “ggplot2” (Lozupone et al., 2012).

To identify the influence of preservation methods on dominant and rare microbial taxa, we followed the definition from Soliveres et al. (2016) to extract dominant communities (the top 10% of OTUs in terms of abundance) and rare communities (the bottom 10% OTUs) from the OTU tables generated with Mothur, respectively. PERMANOVA, alpha and beta diversity analyses were applied following the methods described above.

To compare the two datasets using different primer pairs on soil bacterial communities, both datasets using two sets of primers (341F/805R and 799F/1193R) with 12,199 and 16,229 bacterial raw OTUs, respectively, were rarefied to 8,000 sequences per sample (Figures S2C, D) with 9,317 and 13,737 OTUs, respectively. Alpha diversity analysis and correlation between two datasets, as well as mantel test based on Bray–Curtis measures estimating the beta diversity correlation between two datasets were conducted in R. Microbial composition was also analysed with R package “phyloseq”.

RESULTS

Effect of Preservation Methods on Plant Microbiomes

In leaf preservation approach, a total number of 8,218 bacterial and 3,215 fungal OTUs were analysed in the preservation experiment. Five plant DNA samples were dropped due to low DNA quality and poor sequencing reads, which end up with 121 samples in total. Generally, there were no significant difference of species richness and evenness among the different preservation treatment observed from the alpha indices (Chao1, Shannon and Simpson, Figure 1; $P > 0.05$) with a few exceptions due to low diversities (In bacterial communities, Shannon index – Dry \neq Ice in cotton leaf, Dry \neq Fro = Ice in cotton stem, Dry = Fro \neq Ice in lucerne leaf; Simpson index – Dry \neq Ice in cotton leaf, Dry \neq Fro = Ice in cotton stem, Fro \neq Ice in lucerne leaf. In fungal communities, Chao1 index – Fro \neq Ice = Dry in cotton stem; Shannon index – Dry = Fro \neq Ice in kangaroo leaf;

Simpson index – Fro \neq Ice in kangaroo leaf, $P < 0.05$). When comparing the bacterial structure (Bray–Curtis dissimilarity) between sample groups and preservation treatments from PERMANOVA tests (Table 1), no significant differences ($P > 0.05$) were found between preservation treatments except for lucerne leaves wherein we found some small differences for ice incubation and frozen, ice incubation and air-dry treatments ($P < 0.05$), respectively, and for cotton roots between ice incubation and air dry treatments ($P < 0.05$). PERMDISP tests indicated that differences in lucerne leaves ($F = 5.128$, $df1 = 2$, $df2 = 15$, $P = 0.058$) and cotton roots ($F = 1.167$, $df1 = 2$, $df2 = 13$, $P = 0.643$) were likely driven by preservation methods. For bacterial identity (presence/absence, Jaccard dissimilarity), no significant difference was found between preservation treatments (Table 1A). PCoA plots showed difference of bacterial abundances and identities assembled on different plant species and tissue (Figure 2A), but differences between preservation methods within each plant species and tissue were found matching the PERMANOVA test (Figure 3A). In the subset of dominant bacterial communities, no significant difference was found in Bray–Curtis dissimilarity except in lucerne leaves between ice incubation and air-dry preservation methods, and no significant difference was found across all samples in Jaccard dissimilarity (Table 2A). In the subset of rare microbial communities, no significant difference was found across all samples in either Bray–Curtis or Jaccard dissimilarities (Table 2A). In bacterial structure and composition, no clear patterns can be found between preservation methods at the phylum level (Figure 4A).

In fungal community, no significant difference was found in either fungal community structure based on Bray–Curtis dissimilarity or composition based on Jaccard dissimilarity (Table 1B). PCoA plots showed similar fungal abundances and identities across all plant species except cotton (Figure 2B), but differences between preservation methods within each plant species and tissue were found matching the PERMANOVA test (Figure 3B). Regardless of the plant species and tissue, different preservation methods did not influence the microbial communities. In the subset of dominant and rare fungal communities, no significant difference was found in either Bray–Curtis or Jaccard dissimilarities across all dominant and rare fungal communities (Table 2B). In fungal structure and composition, no clear pattern can be found between preservation methods under phylum level (Figure 4B).

Assessing the Utility of Plant-Based Bacterial Primer Pairs for Soil Samples

Our results indicate that both primer sets provide similar results, and that overall community compositional data from the plant-based primer set (799F/1193R) were directly comparable to that obtained from the 341F/805R primer set (soil primer sets). In general, the 799F/1193R primer generated higher alpha diversity than 341F/805R (Table 3), but the variation trends were similar (Figure S1). All the diversity metrics, including Shannon diversity, richness (Chao1) and Faith's phylogenetic diversity were highly correlated between the two primer sets (Figure S1). The two primer pairs generated the same

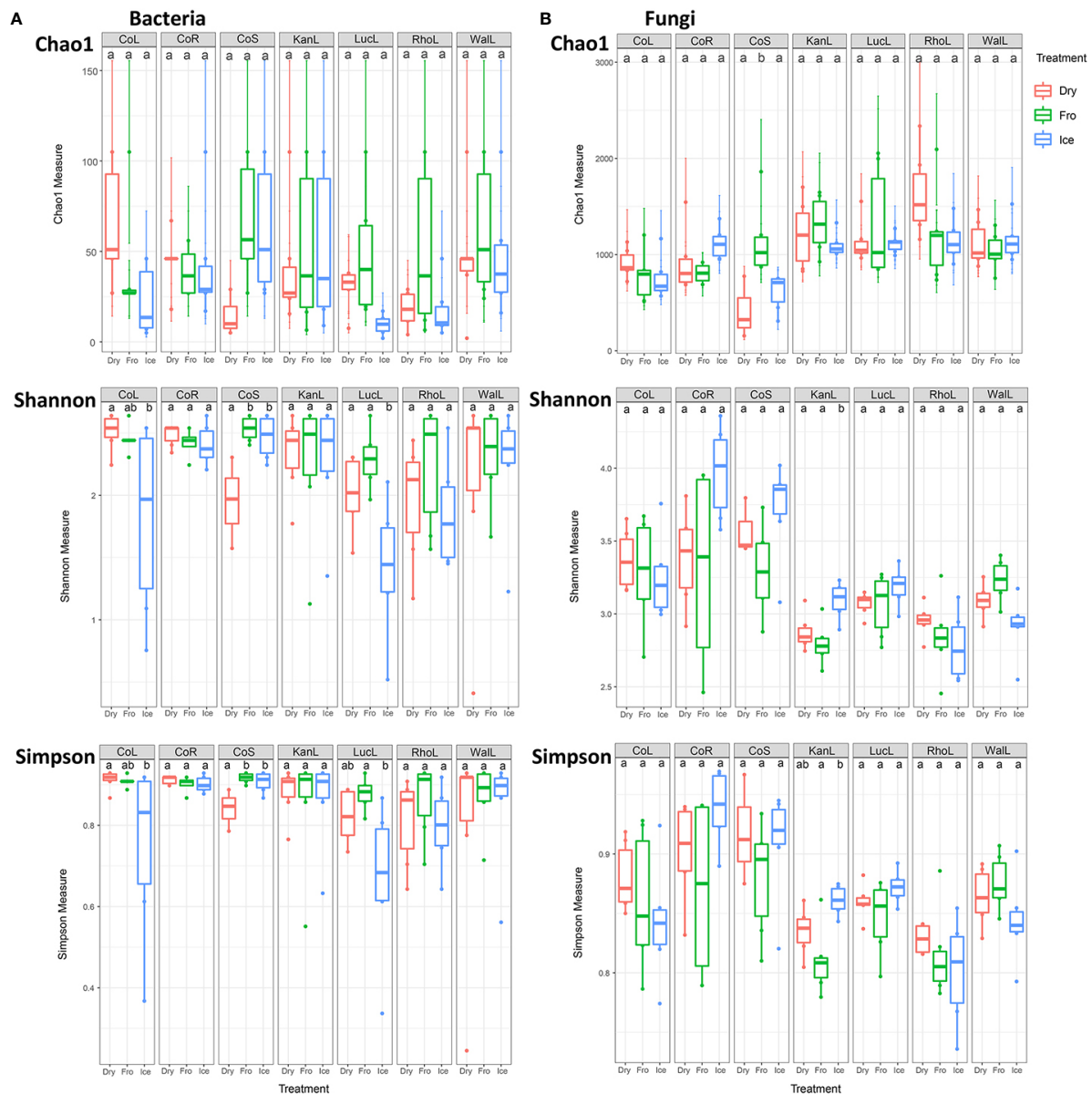


FIGURE 1 | Alpha diversity (Chao1, Shannon and Simpson) indices of bacterial **(A)** and fungal **(B)** communities under different preservation methods. Dry = air-dry (red), Fro = snap freezing (green), Ice = ice incubation (blue). CoL = cotton leaf, CoR = cotton root, CoS = cotton stem, KanL = kangaroo leaf, LucL = lucerne leaf, RhoL = rhodes leaf, WalL = wallaby leaf.

abundant phylum (top 12), and these phyla accounted for 97.5% of the total bacterial abundance in both datasets (**Figure 5**). The ranking order of the abundant phylum were similar for both primer pairs except for two bacterial phyla (Chloroflexi and Cyanobacteria) with photosynthesis abilities. At the OTU level, the community compositions were highly correlated between the two datasets, and different primer pairs did not affect the treatment effect on bacterial community composition (**Figure 6**). In the result of mock community amplification, primer pair 341F/805R resulted in 51.5% Bacillales, 19.4% Lactobacillales, 24.7% Enterobacteriales and 4.4% Pseudomonadales, while primer pair 799F/1193R

resulted in 42.5% Bacillales, 25.1% Lactobacillales, 25.6% Enterobacteriales and 6.8% Pseudomonadales (**Figure S3**). The result of bacterial community using two primer sets showed similar proportions compare to standard mock community, indicating the validity of the result.

DISCUSSION

Our study provides strong evidence that preservation strategies have minor, if any impact on the plant leaves, stems and roots

TABLE 1 | Pairwise PERMANOVA analyses of bacterial (A) and fungal (B) communities based on Bray–Curtis and Jaccard measures of square-root transformed relative abundances of plant bacterial communities under different treatments (snap frozen, ice incubation and air dry)..

	(A) Bacterial Community		(B) Fungal Community	
	Bray-Curtis	Jaccard	Bray-Curtis	Jaccard
Kangaroo Leaf	NSD	NSD	NSD	NSD
Rhodes Leaf	NSD	NSD	NSD	NSD
Wallaby Leaf	NSD	NSD	NSD	NSD
Lucerne Leaf	Fro ≠ Ice, Ice ≠ Dry	NSD	NSD	NSD
Cotton Leaf	NSD	NSD	NSD	NSD
Cotton Stem	NSD	NSD	NSD	NSD
Cotton Root	Ice ≠ Dry	NSD	NSD	NSD

NSD, no significant difference Significant results ($P < 0.05$) highlighted with bold.

microbiomes for multiple plant species belonging to contrasting functional groups (e.g. C3, C4, N-Fixers, etc.). Moreover, we found that plant and soil microbiomes might be directly comparable in future studies as widely used plant-based primer set (799F/1193R) produced similar results to those from the primer set used widely in soil microbiome studies (341F/805R) in terms of diversity and community composition across contrasting soil types. These findings imply that multiple approaches are available to accommodate different research logistics and needs without

compromising the reliability of findings. This information is critical to overcome some of the critical logistics challenges associated with large-scale studies on the plant microbiome at the regional and global scales, and also indicate that amplicon sequencing for bacterial communities are robust to primer set bias.

Contrasting Preservation Methods Do Not Alter the Plant Microbiome Structure

Microbiomes associated with plant tissue are variable and could be impacted by multiple factors such as environmental changes, plant-microbe interactions and microbe-microbe interactions (Singh and Trivedi, 2017; Hamonts et al., 2018). Major concerns with sample preservation are mainly associated with increased in temperature (above -20°C) because of the leaf disintegration under high temperature, commonly reported in leaf litter (Dilly et al., 2001; Voříšková and Baldrian, 2013; Shay, 2016). The preservation methods implemented in this study tested a range of preservation temperatures, which overall did not affect the bacterial and fungal communities (except for bacterial community of lucerne leaf and cotton root) associated with different parts of plants, suggesting reliable data could be obtained from all preservation methods used in this study.

In addition to the overall communities, we also investigated the dominant and rare communities separately to avoid

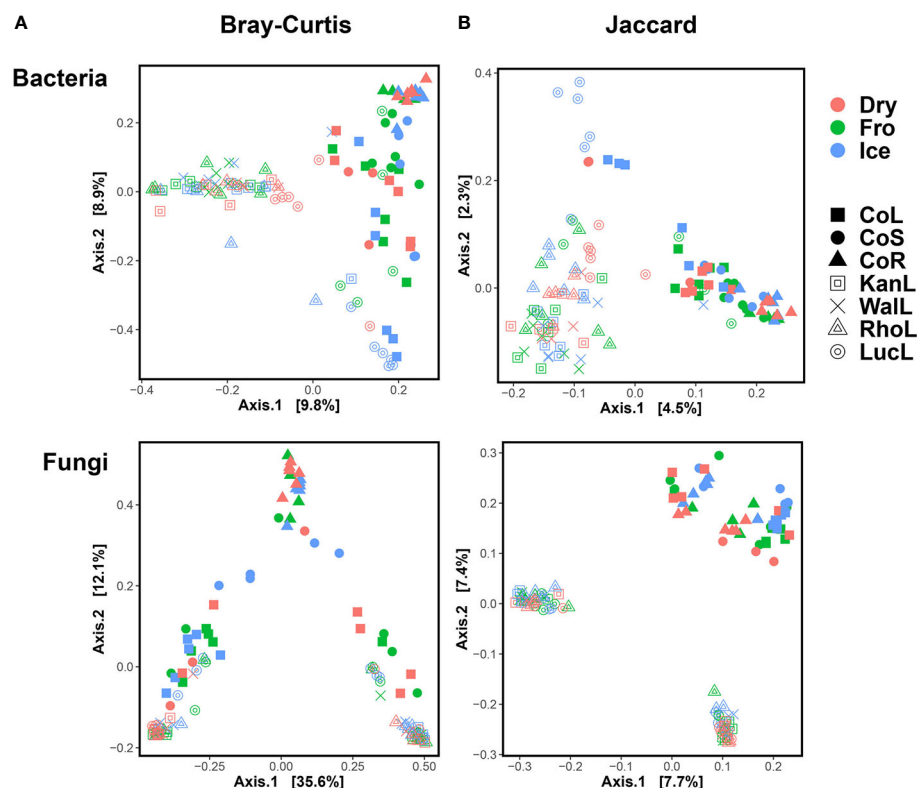


FIGURE 2 | Principal Coordinates Analysis (PCoA) plot using Bray–Curtis and Jaccard distance matrix on bacterial (A) and fungal (B) communities under different preservation methods. Dry = air-dry (red), Fro = snap freezing (green), Ice = ice incubation (blue). CoL = cotton leaf (solid square), CoS = cotton stem (solid circle), CoR = cotton root (solid triangle), KanL = kangaroo leaf (open square), WalL = wallaby leaf (cross), RhoL = rhodes leaf (open triangle), LucL = lucerne leaf (open circle).

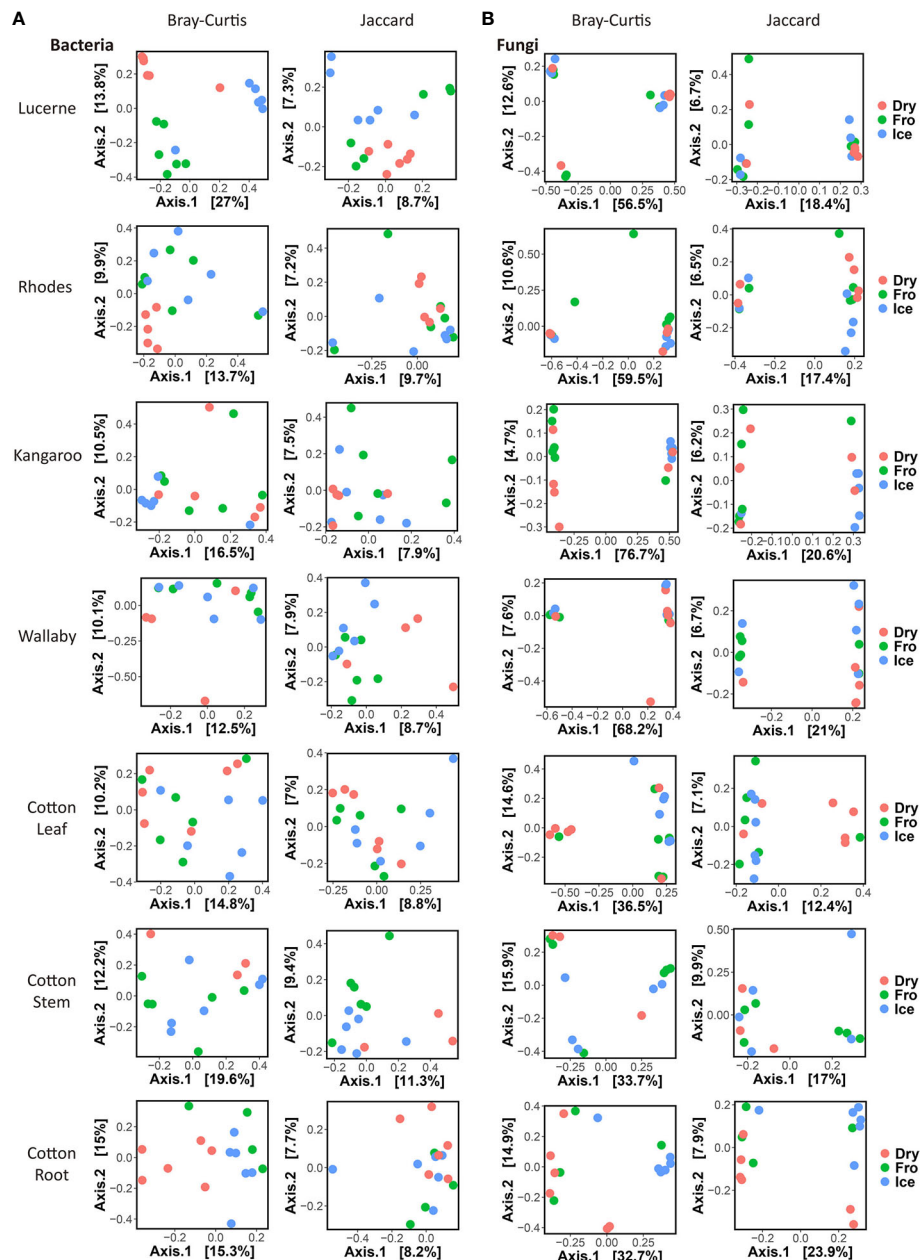


FIGURE 3 | Individual Principal Coordinates Analysis (PCoA) plot using Bray–Curtis and Jaccard distance matrix on bacterial (A) and fungal (B) communities under different preservation methods.

omissions of potential microbial variation from less abundant microbes, given the potential functional role of microbial communities in the ecosystem (Nazaries et al., 2013; Soliveres et al., 2016). Consistent with the overall community patterns, in dominant communities remain unchanged except for bacterial structure of lucerne leaves ($P < 0.05$, Table S1B). In contrast, in rare communities, no significant difference was found between preservation methods across all plant species and tissue. Collectively, the small changes in microbiome of lucerne leaves

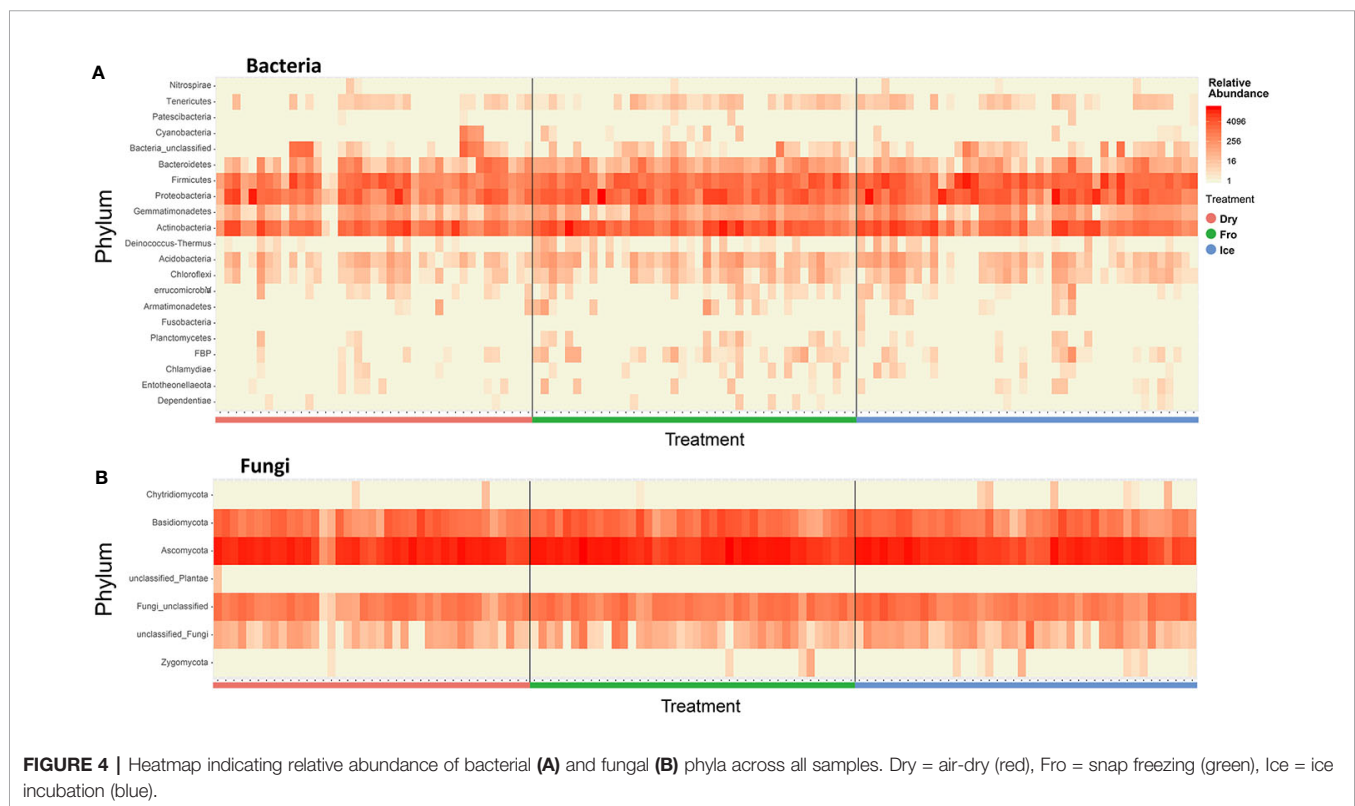
were present only in dominant bacterial communities but not rare communities, indicating that difference observed was mainly driven by shifts in the most abundant species rather than the rare ones.

In microbial composition, variation could be observed between samples, but no significant pattern was found between preservation methods. This result was consistent for both bacterial and fungal communities (Figure 4). Our findings are supported from those of other similar studies on different biological materials such as feces,

TABLE 2 | Pairwise PERMANOVA analyses of dominant and rare bacterial (A) and fungal (B) communities based on Bray–Curtis and Jaccard measures of square-root transformed relative abundances of plant bacterial communities under different treatments (snap frozen, ice incubation and air dry).

	(A) Bacterial Community				(B) Fungal Community			
	Dom.bray	Dom.jac	Rare.bray	Rare.jac	Dom.bray	Dom.jac	Rare.bray	Rare.jac
Kangaroo Leaf	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Rhodes Leaf	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Wallaby Leaf	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Lucerne Leaf	Ice ≠ Dry	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Cotton Leaf	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Cotton Stem	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD
Cotton Root	NSD	NSD	NSD	NSD	NSD	NSD	NSD	NSD

Dom, Dominant; Bray, Bray–Curtis; Jac, Jaccard; NSD, no significant difference. Significant results ($P < 0.05$) highlighted with bold.



soil and insects, which also reported little effect of temperature, storage method and duration on microbial communities (Lauber et al., 2010; Dominianni et al., 2014; Hammer et al., 2015). Therefore, the preservation methods in this study have provided a new perspective to overcome the difficulties of bulk sampling in regional or remote areas.

Plant-Based Primer Sets Are Comparable to Those From Soil Surveys

Primer selection is one of the key factors in microbiome analyses. Primer pairs 341F/805R and 515F/806R are widely accepted for bacterial community analysis from human, insects, soil, plant and marine species (Caporaso et al., 2011; Caporaso et al., 2012; Jakobsson et al., 2014; Delgado-Baquerizo et al., 2016; Walters et al., 2016; Gomez-Polo et al., 2017; Hamonts et al., 2018; Clerissi et al., 2020), while primer pair 799F/1193R has a two-

base pair mismatch for chloroplast (Chelius and Triplett, 2001), which is more suitable for plant microbiome analysis.

However, plant microbiome studies usually require both soil and plant microbiome profiles to connect underground and aboveground microbial communities (Liu et al., 2017; Hamonts et al., 2018), which essentially need consistency with primer selections. Therefore, to priorly remove the contamination of chloroplast from plant tissue, primer pair 799F/1193R is preferred in the plant microbiome analyses.

In our study, the two primer pairs showed similar patterns of relative abundance and composition of bacterial communities the soil samples (Figures 5 and 6). A lower Cyanobacteria abundance were found in the bacterial community using primer pair 799F/1193R because of the chloroplast mismatch, which was evidenced in previous studies (Beckers et al., 2016; Thijs et al., 2017). Despite the minor variation of a few bacterial phyla between the communities

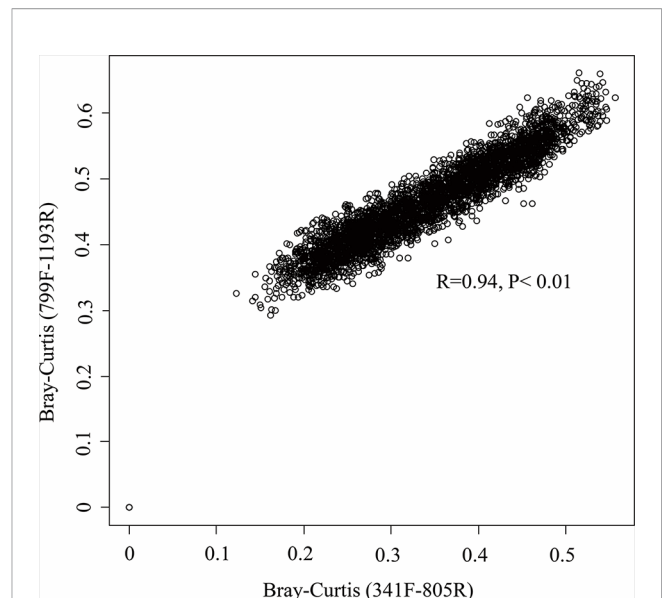
TABLE 3 | Alpha diversity measure (Mean \pm SD) of soil bacterial communities using two different primer pairs.

Primer pair	Shannon	Faith's PD	Chao1
341F/805R	9.03 \pm 0.41	121.2 \pm 15.3	2357.0 \pm 300.4
799F/1193R	9.66 \pm 0.40	136.6 \pm 14.4	4162.5 \pm 377.5

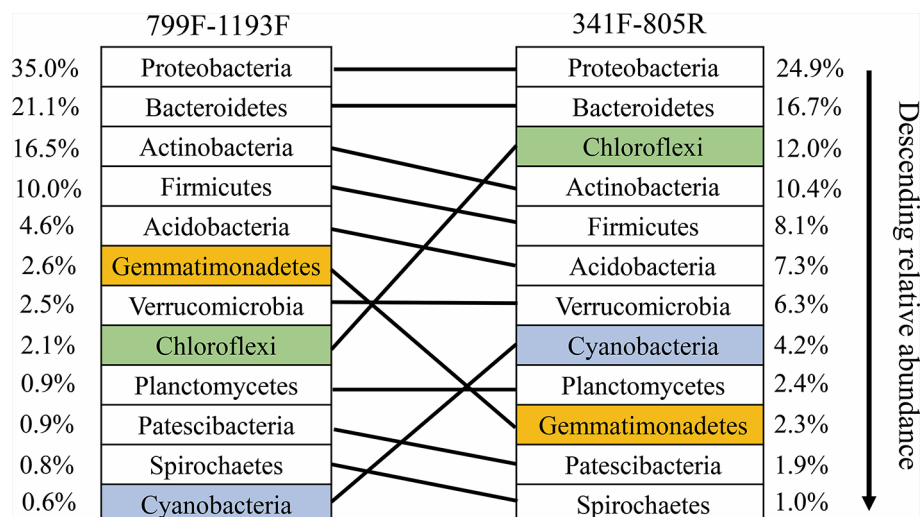
using two different primer sets, the overall bacterial structures were highly correlated ($R^2 = 0.94$, $P < 0.01$, **Figure 6**), which enhanced the previous finding of higher coverage using primers targeting V3–V4 and V5–V7 hypervariable regions (Thijs et al., 2017). This result suggests that datasets using these two primer pairs on microbiome studies are comparable, and primer pair 799F/1193R use for soil microbiome studies is valid.

CONCLUSION

In this study, we 1) sampled multiple plant species with different functional pathways and leaf architectures to identify the impact of different preservation method on plant microbiome, and 2) evaluated the validity using plant specific primer pair 799F/1193R on soil microbiome approach. The preservation methods used in this study did not impact either the bacterial community or the fungal community, and this pattern was consistent across most of the plant species. While more robust preservation methods to be implemented in the future is possible, the result from this study could significantly help large-scale sampling at regional and global scales, particularly in remote areas, with air-dry or ice incubation method.

**FIGURE 6 |** Relationship between the community composition as determined by the primer pairs 799F/1193F and 341F/805R. Mantel correlation was performed on the Bray–Curtis matrix at the OTU level.

The two different pairs of primers on bacterial plant microbiome analysis resulted in similar bacterial abundance and composition, indicating that the mismatch primer pair 799F/1193R designed for plant microbiome analysis, could also be used on other non-plant samples when Cyanobacteria was not considered. Our result facilitated the sampling on global-

**FIGURE 5 |** Ranked relative abundant of the top-12 dominant bacterial phyla as determined by the primer pairs 799F/1193F and 341F/805R, respectively. The same phylum amplified by the two primer pairs are linked by line.

scaled plant microbiome studies and enables researchers to perform combined soil and plant microbiome analyses.

DATA AVAILABILITY STATEMENT

All raw sequence data related to this study are available in the European Nucleotide Archive (The European Bioinformatics Institute, EMBL-EBI) database (Accession No. PRJEB38041).

AUTHOR CONTRIBUTIONS

ZQ, JW, MD-B, PT, EE, and BS conceived and designed the study. ZQ, JW, Y-MC, and HZ collected the samples. ZQ, Y-MC, and JW processed the samples and data analyses. ZQ wrote the

first draft of the manuscript which was revised by all co-authors. MD-B, PT, and EE revised the manuscript. All authors contributed to the article and approved the submitted version.

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Proteome Comparison Between Natural Desiccation-Tolerant Plants and Drought-Protected *Capsicum* *annuum* Plants by *Microbacterium* sp. 3J1

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Desiccation-tolerant plants are able to survive for extended periods of time in the absence of water. The molecular understanding of the mechanisms used by these plants to resist droughts can be of great value for improving drought tolerance in crops. This understanding is especially relevant in an environment that tends to increase the number and intensity of droughts. The combination of certain microorganisms with drought-sensitive plants can improve their tolerance to water scarcity. One of these bacteria is *Microbacterium* sp. 3J1, an actinobacteria able to protect pepper plants from drought. In this study, we supplemented drought-tolerant and drought-sensitive plant rhizospheres with *Microbacterium* sp. 3J1 and analyzed their proteomes under drought to investigate the plant-microbe interaction. We also compare this root proteome with the proteome found in desiccation-tolerant plants. In addition, we studied the proteome of *Microbacterium* sp. 3J1 subjected to drought to analyze its contribution to the plant-microbe interaction. We describe those mechanisms shared by desiccation-tolerant plants and sensitive plants protected by microorganisms focusing on protection against oxidative stress, and production of compatible solutes, plant hormones, and other more specific proteins.

Keywords: drought tolerance, *Microbacterium* sp. 3J1, *Capsicum annum*, comparative proteomics, actinobacteria

INTRODUCTION

Water scarcity is one of the most important limiting factors in agricultural production (Niles et al., 2015). Only a significant increase in food production can counterbalance the exponential growth of the population to supply enough food in the near future (Sapkota, 2019). To achieve this increase in food we have to ensure that crops are not lost due to more frequent droughts. Furthermore, we can recover previously abandoned farmland due to lack of water resources. We can use different approaches to grow plants for food purposes in these environments suffering from water limitation. One of these approaches consists of imitating the strategies followed by plants

that naturally tolerate the lack of water, such as desiccation-tolerant plants and genetically modify the desiccation-sensitive plants (Lamaoui et al., 2018). Another approach that have arisen recently is the use of desiccation-tolerant microorganisms with the capacity to protect plants that would otherwise be sensitive to drought (Nadeem et al., 2014; Enebe and Babalola, 2018).

Our group have identified a collection of desiccation-tolerant microorganisms, among them several actinobacteria stand out for their ability to tolerate desiccation due to the production of protective molecules called xeroprotectants (Narváez-Reinaldo et al., 2010; Julca et al., 2012; Santacruz-Calvo et al., 2013). In addition, some of these microorganisms have the ability to colonize plant roots and protect these plants, such as pepper and tomato, against drought (Manzanera et al., 2015a,b; Vílchez et al., 2016). A correlation between microorganisms with the largest capacity to protect plants from drought and their production of trehalose was found. *Microbacterium* sp. 3J1 was the microorganism with the highest production of trehalose and the one showing the highest protection of plants (Vílchez et al., 2016). By studying the metabolome of the interaction between the pepper plant root and *Microbacterium* sp. 3J1 under drought conditions, we have identified a change in the content of glutamine and α -ketoglutarate that resulted in the alteration of the C and N metabolism. As a result of this alteration, the concentration of sugars and amino acids was also modified due to the presence of the bacteria. In addition, antioxidant molecules, metabolites involved in the production of plant hormones such as ethylene, and substrates used for lignin production were altered in response to the presence of *Microbacterium* sp. 3J1 under drying conditions (Vílchez et al., 2018).

Similarly to desiccation-tolerant microorganisms there are some plants, such as *Xerophyta viscosa*, *Selaginella tamariscina*, *Craterostigma plantagineum*, and *Boea hygrometrica*, able to withstand desiccation by arresting their metabolism during drying conditions and resuming such metabolism once water becomes available again (Challabathula and Bartels, 2013). This process is termed anhydrobiosis and the plants are known as desiccation-tolerant plants or resurrection plants. To survive desiccation, these plants modulate the expression of a set of proteins involved in the decrease of photosynthesis, sugars accumulation, production of antioxidant molecules, or in the increase of the flexibility of cell walls and membranes (Challabathula and Bartels, 2013; Ambastha and Tiwari, 2015).

The study of proteomes is a versatile tool to understand the physiological processes involved in the tolerance of plants to absence of water. The study of proteomics instead of transcriptomics allows us to take into consideration transcript instability, post-transcriptional modifications and translational regulation of mRNA affecting gene expression. Changes in the quality and quantity of expressed proteins can be found with proteomic studies. To our knowledge, this is the first time the proteome of plants (*Capsicum annuum*) protected by a microorganism (*Microbacterium* sp. 3J1) under drought conditions is described and compared with the proteome of naturally desiccation-tolerant plants. The comparison of both types of proteomes may form the basis for the development of

alternative strategies to protect desiccation-sensitive food plants frequently affected by droughts.

RESULTS AND DISCUSSION

Experimental Set-Up

We identified the interaction between *Microbacterium* sp. 3J1 and pepper roots for the protection of the plant against drought. Therefore, we decided to focus on the analysis of *C. annuum* in the presence and absence of the microorganism, in both cases under drought conditions. In addition, a comparison of the proteome profile of the 3J1 strain between drought and non-stressing conditions was performed to find out a potential contribution of *Microbacterium* sp. 3J1 to the pepper plant.

We decided to sample root material from 28-day old plants previously exposed to drought for 14-days to compare the proteome resulting from different microbiome compositions. *Microbacterium* sp. 3J1 was added to the plant 14 days after germination (Figure 1A). Then we subjected this material to the mapping of the roots proteome. In addition, the relative water content (RWC) of the plants was recorded, and total soluble protein was extracted and analyzed by two-dimensional polyacrylamide gel electrophoresis (2D SDS-PAGE or 2-DE).

For the *Microbacterium* sp. 3J1 proteome, TSA cultures of the microorganism were supplemented with 5% and 50% polyethylene glycol (PEG), as indicated in the Materials and Methods section, to simulate the water activity of a non-stressed soil and of a soil subjected to drought-stress, respectively, (Figure 1B).

Analysis of Differential Proteins in *Microbacterium* sp. 3J1-Inoculated and Non-inoculated *C. annuum* Roots Under Drought

After 14 days in the absence of watering, the RWC of non-inoculated plants was 0.4, while the RWC for inoculated plants was 0.68. These results show that plants were protected by the presence of *Microbacterium* sp. 3J1 (Figure 2). Inoculated and non-inoculated plants were analyzed by 2-DE to understand the proteome response of pepper plants subjected to drought to the presence of *Microbacterium* sp. 3J1. The root protein maps produced from three independent protein extractions showed a high reproducibility based on the analysis using the PDQuest software.

Figure 3 shows representative gels of proteins extracted from the non-inoculated and inoculated plants. In conjunction, a total of 749 protein spots were reproducibly detected using PDQuest software from the non-inoculated samples and from the inoculated samples ($n = 3$). From a spot-to-spot comparison and based on statistical analysis, a total of 66 spots exhibited at least 2-fold ($p < 0.05$) difference in abundance between the non-inoculated and the inoculated plants (Figure 3A). Among 66 differential proteins, 30 spots showed qualitative changes (3 qualitative spots corresponded to inoculated-roots, whereas 27 spots were identified in non-inoculated roots. The rest 36 spots

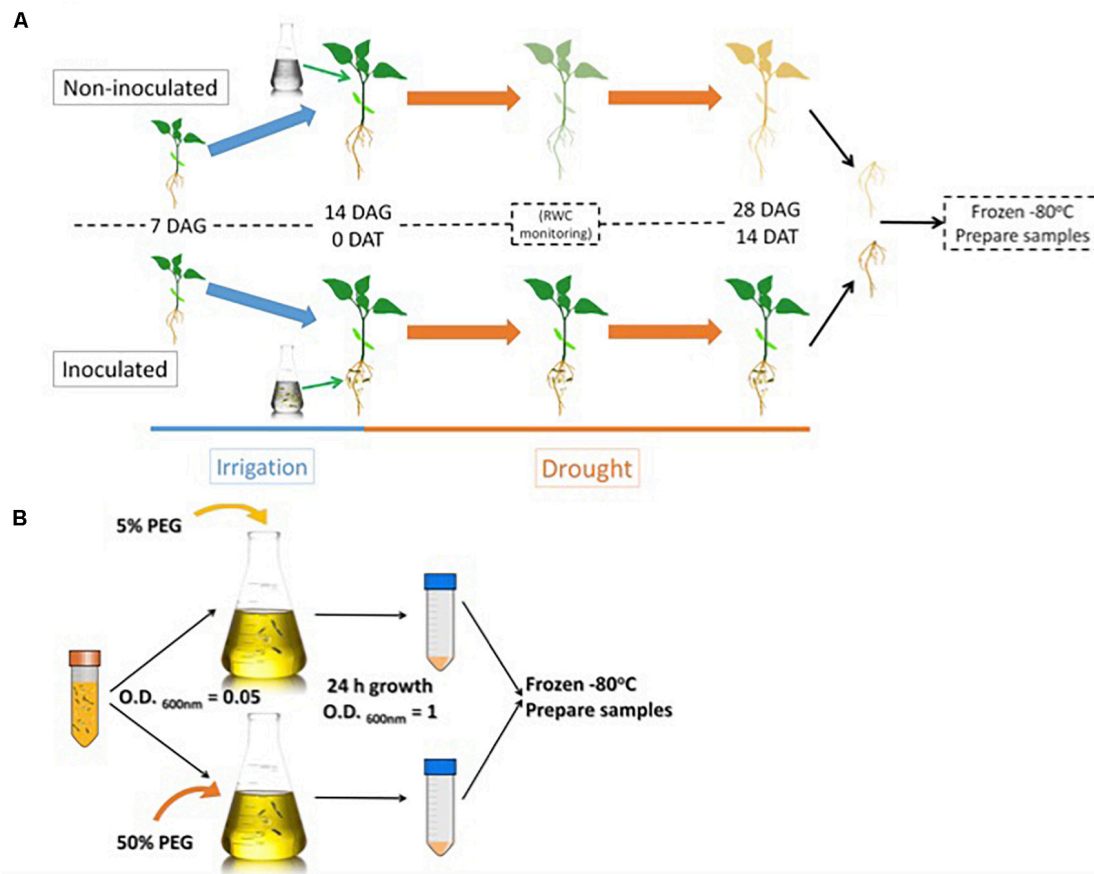


FIGURE 1 | Root sample preparation workflow **(A)** and *Microbacterium sp. 3J1* sample preparation workflow used in the proteomic study **(B)**. DAG: days after germination; DAT: days after treatment. *Microbacterium sp. 3J1*-inoculated and non-inoculated pepper plant roots were sampled and preserved 14 DAT under drought conditions. Tests were performed with at least eight plants in triplicate. After freezing the samples, workflow continues with protein isolation and 2D-PAGE. *Microbacterium sp. 3J1* cultures were grown until OD_{600 nm} of approximately 1 in TSB supplemented with 5 or 50% PEG. Then samples from three different cultures were centrifuged and frozen until use. Protein isolation and 2D-PAGE (in triplicate) were performed using the frozen samples.

were present in both conditions, showing quantitative changes. A total of 27 spots out of these 36 differentially expressed spots were identified by MALDI TOF/TOF (**Figure 3B** and **Table 1**). Therefore despite the time consuming and laborious involved in 2D-PAGE proteomics compared to shotgun proteomics we decided to use 2D-PAGE proteomics for a better comparison with most of the proteomic studies reported on desiccation-tolerant plants.

The number of protein spots detected for the *Microbacterium sp. 3J1* protection of *C. annuum* against drought was within the range of protein spots described in previous studies on desiccation-tolerant plants. These plants included *Haberlea rhodopensis*, which showed a similar number of protein spots (152 vs. 148) when comparing hydrated control plants with those dehydrated (with 16% water content), showing 33 proteins specific for the desiccated *H. rhodopensis* plants only (Mladenov et al., 2015). For *X. viscosa*, 428 (± 52) protein spots were identified using also the same software, although a lower difference in water content, changing from 65% to 35% RWC, was tested (Ingle et al., 2007). In the case of *B. hygrometrica*,

the change in RWC from 100% to 2.4% was more drastic and a total of 223 protein spots were reproducibly detected (Jiang et al., 2007).

Proteins Identified Using MALDI-TOF/TOF-MS

To understand the function of differentially expressed proteins in *C. annuum* roots exposed to drought in the presence of *Microbacterium sp. 3J1*, the 66 differentially expressed spot proteins were analyzed by gel excision followed by a trypsin digestion and MALDI TOF/TOF-MS analysis in order to obtain the peptide mass fingerprint (PMF) data. Only 27 out of the 66 analyzed proteins could be identified (**Table 1**). The reason for the low number of reported identified proteins is based on the combination of two facts. The first one is that there are only 1099 annotated proteins for Viridiplantae and *C. annuum* in the NCBI database and 62 in SwissProt. Therefore it might be possible that some of the selected proteins are not reported in the databases and therefore are considered as non-identified. In addition we

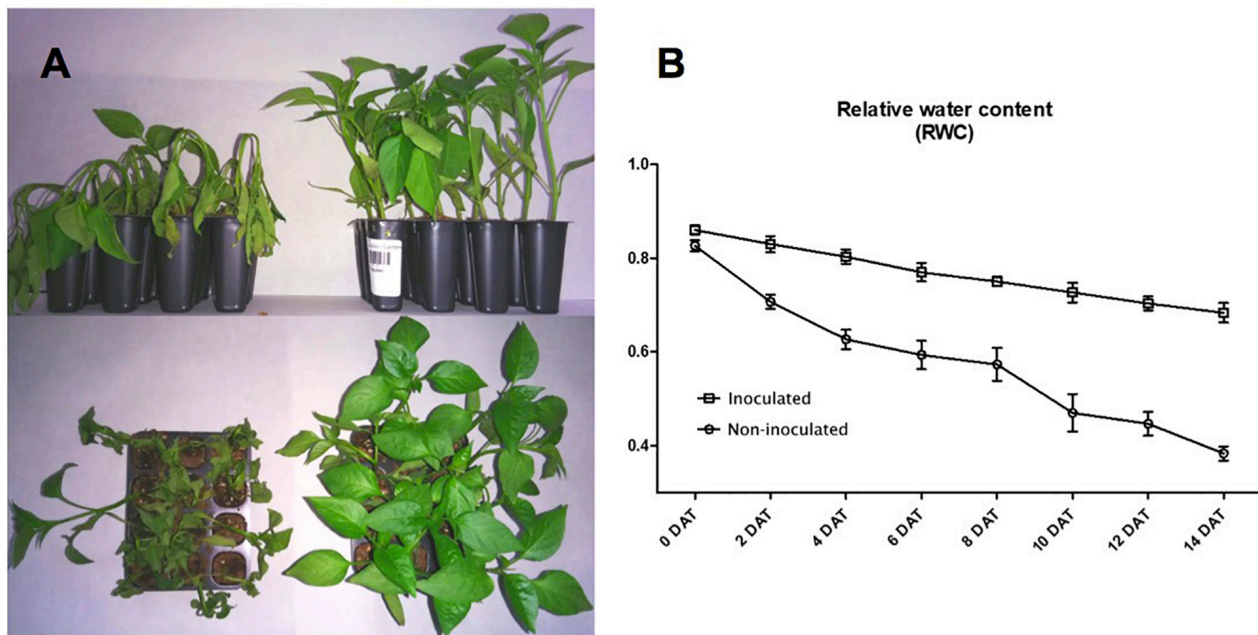


FIGURE 2 | *Microbacterium* sp. 3J1-inoculated and non-inoculated *C. annuum* used in this study. **(A)** Phenotypes showed at 28 days after germination (DAG) of seedlings, at 14 days after treatment (DAT) with *Microbacterium* sp. 3J1 (left) and in absence of the microorganism as a non-inoculated condition (right), both in the absence of watering. **(B)** Drought critical point after treatment was determined by tracking the relative water content (RWC). Tests were performed with at least eight plants in triplicate. Population standard deviation (PSD) was used to determine the inner error bars. Statistic differences were found from 2 DAT. For statistic analysis ANOVA with *post hoc* Tukey's test ($P \leq 0.05$) was used for comparison of datasets obtained at each sampling time.

wanted to show only data corresponding to high confidence, therefore only proteins with high enough coverage, and some of the non-identified proteins corresponded to low coverage results. The low coverage of some of the non-identified proteins could be most likely due to difficulties associated with separating and detecting larger proteins or due to the co-migration of proteins to the same spot with proteins of similar isoelectric points and denatured molecular weight becoming focused at the same position of the gel.

These proteins were grouped in osmoprotectant producing proteins, reactive oxygen species (ROS) scavengers, plant-hormones, structural proteins, or signaling proteins. Similarly to the proteome of desiccation-tolerant plants exposed to dehydration, *C. annuum* exposed to drought showed a diverse nature of differential proteins in the presence of *Microbacterium* sp. 3J1 compared to the non-inoculated plant. We consider this result to be indicative of the large impact of *Microbacterium* sp. 3J1 on plant cell function during dehydration. This is consistent with the diverse nature of differential proteins observed for desiccation-tolerant plants during dehydration processes.

Osmoprotectants

As a result of the proteome analysis, we have identified the decrease in fructose biphosphate aldolase and NAD malate dehydrogenase in *C. annuum* plants exposed to drought in the presence of *Microbacterium* sp. 3J1 (spots 13 and 18, respectively) compared to non-inoculated plants. These enzymes are involved in the production of glyceraldehyde 3-phosphate and

oxaloacetate, respectively. Fructose-1,6-bisphosphate aldolase is a key metabolic enzyme that reversibly catalyzes the aldol cleavage of fructose-1,6-bisphosphate into dihydroxyacetone phosphate and glyceraldehyde-3-phosphate, either during the glycolytic pathway, or gluconeogenesis and during the Calvin cycle (Berg et al., 2010). These reactions are involved in carbon fixation and sucrose metabolism and they are present in the chloroplast stroma and in the cytosol of green plants, allowing the plant to fix carbon dioxide into glyceraldehyde-3-phosphate, which can then be incorporated into other sugars (Anderson et al., 2005; Lv et al., 2017). The fact that the pepper plant produces increased amounts of these two enzymes points to the requirement of sucrose in the absence of *Microbacterium* sp. 3J1, whereas such requirement is no longer needed in the presence of this bacterial strain. We observed the accumulation of fructose (more than 8-fold), fructose-6-phosphate (approximately 18-fold increase), and trehalose (approximately 85-fold increase) in a recent metabolomic characterization of the pepper plant subjected to drought in the presence of *Microbacterium* sp. 3J1, compared to the non-inoculated plant. This result points to an alternative use of sugars as more efficient osmoprotectant (Vilchez et al., 2018). The concentration of other sugars was also increased in response to the microorganism when pepper plants were subjected to drought. Similarly, one of the mechanisms that desiccation-tolerant plants use to respond to drought consists of the accumulation of osmoprotectants, osmoregulators, and osmolyte substances. For instance *X. viscosa*, *C. plantagineum*, *Sporobolus stapfianus*, and other

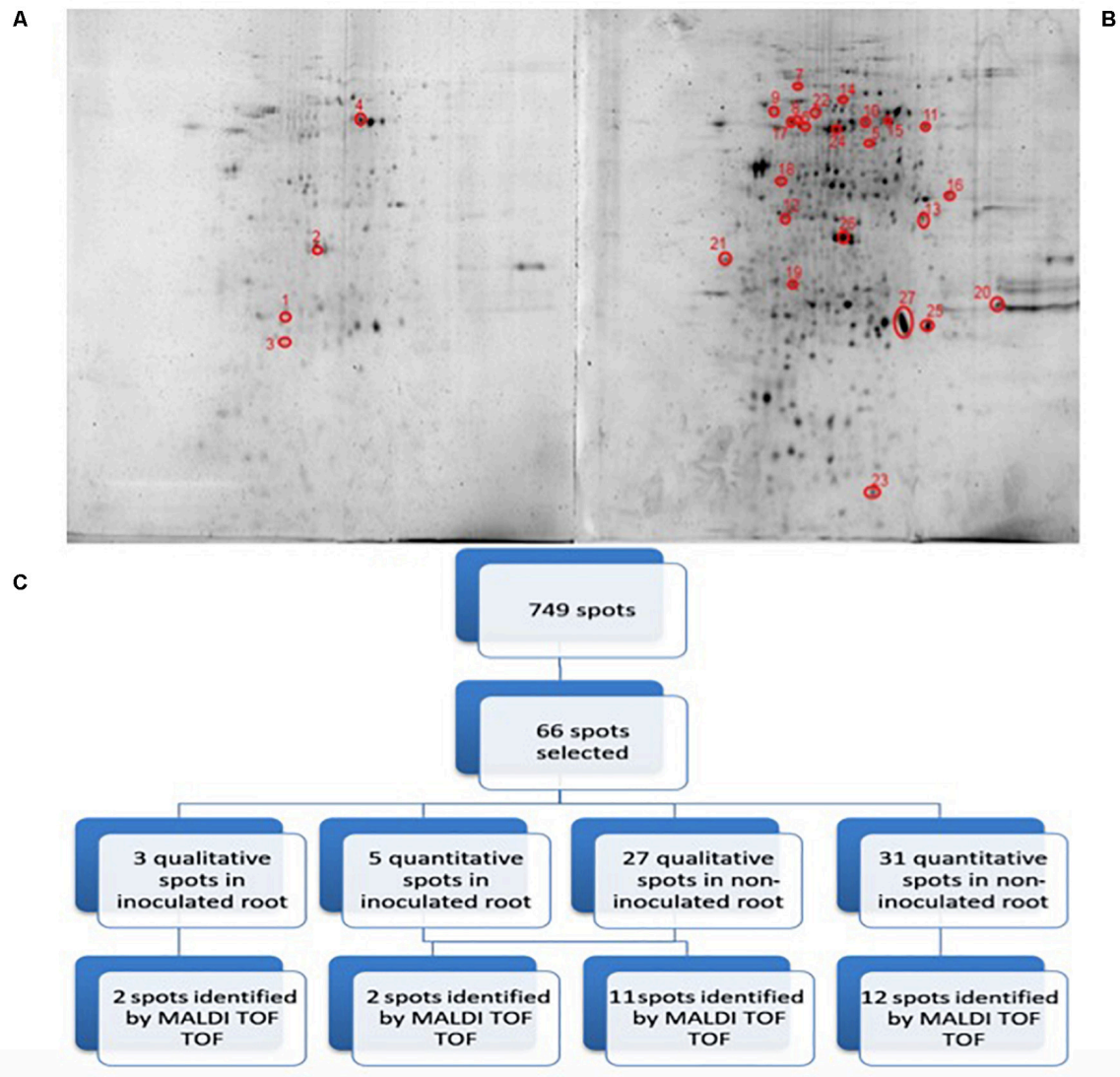


FIGURE 3 | 2D-PAGE image analysis of non-inoculated and inoculated plants subjected to drought. Upper pictures show differences in pepper root proteomes of non-inoculated (A) and inoculated with *Microbacterium* sp. 3J1 (B) seedlings obtained under drought conditions. Three biological replicates (three different plants) were used. Lower diagram shows the spot selection procedure and the final identified ones (C). Spots detection and selection were performed with PDQuest software v8.0. Red circles and numbers correspond to selected protein spots that were finally identified by MALDI TOF/TOF and described in Table 1. Pictures were selected as the most representatives from at least three 2D-PAGE replicates performed for each condition.

resurrection plants modulate their carbohydrate metabolism for the production of sucrose (Bianchi et al., 1991; Whittaker et al., 2001; Ingle et al., 2007; Yobi et al., 2013). However, *Selaginella lepidophylla* accumulates oxaloacetate, fumarate, succinate, and alpha-ketoglutarate (intermediates of the tricarboxylic acid cycle) during dehydration, therefore the importance of these metabolites for the metabolic flux through these pathways during dehydration or in preparation for rehydration is suggested (Yobi et al., 2013). Nevertheless, trehalose is a minor component in a few desiccation-tolerant angiosperm species despite its importance in desiccation tolerance among lower organisms (Ghasempour et al., 1998b). Despite the differences in the profile of osmoprotectants produced by desiccation-tolerant

plants and by *Microbacterium* sp. 3J1-inoculated plants, Ingle et al. have described the increase in the abundance of proteins (phosphopyruvate hydratase) involved in the control of glycolysis-gluconeogenesis pathways in the desiccation-tolerant plant *X. viscosa* (Ingle et al., 2007). Oliver et al. also described the increase in enzymes associated with glycolysis including aldolases, phosphoglycerate kinase, and glyceraldehyde-3-phosphate dehydrogenase in *S. stapfianus* subjected to drying conditions (Oliver et al., 2011). All the increased proteins: phosphopyruvate hydratase (in *X. viscosa*), phosphoglycerate kinase and glyceraldehyde-3-phosphate dehydrogenase (in *S. stapfianus*), and fructose biphosphate aldolase and NAD malate dehydrogenase (in *C. annuum* plants exposed to drought

in absence of *Microbacterium* sp. 3J1) would allow an increase in flux through the gluconeogenic route during drought, providing an increased pool of hexose phosphate substrates to deal with the higher demand of sucrose in both types of plants (natural desiccation-tolerant plants and 3J1-protected pepper plants) under drying conditions.

Energy Metabolism

The addition of *Microbacterium* sp. 3J1 to pepper plants subjected to drought resulted in the production of two proteins (spots 1, 3) not found in non-inoculated plants, the disappearance of three proteins (spots 6, 7, and 10) and the decreased in the amount of two proteins (spots 17, 22) related to the production of ATP. This change in the expression pattern of proteins involved in the production of ATP may produce a different cell concentration of ATP. The availability of ATP during drought is critical because this metabolite is needed for the production of osmoprotectants. The increased amount of certain proteins involved in the production of ATP substituting other ATP producing proteins has been reported for several resurrection plants such as *B. hygrometrica* (Jiang et al., 2007), *S. stapfianus* (Oliver et al., 2011), *X. viscosa* (Ingle et al., 2007), *Tortula ruralis* (Oliver et al., 2004), and for *S. tamariscina* (Wang et al., 2010), where the increased production of ATPases was suggested to play a role in protein folding or unfolding and protein degradation in response to drought stress.

Despite previous studies have reported that phosphorylation of high mobility group proteins reduced their binding to DNA, inhibiting replication and transcription in drought-sensitive plants such as maize (Zhao et al., 2009), we have described the potential role of DNA as xeroprotectant, i.e., as dehydration protectant (García-Fontana et al., 2016). Therefore we do not rule out a potential role of the over production of ATP in DNA production for DNA repair and, we do not discard that ATP can function as xeroprotectant by the production of DNA in the plant. The increase in proteins involved in DNA related processes have been reported for some desiccation-tolerant plants, such as *S. stapfianus* (Oliver et al., 2011). This increase suggests a shared mechanism between desiccation-tolerant plants and *C. annuum* protected by *Microbacterium* sp. 3J1.

Oxidative Metabolism

The proteome analysis of *Microbacterium* sp. 3J1-inoculated *C. annuum* plants subjected to drought is marked by the decrease in abundance of proteins associated with oxidative metabolism, and more specifically, with enzymes involved in the protection against ROS, pointing to a protection against ROS by alternative methods. ROS causes deleterious effects on the respiratory system, metabolic pathways, genomic stability, membranes, and organelles during drought events. The increased production of ATP occurs in conjunction with the protection against ROS, and in this sense, we have found increased production of certain ATP producing enzymes as above described. Glutathione protects against oxidative damage by quenching activated oxygen species and participates in the metabolism of hydrogen peroxide (Neubauer and Yamamoto, 1994). In addition to those proteins, we have identified an increased production of proteins involved

in glutathione production such as glutathione-S-transferase and glutathione S-transferase GST1 (spots 25, 27). In the absence of *Microbacterim* sp. 3J1, the plant produces higher quantities of ROS scavengers, showing a special need to fight ROS, something that does not seem to be required in the presence of the 3J1 strain. The production of ROS scavengers is a common feature among desiccation-tolerant plants subjected to drying conditions (Kranter and Birtic, 2005; França et al., 2007; Oliver et al., 2011; Challabathula et al., 2018; Bentley et al., 2019). Jiang and coworkers identified increased production of glutathione S-transferase in response to dehydration in *B. hygrometrica*, in special during the early stages of dehydration using proteome assays (Jiang et al., 2007). The participation of glutathione to protect desiccation-tolerant plants has been described in many species, including *H. rhodopensis* (Georgieva et al., 2017), *B. hygrometrica* (Jiang et al., 2007), and *S. stapfianus* (Oliver et al., 2011), to name a few. Apart from glutathione-producing enzymes, other proteins such as ascorbate peroxidase (ascorbate acts as a scavenger of hydrogen peroxide), or superoxide dismutase have been reported as proteins involved in oxidative mechanisms in several desiccation-tolerant plants, such as *X. viscosa* (Sherwin and Farrant, 1998; Farrant, 2000). However, we have only found a decrease in the proteins needed for the production of glutathione for this type of antioxidant proteins in response to the presence of *Microbacterim* sp. 3J1.

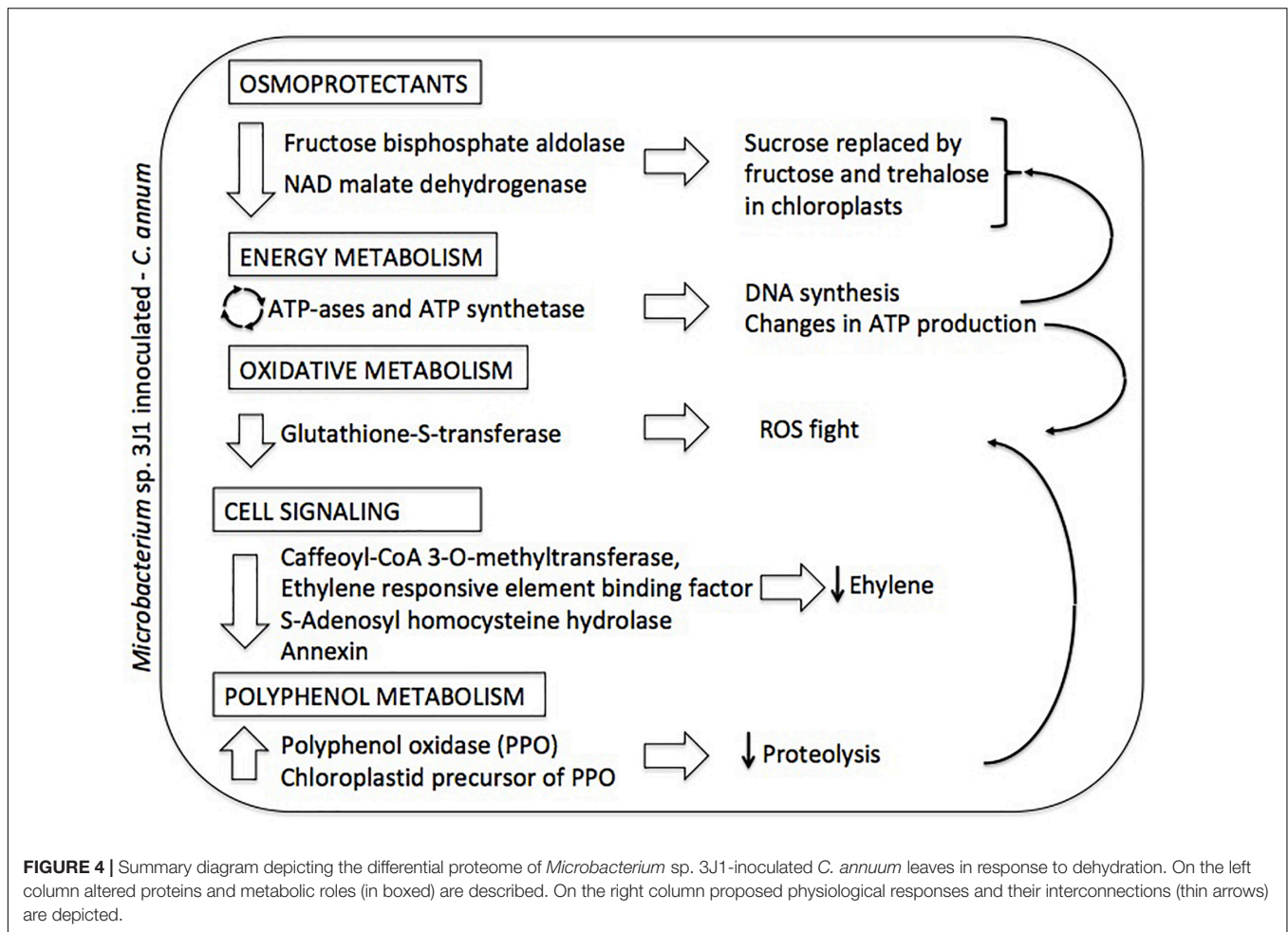
Cell Signaling

An appropriate signaling system and the production of regulatory molecules, such as plant hormones, are needed for a coordinated response between the microorganism and the plant for its protection against drought. Therefore, we would expect the detection of proteins involved in the production of such molecules. A decrease in four proteins involved in the production of plant hormones was found in *Microbacterium* sp. 3J1-inoculated plants. These four proteins are caffeoyl-CoA 3-O-methyltransferase, ethylene responsive element binding factor, S-Adenosyl homocysteine hydrolase, and annexin (spots 19, 21, 24, and 26, respectively). Caffeoyl-CoA 3-O-methyltransferase (spot 19), catalyzes the conversion of S-adenosyl-L-methionine (SAM), and caffeoyl-CoA into S-adenosyl-L-homocysteine (SAH), and feruloyl-CoA, thus reducing the concentration of SAM. The production of ethylene in plants is mainly based in the presence of SAM, and therefore a decrease in caffeoyl-CoA 3-O-methyltransferase would translate into a reduction of ethylene and a concomitant release from senescence of the plant due to lack of water. The S-adenosyl homocysteine hydrolase (spot 24) is therefore involved in the reversible production of S-adenosyl homocysteine, contributing to the reduction of SAM and, consequently, reducing the ethylene production. The production of both enzymes has been described to increase in the drought-tolerant plant *C. dactylon* (Bohler et al., 2013). The SAM to SAH ratio is involved in the enhancement of drought tolerance where methylation is a key step (Espartero et al., 1994). In addition, we have previously described a reduction in the production of ethylene in pepper plants subjected to drought when inoculated with *Microbacterium* sp. 3J1 (Vílchez et al., 2018). This is of special

TABLE 1 | List of proteins from the proteome analysis showing significantly different amounts between inoculated and non-inoculated plants.

POS ^a	Protein name	ID ^b	Function	T MW/PI ^c	E MW/PI ^d	SPOT	PDQUEST analysis		MALDI TOF-TOF			PMF			
							p ^e	App ^f	Score ^g	Sec Cov ^h	No. of pep ⁱ	Score ^j	Sec Cov ^k	No. of pep ^l	D ^m C ⁿ
1	ATP-synthetase	gi413947582	R	55.4/6.2	35/6.2	Quali	–	3/3	105/44	2,90	1/5				0 I
2	Polyphenol oxidase	gi1146424	R, O	66.8/6.6	67/6.5	Quanti	0.0022	3/3	46/44	2,50	1/5				0 I
3	V-ATPase (hp)	gi147791359	R	73/5.2	35/6.3	Quali	–	3/3	163/45	2,30	2/5				0 I
4	Chloroplast Polyphenol Oxidase precursor	gi251851953	R, O	67,6/7,2	67/7.1	Quanti	0.0239	3/3	123/45	4,20	2/5				0 I
5	7-Gamma Aminobutyrate Transaminase 3,Chloroplastic-like	gi470136321	X, R, O	55,6/6,9	55/7	Quali	–	3/3	80/44	2,80	2/5				0 NI
6	ATP Synthase Subunit beta mitochondrial	gi114421	R	59,8/5,9	60/6	Quali		3/3	148/44	5,0	2/5	128/75	35	14/47	0 NI
7	ATPase IV-type proton Catalytic subunit alpha Vacuolar proton pump	gi147791359	R	73/5,2	73/5.9	Quali		3/3	228/43	6,7	3/5				0 NI
8	ATP synthase Subunit beta mitochondrial	gi114421	R	59.9/5.9	60/5.9	Quali		3/3	182/45	9.8	4/5	134/75	31	16/44	0 NI
9	Disulfide-Isomerase like	gi565396585	X, R	55.6/4.9	55/5.9	Quali		3/3	57/44	2.6	1/5				0 NI
10	ATPase alpha F1 subunit Mitochondrial	gi57013987	R	55.2/5.8	60/6.7	Quali		3/3	274/44	12.2	5/5	223/75	45	21/48	0 NI
11	Serine Hydroxymethyl Transferase 4	gi11762130	R, O, X	51.8/7.1	58/7.6	Quali		3/3	85/45	3.4	1/5				0 NI
12	Succinyl-CoA Ligase Subunit beta	gi146215968	O, R	45.3/6.4	55/6.5	Quali		3/3	115/43	3.3	2/5				0 NI
13	Fructose Bisphosphate Aldolase 3 Chloroplastic-like	gi460386382	R, O	43/8.9	45/7.7	Quali		3/3	45/43	6.8	2/5				0 NI
14	2,3 Bisphosphoglycerate Independent Phosphoglycerate Mutase	gi565402329	O	61.2/5.4	62/6.5	Quanti	0,00017	3/3	71/45	2.1	1/5				0 NI
15	Dehydrogenase	gi10178084	R	61.7/6.3	59/7.2	Quanti	0.0054	3/3	44/44	2.2	1/4				0 NI
16	Glyceraldehyde 3 phosphate Dehydrogenase	gi18072801	R	34.5/6.2	50/7.9	Quanti	0.0017	3/3	22/11	4.7	1/4				0 NI
17	ATP synthase Subunit beta Mitochondrial like	gi114421	R	59.8/5.9	59/5.9	Quanti	0.026	3/3	293/45	10.4	5/5	171/75	40	24/71	0 NI
18	NAD-Malate Dehydrogenase	gi5123836	R, O	43.3/8.0	45/6.3	Quanti	0.048	3/3	170/44	4.4	1/5				0 NI
19	Caffeoyl -CoA 3-O- Methyltransferase	gi193290676	P, R	27.8/7.5	38/6	Quanti	0.001	3/3	46/11	4.0	1/4				0 NI
20	Chitinase	gi237662969	S, R, Other	27.7/7.5	36/8.5	Quanti	0.0003	3/3	74/43	5.1	1/5				0 NI
21	Ethylene Responsive Element Binding Factor	gi40353323	P	21.7/6.2	43/5.3	Quanti	0.02	3/3	10/9	2,1	1/5				0 NI
22	F1-ATPase alpha Subunit	gi3273590	R	45.1/8.5	61/6.5	Quanti	0.003	3/3	71/43	7.4	2/5				0 NI
23	Nucleoside Diphosphate Kinase	gi12230332	R	16.3/6.4	16/6.9	Quanti	0.05	3/3	28/10	10.8	1/5				0 NI
24	S-Adenosylhomocysteine Hydrolase	gi407930077	P	53.1/5.6	56/6.4	Quanti	0.024	3/3	82/11	7.6	3/5	54/43	17	7/36	0 NI
25	Glutathione S-Transferase	gi18150415	R	23.4/5.6	35/7.8	Quanti	0.0158	3/3	46/45	3.8	1/2				0 NI
26	Annexin	gi1071660	R, P	35.8/5.7	48/6.5	Quali		3/3	378/12	20	4/5	238/43	56	22/68	0 NI
27	Glutathione S-transferase GST1	gi61889381	R	23.8/6.4	35/7.3	Quali		3/3	88/44	14	3/5	97/75	44	10/40	0 NI

^aPosition of the spot in the 2D-Gel representative map; ^bProtein accession number in SWISSPROT Database; ^cTheoretical molecular weight (kDa)/isoelectric point; ^dEstimated experimental molecular weight (kDa)/isoelectric point in 2D gel; ^ep value of PDQuest analysis according to the ANOVA model for quantitative (Quanti) spots. Qualitative spots are showed as Quali; ^fnumber of maps in which the spot appears from a total of 3; ^gMALDI TOF/TOF protein score over a minimum significant score; ^hamino acid sequence coverage for the identified protein in percentage; ⁱnumber of peptides matched by mass-mass spectrometry; ^jPMF protein score over a minimum significant score; ^kamino acid sequence coverage for the identified protein in percentage; ^lnumber of peptides matched by PMF; ^mDecoy; and ⁿCondition in which the spot was overexpressed: I (Inoculated plants), and NI (non-inoculated plants). Function codes: Involved in Structures, E; Signaling, X; ROS scavenging, R; Phytohormones/Ethylene control, P; or Osmoprotectants, osmoregulators, osmolites, O.



relevance since we have observed the decrease in the ethylene responsive element-binding factor (spot 21) in pepper plants subjected to drought when *Microbacterium* sp. 3J1 was present. In resurrection plants, such as *X. viscosa*, it has been suggested that ethylene could be a signal for their tolerance to drought through the expression of *XVT8* (a gene encoding a glycine-rich protein, with significant identity to dehydrins; Ndima et al., 2001). However, the abscisic acid (ABA) is a more common plant hormone for the response to drought in *X. viscosa* (Ingle et al., 2007). ABA controls the stomata closure and the accumulation of metabolites, increases the activity of antioxidant enzymes and the expression of genes encoding protective proteins in response to dehydration and it has been described as a major component in the hormonal control of the acquisition of desiccation tolerance in *S. stapfianus* (Gaff et al., 2009), and in other resurrection plants (Oliver et al., 2010; Oliver et al., 2011). The existence of a non-ABA signaling pathway has been proposed for *S. stapfianus*, where brassinosteroids, and methyl jasmonate could play a role (Ghasempour et al., 1998a, 2001).

Polyphenol Metabolism

We found an increase in the production of polyphenol oxidase (PPO) and the chloroplastid precursor of PPO (spots 2 and 4,

respectively) in plants subjected to drought in the presence of *Microbacterium* sp. 3J1. An increase in PPO has been described to prevent of proteolytic activity for the conservation of the proteins during the desiccation state in *Ramonda serbica* and *B. hygrometrica* (Veljovic-Jovanovic et al., 2006; Jiang et al., 2007). The PPO has also been described as ROS scavenger in *H. rhodopensis*, *M. flabellifolia*, and *Ramonda serbica* (Moore et al., 2005; Georgieva et al., 2007; Veljovic-Jovanovic et al., 2008). A potential role of PPO in Mehler-like reaction for detoxification of ROS has been suggested because most PPOs are localized in the chloroplast thylakoid lumen; in addition, the chemical structure of PPOs makes them ideal for free radical scavenging activities in the chloroplast (Sherman et al., 1995; Rice-Evans et al., 1997). Therefore, we suggest that the presence of *Microbacterium* sp. 3J1 in pepper plants subjected to drought use PPO instead of glutathione to prevent the damage produced by ROS (Figure 4).

Microbacterium sp. 3J1 Proteome in Response to Drought

In order to assess the potential impact of the proteins produced by *Microbacterium* sp. 3J1 on the pepper plant during drought, we characterized the differential protein expression of this soil microorganism in response to severe drought by addition of

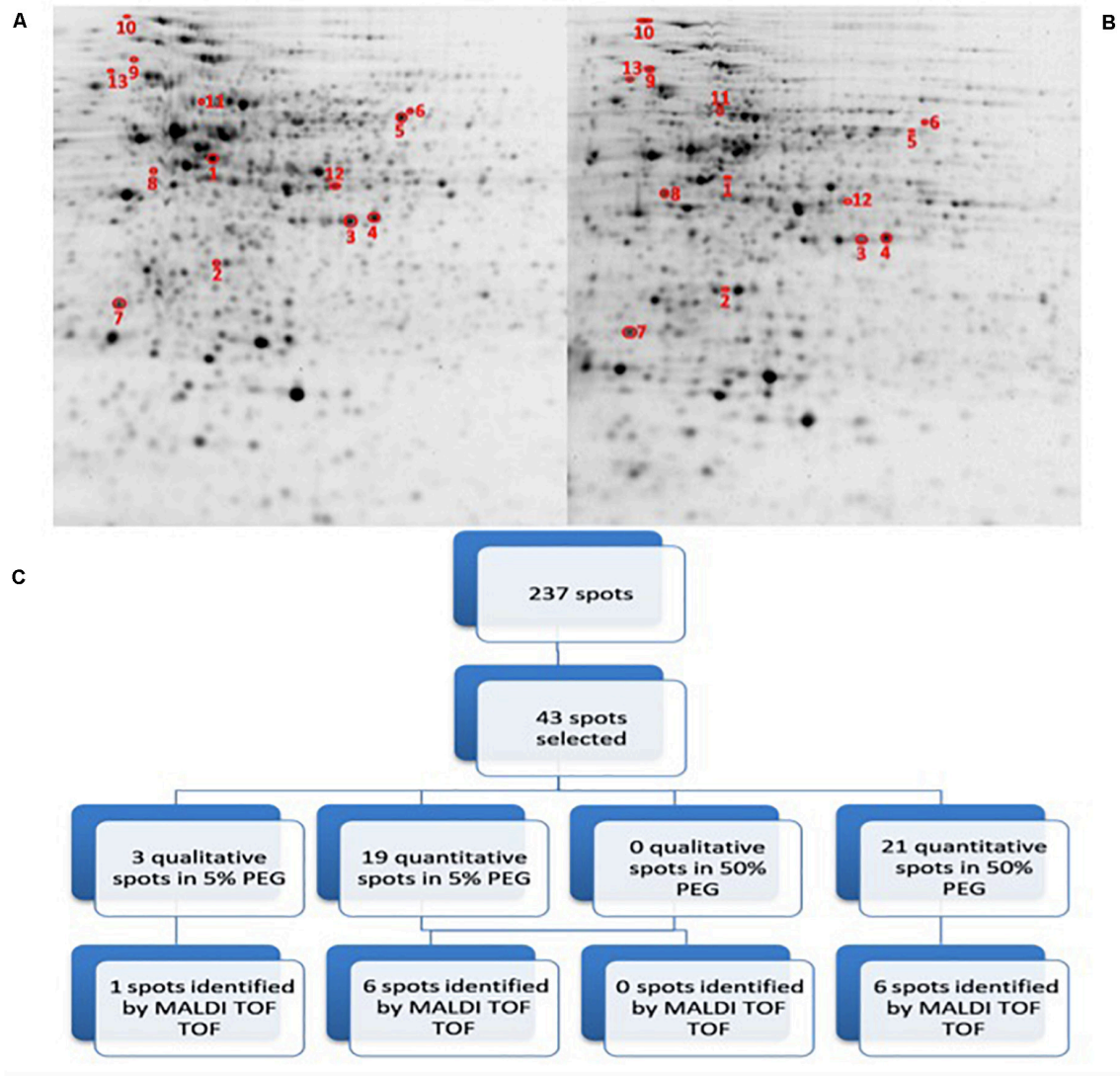


FIGURE 5 | 2D-PAGE image analysis of drought-subjected *Microbacterium* sp. 3J1 cultures. Upper pictures show differences in *Microbacterium* sp. 3J1 proteome when 5% (A) or 50% PEG (B) were supplied to TSB growth medium. Three biological replicates (three different cultures) were used. Lower diagram shows the spot selection procedure and the final identified ones (C). Spots detection and selection were performed with PDQuest software v8.0. Red circles and numbers correspond to selected protein spots that were finally identified by MALDI TOF/TOF and described in Table 2. Pictures were selected as the most representatives from at least three 2D-PAGE replicates performed for each condition.

PEG. The addition of PEG results in the sequestration of water molecules reducing the hydric potential of the media. To simulate the change from a non-stressed soil to a severe drought-stressed soil, liquid cultures of *Microbacterium* sp. 3J1 were supplemented with 5 and 50% (w/v) PEG 8000, respectively. The 2-DE was performed following the previously described conditions. A fold-change of at least 2 was considered as the minimum level of differentially expressed proteins when analyzing the 2-DE images. A total of 237 protein spots were analyzed as statistically significant (p -value < 0.05) and 43 protein spots were selected and analyzed by MALDI-TOF/TOF-MS- (Figure 5). A total of 40 quantitative (19 up- and 21 down-regulated) proteins and 3 qualitative spots

were differentially expressed proteins between 5 and 50% PEG conditions. Among these 43 analyzed spots, 13 were successfully identified (Table 2). Similarly to the *C. annuum*'s proteome, *Microbacterium*'s proteome is poorly described and therefore it might be possible that some of the selected proteins are not reported in the databases and again are considered as non-identified. Also some of the non-identified proteins corresponded to low coverage results.

All differentially identified proteins were analyzed via the NCBI BLAST database and the function of these proteins was explained by inputting the Uniprot accession number into the Uniprot database as shown in Table 2 (Boutet et al., 2016; UniProt Consortium, 2019). The identified proteins were mainly involved

TABLE 2 | List of proteins from the *Microbacterium* sp. 3J1 proteome analysis showing significantly different amounts under hydric stress (50% PEG) and non-stressed conditions (5% PEG).

POS ^a	Protein	ID ^b	Function	T	PM/PI ^c	E	PM/P ^d	TYPE	PDQuest Analysis		MALDI TOF-TOF		PMF		C ⁿ			
									p-value ^e	Maps ^f	Qualif ^g	% Cov ^h	No of pep ⁱ	Calif ^j		% Cov ^k	No of pep ^l	D ^m
1	Isocitrate dehydrogenase (NADP)	IDH_BACSU	O, E	46.4/5.02	51/5.05	Qt			0.00324	3/3	97/38	4.5	3/5			5% PEG		
2	L-aspartate Oxidoreductase	gi 551257345	R, O	34.5/5.06	39.3/5.06	Qt			0.005	3/3	35/30	4.3	1/5			5% PEG		
3	Keto-acid reductoisomerase	ILVC_BACSK	R, P, O	37.2/5.32	42/5.32	Qt			0.00667	3/3	121/38	5.6	1/4			5% PEG		
4	L-lactate dehydrogenase	LDH_BACME	R, O	35/5.58	40/5.58	Qt			0.00017	3/3	166/39	10.7	4/5			5% PEG		
5	Acyl-CoA dehydrogene	gi 497462210	R	40.3/4.93	45.2/4.93	Qt			0.00808	3/3	37/36	2.9	1/4			5% PEG		
6	Glutamate synthase (NADPH)	gi 652424279	O	54.8/7.52	55.3/7.92	Qt			0.00016	3/3	53/39	2	1/5			5% PEG		
7	Triosephosphate isomerase	TPIS_BACMD	R	26.9/4.63	40.5/4.63	Qt			0.025564	3/3	65/39	6	1/5			50% PEG		
8	Alanil-ARNt synthase	gl 493548752	X, O	97.2/5.36	88.4/5.36	Qt			0.0007186	3/3				73/70	9.7	8	0	
9	Chaperone DnaK	DNAK_BACME	R	65.2/4.69	68.3/4.69	Qt			7.8017E-05	3/3				70/70	9.8	4/5	0	
10	Aconitate hydratase	gl 657019280	O, E	101.7/4.75	101.5/4.75	Qt			0.0002732	3/3	99/30	3.2	3/5			50% PEG		
11	1-pyrroline-5-carboxylate dehydrogenase	ROCA_GEOTN	X, R	56.5/5.54	62.8/5.54	Qt			0.0037244	3/3	50/40	2.25	2/5			50% PEG		
12	Glyceraldehyde-3-phosphate dehydrogenase 1	G3P1_BACOE	O, E	35.8/5.27	45.2/5.27	Qi			-	3/3	130/40	7.2	2/5			5% PEG		
13	Glyceraldehyde-3-phosphate dehydrogenase 1	ATPB_BACAA	R	51.2/4.94	55.6/4.94	Qt			0.00017	3/3	173/40	11.1	2/5			5% PEG		

^aPosition of the spot in the 2D-Gel representative map; ^bProtein accession number in SWISSPROT Database; ^cTheoretical molecular weight (kDa)/isoelectric point; ^dEstimated experimental molecular weight (kDa)/isoelectric point in 2D gel; ^ep value of PDQuest analysis according to the ANOVA model for quantitative (Quant) spots. Qualitative spots are showed as Qualif; ^fnumber of maps in which the spot appears from a total of 3; ^gMALDI TOF/TOF protein score over a minimum significant score; ^hamino acid sequence coverage for the identified protein in percentage; ⁱnumber of peptides matched by mass-mass spectrometry; ^jPMF protein score over a minimum significant score; ^kamino acid sequence coverage for the identified protein in percentage; ^lnumber of peptides matched by PMF; ^mDecoy; and ⁿCondition in which the spot was obtained: PEG 5%, PEG 50%. Function codes: Involved in Structures, E; Signaling, X; ROS scavenging, R; Phytohormones/Ethylene control, P; or Osmoprotectants, osmoregulators, osmolites, O.

in (a) osmotic protection, (b) energy metabolism, (c) antioxidant protection, and (d) cell signaling.

Regarding the proteins involved in osmotic protection, 8 proteins were differentially expressed (spots 1–4, 6, 8, 10, and 12), including the decreased expression of L-aspartate oxidoreductase in media supplemented with 50% PEG (spot 2) compared to medium with 5% PEG. This enzyme is involved in the conversion of L-aspartate into oxaloacetate, resembling the catalytic step produced by the increased expression of malate dehydrogenase observed in *C. annuum* plants subjected to drought in the presence of *Microbacterium* sp. 3J1. In the presence of water stress conditions, we have also found a decreased production of NADPH-isocitrate dehydrogenase (spot 1) and an increased production of aconitase (spot 10), involved in the citric acid cycle, for the reversible catalytic conversion of citrate to isocitrate via *cis*-aconitate. The decreased production of NADPH-isocitrate dehydrogenase that catalyzes the irreversible conversion of isocitrate into α -ketoglutarate in conjunction with the increased production of aconitase in response to water stress conditions would be translated into a reduction of α -ketoglutarate by the partial inversion of the first steps of the citric acid cycle. The metabolomic analysis of the pepper plant subjected to drought also showed a dramatic decrease in α -ketoglutarate due to the presence of *Microbacterium* sp. 3J1. This decrease seems to respond to a decreased production of α -ketoglutarate and to the conversion of α -ketoglutarate into glutamine for the drastic change in the C and N metabolism of the plant due to the presence of the microorganism (Vilchez et al., 2018). In the inoculated plant subjected to drought, the concentration of glutamine is increased compared to the non-inoculated plant; however, no alteration in the glutamate was found. This might be explained by the observed decreased production of NADPH-glutamate synthase (spot 6) in *Microbacterium* sp. 3J1, when exposed to drying conditions (50% PEG). The produced glutamate from glutamine can be transformed into proline, a well described osmoprotectant, by enzymatic and non-enzymatic transformation. The last of the enzymatic steps requires the participation of the 1-pyrroline-5-carboxylate dehydrogenase, one of the increased proteins in *Microbacterium* sp. 3J1 in response to higher water stress (spot 11). The production of both proline and aspartate contribute to the production of osmoprotectants. Glutamate metabolism for the production of other amino acids such as alanine, valine, and isoleucine, could be reduced also by the observed decreased production of keto-acid reductoisomerase (spot 3) under water stress. An increased production of alanyl-tRNA synthetase (spot 8) was found. This is a key enzyme for the availability of amino acids also overproduced by *Microbacterium* sp. 3J1 in response to water stress. A disappearance of glyceraldehyde-3-phosphate dehydrogenase (spot 12) was also found in response to water stress in *Microbacterium* sp. 3J1. This enzyme catalyzes the reversible conversion of glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate, contributing to the metabolic flux of the glycolysis-gluconeogenesis pathways described in the inoculated peppers in presence of *Microbacterium* sp. 3J1. This could also be the case of the increased production of triose phosphate isomerase (spot 7) found in *Microbacterium* sp. 3J1 under water

stress, since this enzyme catalyzes the reversible conversion of glyceraldehyde-3-phosphate into dihydroxyacetone phosphate, allowing the availability of free sugars for the production of osmoprotectant, as described for 3J1-inoculated pepper plants under drying conditions.

A decreased production of the L-lactate dehydrogenase (spot 4) was observed in response to water stress. This protein reversibly catalyzes the inter-conversion of pyruvate and lactate, which may serve for the connection of the altered glycolysis-gluconeogenesis pathways with the altered citric acid cycle, therefore allowing an increased production of glucose.

Finally, an increased production of the chaperone protein DnaK (spot 9) was found in response to water stress by increasing the PEG concentration in the media from 5% to 50%. The DnaK protein actively participates in the response to hyperosmotic shock and it is also involved in chromosomal DNA replication. As previously described by our group, an increased production of DNA by *Microbacterium* sp. 3J1 was found in response to drought, where the DNA molecules seem to have a role as osmoprotectant as proved *in vitro* for the protection of proteins against desiccation (García-Fontana et al., 2016).

An increased production of the β subunit of the ATP synthetase (spot 13) was found in response to water stress. We associate this increased production with the energy metabolism of *Microbacterium* sp. 3J1 in an analogous manner to the need of ATP during droughts in plants. Another finding was the decreased production of acyl-CoA dehydrogenase in response to water stress (spot 5). Acyl-CoA dehydrogenases are enzymes catalyzing the α , β -dehydrogenation of acyl-CoA esters in fatty acid and amino acid catabolism. We associate this reduced production of the acyl-CoA dehydrogenase under drying conditions with being part of the shut down of the lipid metabolism within the general arrest of metabolism found in anhydrobionts during the desiccated state. A reduced production of the acyl-CoA dehydrogenase was observed in *Bradyrhizobium japonicum*, a desiccation-tolerant microorganism, in response to other stressors, such as acidic pH (Puranamaneewiwat et al., 2006), or in *Acinetobacter baumannii* and in the cyanobacteria *Leptolyngbya ohadii* among other desiccation-tolerant microorganisms in response to desiccation (Gayoso et al., 2014; Oren et al., 2017).

CONCLUSION

The desiccation-tolerant bacteria *Microbacterium* sp. 3J1 has been characterized at biochemical and metabolomic levels due to its response to drought. Despite the ability of *Microbacterium* sp. 3J1 to protect a diverse range of plants from drought, its response to drought was not characterized at the proteomics level to date. The ability to tolerate desiccation has been described in several microorganisms, and many of them have been described as able to protect plants from drought. However, the information available on the proteomics of desiccation-tolerant microorganisms and their role in protecting plants from drought is scarce. In the present study, we observe a parallel pattern between the protection of plants from drought conducted by *Microbacterium* sp. 3J1 and the mechanisms that

naturally desiccation-tolerant plants use to survive desiccation. Despite of the large variety of mechanisms used by plants, a common strategy consisting of increasing the production of osmotic protectants, increasing energy metabolism, improving the antioxidant protection and reshaping the cell signaling in the plant via plant hormone production was found. We describe a similar pattern in pepper plants protected from drought by *Microbacterium* sp. 3J1, where all these molecular strategies are altered by an altered expression of proteins. The presence of *Microbacterium* sp. 3J1 in pepper plants subjected to drought seems to respond to the mechanism that the bacterium itself uses to survive drought, where the microorganism seems to take control of the plant metabolism for a higher chance of survival. We believe that this knowledge may form the basis for the development of alternative strategies to protect desiccation-sensitive food plants such as *C. annuum*, which are affected by droughts frequently.

MATERIALS AND METHODS

Bacteria and Plant Growth Conditions

Green Pepper *Capsicum annuum* L. cv. Maor plants were produced by the specialist grower SaliPlant S.L. (Granada, Spain). Plants were grown in green houses under constant relative humidity (50–60%). The plants were illuminated with a 12-h light/dark cycle. Once plants were purchased, they were incubated in the lab under similar humidity and light/dark cycle. For the light cycle, 200 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ were used. In addition, dawn–dusk cycles were included with 150 $\mu\text{mol photons}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$. The temperature ranged from 18 to 20°C during the dark period and from 20 to 25°C during the light period.

For inoculation, half of the plants were treated with 4 mL of bacterial suspension (10^8 – 10^9 CFU/mL) per plant in sterile saline solution, while the other half of the plants were used as non-inoculated controls and watered with the same volume of sterile saline solution.

Plants were regularly watered and allowed to acclimate for at least 2 weeks after inoculation before simulation of drought was implemented.

For inoculation, *Microbacterium* sp. 3J1 was used as a rhizobacteria drought-tolerance enhancer. These bacteria were grown in tryptic soy broth (TSB) at 30°C and 180 rpm (Manzanera et al., 2004).

Dehydration Treatment and Determination of the Relative Water Content (RWC)

Three groups of *C. annuum* plants, each consisting of three individual plants, were dried down by withholding water. A control group of plants was watered with an equivalent volume of saline solution in the absence of inoculant. Plants of each condition (with and without inoculant) were extracted from the substrate and completely cleaned of soil by several washes in distilled water. Roots tissues were cut and preserved until sample preparation and protein extraction by wrapping

in aluminum foil and storing at -80°C after treatment with liquid nitrogen.

Fresh weight (FW), dry weight (DW), and fully turgid weight (FTW) of the whole plants free from soil were recorded. The RWC was calculated according to Vilchez et al. (2016) and to Mayak et al. (2004) using the following equation $\text{RWC} = (\text{FW} - \text{DW}) \times (\text{FTW} - \text{DW})^{-1}$. In addition, root length (RL) and stem length (SL) were measured.

Polyethylene glycol 8000 was added to the microorganism culture to simulate dehydration. *Microbacterium* 3J1 were grown in TSB supplemented with 5% (w/v) PEG 8000, at an initial absorbance of 0.05 at 600 nm wavelength. The cultures were incubated for 24 h and then split into two. Half of cultures were supplemented with 50% PEG 8000 to simulate severe drought. The cells were further incubated at 30°C and 180 rpm until stationary phase was reached and then, harvested by centrifugation at 15,000 g for 30 min. The cell pellets were stored at -80°C until use.

Isolation of Total Protein

Frozen pellets, consisting of 0.5 g of plant tissue or cells collected from 10 ml of *Microbacterium* sp. 3J1 cultures were resuspended in 1 mL lysis buffer containing 500 mM Tris-HCl pH 6.8, 4% sodium dodecyl sulfate (SDS; w/v), 30% glycerol (v/v), 1 mM EDTA, 5 mM phenylmethanesulfonyl fluoride (PMSF), 200 mM dithiothreitol (DTT), 0.01% Benzonase® Nuclease (v/v; Roche). Three biological replicates (three different plants and three different bacterial cultures) and technical replicates (three different 2D-PAGE) were used.

For the plants and bacterial pellets, cells were broken using FastPrep (three cycles of 40 s at 6.5 m/s). The lysate was centrifuged for 15 min at 20,000 g and 4°C , and the supernatant was recovered for soluble protein quantification by Bradford Method. Soluble proteins (150 μg) were precipitated. Protein precipitation was performed by adding 50% (v/v) ice cold trichloroacetic acid (TCA) to obtain a final concentration of 10% TCA, which was then incubated on ice for 15 min. The samples were centrifuged for 15 min at 20,000 g and 4°C , and then the protein pellet was washed three times with 1 ml of chilled acetone and it was centrifuged at 4°C for 5 min at 20,000 g. Finally, the dry samples were stored at -20°C until use.

Two-Dimensional Polyacrylamide Gel Electrophoresis (2D-PAGE)

Total soluble protein was dissolved in 300 μL of rehydration buffer (RH) 8 M Urea, 2 M Thiourea, 2% (w/v) Chaps, 0.5 mM PMSF, 20 mM DTT, 0.5% (v/v) Bio-Lyte Ampholyte (BioRad), and trace amounts of bromophenol blue, at 15°C 2 h and 200 rpm. The protein samples were then centrifuged for 15 min at 20,000 g and 4°C .

Supernatants, containing 150 μg soluble protein fraction, were separated by isoelectric focusing (IEF) using a 17-cm non-linear pH 3.0–10.0 immobilized pH gradient (IPG) for plant proteins and a non-linear pH 4.0–7.0 IPG strips (ReadyStrips, BioRad) for bacterial proteins in the first dimension. IEF was performed at 20°C . Strips were first actively rehydrated at 50 V for 14 h which accumulated to 288 V/h. IEF was performed using a Protean

i12 IEF System (BioRad) with the following program: and initial step of 100 V for 5 h, followed by four step gradients of 500 V for 30 min, 500 V for 7 h, 1,000 V to 500 V/h, and 8000 V to 13,500 V/h. At the end, a step of 8,000 V to 45,000 V/h was reached. A total of 64,076 V/h were accumulated at the end.

For the second dimension, IPG strips were equilibrated for 15 min in 5 mL of reducing equilibration buffer [75 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 10 mg/mL of DTT, and traces of bromophenol blue] followed by 15 min incubation in 5 mL of alkylating equilibration buffer [75 mM Tris-HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 25 mg/mL of iodoacetamide and traces of bromophenol blue]. The second dimension SDS-PAGE was performed on a PROTEAN II xi Basic Electrophoresis System (BioRad) at 1 mA/gel and 100 V at 15°C , overnight until the bromophenol blue reached the bottom of the gel. The entire electrophoresis unit was protected from direct light during the run.

After electrophoresis, gels were stained for 2 h with fluorescent Oriole Solution (BioRad), protected from direct light and under constant shaking.

Image Acquisition and Image Analysis

Stained gels were immediately observed at excitation/emission wavelengths of 270/604 nm using ChemiDoc MP Imaging System (BioRad). The gel images were captured using ImageLab software, using the automatic exposure and 24.5 cm width.

Images were analyzed with PDQuest Basic Software (BioRad, Hercules, CA, United States). Spots differentially expressed were selected by two different criteria: Qualitative spots (presence/absence), and quantitative spots ($p = \leq 0.05$) with a fold difference of 2 compared to the control.

Gel Excision and In-Gel Digestion of Proteins

The spots containing the proteins of interest were cut using the EXQuest Spot Cutter (BioRad).

Gel pieces containing the proteins of interest were digested using the DigestPro MS (Intavis) instrument. Proteins were reduced with DTT 10 mM in BCA 50 mM (56°C 1 h), alkylated with iodoacetamide (IA) 55 mM in BCA 50 mM (30 min), and enzymatically digested with Trypsin Gold Mass Spectrometry Grade (Promega) for 10 h at 37°C . Peptides were eluted with trifluoroacetic acid (TFA) 0.2% and acetonitrile (AcN) 30%.

Mass Spectrometry (MS) Identification of Proteins

Tryptic digests of each spots were desalted with Zip Tip μC18 Pipette Tips (Millipore, Bedford, CA, United Kingdom) according to the recommended protocol and eluted directly on the AnchorChip target plate (Bruker-Daltonics, Hamburg, Germany) with α -cyano-4-hydroxycinnamic acid (CHCA) matrix. The MS and tandem MS (MS/MS) spectra were obtained using an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker-Daltonics) in auto-mode using Flex Control v3.4 (Bruker-Daltonics) and processed using ProteinScape v3.1.3 (Bruker-Daltonics). Peptide spectra were acquired in reflectron

mode with 2,000 laser shots per spectrum. Spectra were externally calibrated using peptide calibration standard (Bruker). A total of 2,500 laser shots were accumulated for MS/MS data. The PMF and MS/MS search were performed on NCBI database for Viridiplantae and *Capsicum annuum* searching for plant proteins. Bacterial proteins of *Microbacterium* sp. 3J1 were searched in databases for *Microbacteriaceae*, *Micrococci*, and *Actinobacteria*. SwissProt database was used for all organisms, using MASCOT 2.4.0 software integrated together with ProteinScape software (Bruker-Daltonics). The parameters used for the search engine were: monoisotopic peptide mass accuracy of 50 ppm, fragment mass accuracy to ± 0.5 Da; maximum of only one missed cleavage; carbamidomethylation of cysteine as a fixed modification and partial oxidation of methionine as a variable modification. There were no restrictions regarding the molecular weight (MW) and isoelectric point (pI) of the proteins. Filtering of peaks was done for known autocatalytic trypsin peaks; the signal to noise threshold was set to 2. The significance threshold was set at $p < 0.05$.

AUTHOR'S NOTE

This manuscript has been released as a pre-print at BioRxiv (García-Fontana et al., 2020).

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DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: https://datadryad.org/stash/share/weamto_bvAkLYgWdvB6gSpKLQIXpM7EolKZ2LR0PARK.

AUTHOR CONTRIBUTIONS

MM designed the experimental procedures, wrote the manuscript, and analyzed the results. CG-F and JV performed the experiments, did the statistical analysis, and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Towards Better Understanding of the Interactions and Efficient Application of Plant Beneficial Prebiotics, Probiotics, Postbiotics and Synbiotics

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INTRODUCTION

It is well known that a gram of soil contains thousands of individual microbial taxa including bacteria, fungi, protists, oomycetes and viruses. Many of them play the main role in ecosystem functioning determining soil fertility and provide plant growth promotion and disease suppression, (van der Heijden et al., 2008; Glick, 2012; Serna-Chavez et al., 2013; Maron et al., 2018). However, after many years of chemical fertilization, soils lost their natural fertility, plant diversity and microbial richness (Huang et al., 2019). In addition, an increasing number of stress factors are observed such as salinity, alkalinity/acidity, contamination, nutrient deficiency or overload of chemical fertilizers, drought, soil erosion due to climate change, and various biotic factors (Fitzpatrick et al., 2019). The use of plant beneficial microorganisms (PBM) to mitigate these problems in cultivated crop production is now a common practice particularly in the modern, sustainable agriculture and in the context of increasing world population and environmental and climate concerns (Shilev et al., 2019). During the last 20–30 years, a large number of microorganisms have been isolated, characterized and tested as biofertilizers and biocontrol agents in controlled and natural conditions. The results confirmed the beneficial effect of the selected microorganisms on plant growth and health, enhancing nutrient content and improving soil properties. Now, the emphasis of the scientific activity in the field of microbial inoculants is on developing environmentally friendly and efficient microbial formulations and analyse how the introduced microorganisms affect microbial community, diversity, and the specific plant–microorganisms interactions, which determine the plant holobiome functioning (Berg et al., 2017). Therefore, at this moment, at least two major lines of research can be distinguished: the first one deals with holobiome/hologenome studies including molecular mechanisms and genetic regulation (and epigenetic mechanisms) of beneficial microbiota (Corbin et al., 2020) and, another important line of research on the process of establishing a plant beneficial microbiome includes development of efficient single or multiple microbial inoculants. A combination of pro- and postbiotics could be applied to manage and stimulate the existing beneficial microbiome.

WHAT IS IMPORTANT TO KNOW BEFORE SELECTING A PBM?

There are many interrelated points in our understanding of the role of PBM that should be taken into consideration when designing inocula of PBM and applying them in the field. Firstly, the coexistence of all multicellular eukaryotes and microorganisms forming a holobiome and hologenome was evolutionary proved. The vast majority of recent studies including in the field of plant–microbe interactions, have confirmed the role of beneficial microorganisms in host development, metabolism, stress adaptation, and health. It appears that hosts can attract microorganisms with specific plant–beneficial characteristics (Rodrigo et al., 2017). Secondly, due to chemicalization of soils, climate and environmental changes, there is a clear decline in the soil microbial diversity and in the number of PBM: plants are less able to attract, select, and outsource their colonizers as the link between them is broken (Hardoim et al., 2015). Therefore, based on previous physical, chemical, and biological/biochemical analysis of the soil–plant system and microenvironment, we should introduce microbial inoculants composed by a single or multiple microorganism(s) (Qiu et al., 2019). Thirdly, in some cases, microbial formulated products demonstrated excellent plant growth promoting or plant protection effects under greenhouse-controlled conditions, but showed unsatisfactory results in field conditions. Moreover, some studies demonstrated reduced plant growth and increased microbial phytopathogenicity as a result of soil–plant systems inoculation with potentially beneficial microorganisms in conditions of nutrient saturation, changes in the microbial community, or environmental and plant genotype effects (Rayan and Graham, 2002; van der Heijden et al., 2008; Serna-Chavez et al., 2013; Fitzpatrick et al., 2018).

PREBIOTICS, PROBIOTICS, AND POSTBIOTICS

Based on the above considerations, three strategies for microbial management of soil–plant systems could be selected based on prebiotics, probiotics, and postbiotics (Figure 1).

Prebiotics and Synbiotics

Prebiotics are products, which improve microbial diversity and soil microbial health by promoting the growth of soil microorganisms already present within the soil–plant system. Prebiotics are natural products, normally agro-industrial wastes, including biochar, sewage sludge, compost, humus, animal manure, and chitin-bearing wastes, among others, which ameliorate (particularly in degraded soils) the soil structure, biochemical activity, and increase microbial population and diversity (Baker et al., 2011; Vassilev et al., 2013; Strachel et al., 2017). Compost and animal manure, however, can be considered as synbiotic products (Adam et al., 2016) as they contain microorganisms (some of them with beneficial properties); PBM could be additionally inoculated into the compost. Solid-state fermentation (SSF) based inoculants can also be defined as synbiotics. The final SSF products are multifunctional mixtures of mineralized organic matter (with both prebiotic and carrier functions) and plant beneficial microorganism(s) (with probiotic plant growth promoting or biocontrol functions) (Vassilev and Mendes, 2018). When the probiotic microorganism is a P-solubilizing agent, the synbiotic mixture could additionally be enriched with plant available P (Shilev et al., 2019). Similar synbiotic characteristics can be observed in microbial inoculants encapsulated in natural gels in the presence of additives with beneficial microbial stimulating action (Vassilev et al., 2020).

Probiotics

In the field of soil–plant science, probiotics are accepted as beneficial microorganisms, which exert health promoting and nutrient-mobilizing properties, as defined by Haas and Keel (2003). Particularly attractive are bacteria with high enzyme (ACC-deaminase) activity, production of phytohormones (auxins, cytokinins, gibberellins), osmolytic metabolites (e.g. trehalose, glycine betaine) (Shilev et al., 2019). These microorganisms can be found at best on the surface or within the plants (Mendes et al., 2013; Hardoim et al., 2015). Once introduced into soil, probiotics should develop a critical biomass level to exert their plant beneficial traits. As this process is highly dependent on the soil–plant

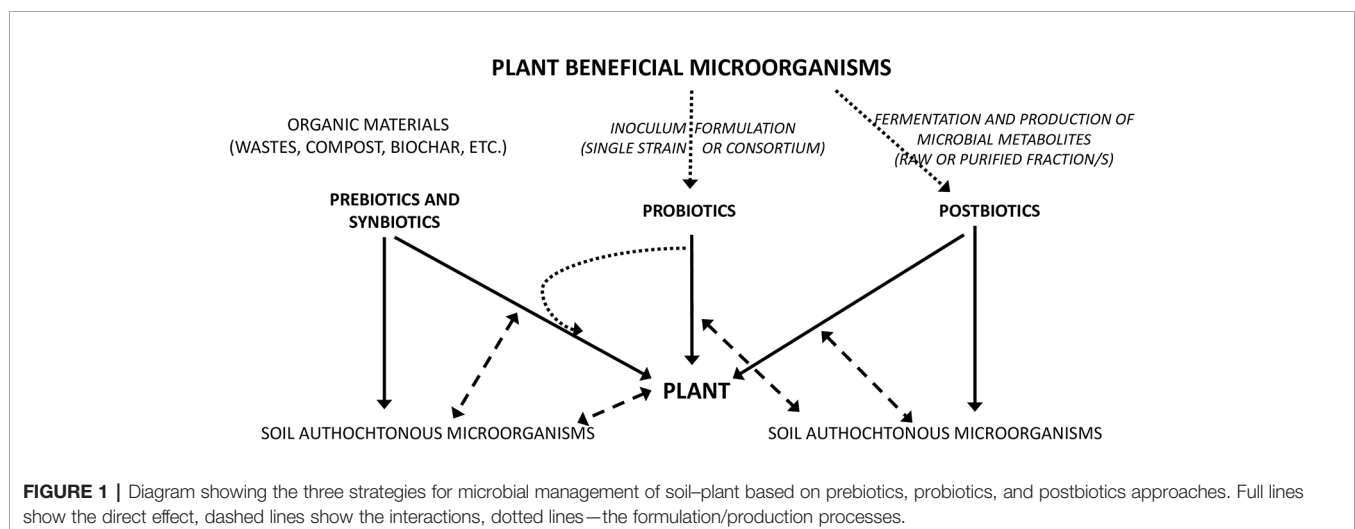


FIGURE 1 | Diagram showing the three strategies for microbial management of soil–plant based on prebiotics, probiotics, and postbiotics approaches. Full lines show the direct effect, dashed lines show the interactions, dotted lines—the formulation/production processes.

characteristics and environmental conditions, it seems difficult for a given single microorganism or a microbial consortium to reach this critical cell number (Woo and Pepe, 2018). Therefore, after a long period of studies on isolation, selection, and characterization of PBM, research scientists are focused on development of economic biotechnological processes for biomass/spores production and formulation that will solve the above problems (Bashan et al., 2016; Parnell et al., 2016; Vassilev and Mendes, 2018). Formulated products can be liquid or solid and should fulfil a number of requirements, the most important of which are to demonstrate high colonizing effectiveness and competitiveness, and increase plant nutrition and health status (Malusa and Vassilev, 2014). One of the most promising formulation techniques is the encapsulation in macro- and micro-beads of polysaccharides which guarantees a continuous deliver of the inoculant into soil preventing the effect of soil and environmental stress factors including indigenous microbial community (Bashan et al., 2016; Qiu et al., 2019). However, a simple gel-entrapment is not sufficient to ensure economical advantages and desired agronomic impact of the formulates (Vassilev et al., 2020). Double/multiple inoculants combined with biostimulants and other additives including seeds (all-in-one smart bio-formulates) should be developed to complete with the traditional chemical fertilizers (Vassilev et al., 2015; Trivedi et al., 2017). Another option, to avoid problems during each phase within production, formulation, storage, and establishment/action of the PBM in soil, is to use their plant beneficial metabolites (postbiotics).

Postbiotics

Postbiotics are metabolic derivatives of PBM, which exert specific, growth promoting and/or biocontrol, effects on plants thus avoiding the risks associated with applying microbial cells. Specific examples of such metabolite include phytohormones, volatiles, and quorum-sensing compounds (Schikora et al., 2016). Which are the risks of using microorganisms in soil-plant systems? Wrong formulation procedures without osmoprotectants, UV-protectors, fillers with nutrient value, and other plant benefiting additives usually provoke inconsistent results under field conditions (Bashan et al., 2016; Vassilev et al., 2020). Further risks include various abiotic and biotic factors, which affect the rate of microbial colonization, the presence of other, more competent, components of the microbial population, the level of plant needs and capacity to attract and feed beneficial microorganism (Fierer, 2017). It is important to note that the protocols for field applications of PBM are not assuring that they will find their niche of establishing and function. Moreover, it is yet not clearly known what kind of metabolites the introduced microorganisms will release in the soil-plant system. This complex set of conditions determines the rate of survival of the inoculants and the performance of their target functions (Kaminsky et al., 2019). Analysing all these aspects, it appears that endophytic microorganisms are better protected from adverse environmental conditions and, in addition, more efficient functionally (Santoyo et al., 2017).

Shall we apply cell-free liquids containing specific or complex metabolites produced by the PBM during fermentation under

controlled conditions? There are two options in developing such kind of biotechnological products. Using cell-free fermentation broth liquids without further downstream operations for separation/purification of specific metabolites is the most economic option and, in some cases mixtures of different microbial cultures demonstrate higher potential even after autoclaving (Mendes et al., 2017; Hussain et al., 2020). Well-established and easy to perform immobilized cell technology methods can be applied to repeatedly/continuously use the metabolic activity of the microorganisms (Kautola et al., 1990), producing plant growth promoting or biocontrol compounds in repeated-batch or continuous fermentation mode thus making the whole process more attractive economically (Vassilev et al., 2017; Mishra and Arora, 2018).

Another approach includes operations such as fragmentation and further use of extracts of the microbial mass or isolation of specific metabolites from the fermentation liquid. However, the application of specific metabolites in soil should be assessed carefully, bearing in mind that in the rhizosphere there is a great variety of microbial and plant metabolites involved in a wide number of interrelated cooperative or antagonistic actions (Basset-Manzoni et al., 2018). Therefore, before applying plant beneficial metabolites directly after the fermentation production process or in purified form, formulation operations should be performed to ensure their efficient release into soil. Encapsulation and nano-encapsulation of microbial metabolites was reported as an effective tool in enhancing proliferation of shoots and rooting (Pour et al., 2019). In this case, the inclusion of carbon nanotubes and SiO nanoparticles in the alginate-gelatin nanocapsules increased the overall beneficial effect of the formulated cell-free product. Nano-formulations by encapsulation are expected to enhance the metabolic stability of the microbial metabolites but their cost-effectiveness can be increased if the principles of the precision agriculture are applied (Duhan et al., 2017).

CONCLUDING REMARKS

Production and application of PBM is now one of the most promising fields of research. The period of searching for easy to cultivate soil microorganisms, their characterization, and testing in controlled conditions was replaced by another one with studies on novel, more efficient and economic fermentation mode of production and formulations. Co-cultivation and formulation of compatible PBM and inclusion of various additives in the formulations become fundamental part of the overall production technology (Vassilev et al., 2014; Vassilev et al., 2015; Vassilev et al., 2020). Another, pivotal point of the new approach to understand and manage the functional and genetic role of soil microorganisms in the soil-plant systems, is the comparison between human gut microbiome and plant microbiome (Adam et al., 2016). Following the human gut example, new strategies for exploitation of PBM appeared based on prebiotic, probiotic, synbiotic, and postbiotic products. A previous analysis of soil physical/chemical characteristics, microbial community dynamics along the plant growth and depending on the climatic specificity is a part of the overall assessment on which

approach will be most efficient. Here, we consciously do not discuss, but should mention, other important issues such as how to control the plant capability of attracting useful microorganisms, the role of core and hub microbiota (Toju et al., 2018), and development of multi-omics tools and interdisciplinary (or artificial intelligence) approaches of management of all soil-microbe spatio-temporal complex data (Alekkett et al., 2017). The advancement in the field of PBM is substantial but there are still largely unexplored options for “biotics” therapeutic treatment of soils and biotechnological optimization of microbiome functioning in agro-soil systems bearing in mind their extreme complexity (Fierer, 2017).

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MV and NV designed and drafted the work. EM and EF-P contributed to the revision of the manuscript.

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Effects of *Enterobacter cloacae* HG-1 on the Nitrogen-Fixing Community Structure of Wheat Rhizosphere Soil and on Salt Tolerance

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The present study investigated the physiological and biochemical characteristics of *Enterobacter cloacae* HG-1 isolated from saline-alkali soil. We further studied the effect of this strain on the salt tolerance of wheat and on the community structure of nitrogen-fixing bacteria in rhizosphere soil. We determined that the investigated strain had high nitrogen fixation activity and produced iron carriers, 1-aminocyclopropane-1-carboxylic acid deaminase, and plant hormones. The metabolites of this strain contained 2,3-butanediol, [R-(R*, R*)], 2-heptanone, and other growth-promoting and antibacterial substances. The strain was also highly salt-tolerant (10% NaCl). After the inoculation of wheat with the HG-1 strain, we recorded increases in root length, plant height, fresh weight, and dry weight of 19.15%, 18.83%, 16.67%, and 17.96%, respectively, compared with uninoculated plants ($P < 0.05$). Compared with the leaves of uninoculated plants, the proline concentration in the leaves of inoculated plants increased by 12.43% ($P < 0.05$), the malondialdehyde level decreased by 27.26% ($P < 0.05$), K^+ increased by 20.69%, Ca^{2+} increased by 57.53% and Na^+ decreased by 31.43% (all $P < 0.05$). Furthermore, we detected that inoculation with the HG-1 strain did not affect the species composition of nitrogen-fixing bacteria in wheat rhizosphere soil at the phylum level. However, the average relative abundance of Proteobacteria was significantly increased, whereas the abundance of Verrucomiobacteria was significantly decreased compared with uninoculated plants. At the genus level, we detected 32 genera in control samples and 27 genera in inoculated samples, and the species diversity and relative abundance of samples inoculated with the HG-1 strain decreased compared with uninoculated plants. Inoculated samples had lower abundances of *Azospirillum*, *Rhodocyclomicrobium*, and *Anabaena*. Our study demonstrated that the inoculation of wheat with *E. cloacae* HG-1 could promote the growth of wheat under salt stress and increase salt stress tolerance. The results of this study investigating the interaction among soil, plants, and microorganisms supplement agricultural microbial databases and could

provide a reference for the development of microbial-based saline soil improvement programs.

Keywords: salt stress, nitrogen-fixing bacteria, plant growth promotion, community structure, high-throughput sequencing

INTRODUCTION

Salinity is one of the major abiotic stresses, with a total of 3% of the world's land mass being affected by salinity (Mukhtar et al., 2018). Global increase in soil salinization constitutes a most devastating environmental threat for crop yield and food quality (Asrar et al., 2017). In China, nearly 37 million hectares of saline-alkali soil, accounting for 4.9% of the nation's arable land (Brandon and Ramankutty, 1994). However, it also remains seemingly unavoidable in the short- to middle-term that global food demand will rise as a result of population growth and environmental degradation (Ervin and Lopez-Carr, 2018). Furthermore, the effects of climate change, such as decreased rainfall and high temperatures, will further increase the degree of salinization (Shrivastava and Kumar, 2015), which will induce an estimated 70% reduction in the cultivation of wheat and other major crops (Acquaah, 2007). The transformation of salt-tolerant crops does not have a high success rate because of the complex mechanism of salt stress in crops (Dodd and Pérez-Alfocea, 2012; Kumari A. et al., 2015) despite considerable efforts in traditional breeding and genetic engineering (Dodd and Pérez-Alfocea, 2012; Joshi et al., 2015; Krishna et al., 2015). However, one study reported that plant growth-promoting (PGP) rhizobacteria (PGPR) significantly promoted the growth of plants under salt stress, which led to an increase in research surrounding PGPR mechanisms and the structure of plant rhizosphere microbial communities (Qin et al., 2016).

PGPR can establish a symbiotic relationship with plants and promote their growth under both normal and stress conditions (Desgarennés et al., 2015). Symbiotic bacteria exist in all plants, and this relationship may be a key factor involved in plant stress tolerance. In fact, local adaptation of plants to their environment is driven by the genetic differentiation among closely associated PGPRs (Rodríguez et al., 2008). Transplanting various plant species in the absence of bacteria is notoriously difficult (Leifert et al., 1989), and this difficulty supports the importance of bacteria to plant growth, including under stressful conditions (Singh and Jha, 2016). The rational use of PGPR is a valuable approach for reducing salt stress in plants (Bisseling et al., 2009). These bacteria exhibit multiple stress-related traits that may contribute to their plant protective capabilities under growth inhibiting levels of salt (Rohban et al., 2009; Siddique et al., 2010; Bharti et al., 2013; Sharma et al., 2016). In naturally salt-affected soil, PGPR without salt tolerance characteristics gradually lose their plant growth-promoting (PGP) characteristics with an increase in salinity (Upadhyay et al., 2009; Etesami and Beattie, 2018). The development and utilization of salt-tolerant PGPR are feasible measures for increasing crop yield under salt stress (Sáenz-Mata

et al., 2016). PGPR are involved in various biological processes such as the mobilization of soluble phosphorus (Zhu et al., 2011); fixation of nitrogen (Yan et al., 2015); increase of antioxidant enzymes levels (Ullah and Bano, 2015); regulation of ion transport protein expression (Etesami, 2018); secretion of extracellular polysaccharides (Qin et al., 2016); and production of plant hormones, iron carriers, and ACC deaminases (Ji et al., 2020). PGPR can also alter the root structure, morphology, hydraulic conductivity, and hormonal status and can release various volatile compounds (such as glutamic acid, proline, and peptide) associated with stress accumulation infiltration, killing pathogens (Arora et al., 2013; Olanrewaju et al., 2017). These mechanisms are related to improvements in plant salt tolerance and an induced system tolerance (IST) (Kaushal and Wani, 2016; Etesami and Beattie, 2018). Rhizosphere soil is a dynamic and complex element in farmland ecosystems (Yang et al., 2016). Soil microorganisms are important drivers of plant diversity and productivity in terrestrial ecosystems (Van et al., 2008). It is roughly estimated that about 25% of plants are dependent on nitrogen-fixing bacteria in natural soils with poor nutrients (Van et al., 2006).

The diversity, relative abundance, and activity of microbial communities play a central role in soil organic matter decomposition, nutrient recycling, system stability, and anti-interference ability (Cardinale et al., 2006; Céline et al., 2007; Enwall et al., 2007; Quadros et al., 2012). However, salinity has a strong filtering effect on bacterial community, and the change of community salt tolerance is partly driven by the change of community composition (Rath et al., 2018). When inoculated with salt tolerant microorganisms, it is likely to cause the recovery of respiration and growth function of microbial community in the soil (Rath et al., 2019). The identification of plant-related bacteria, analysis of their interactions with their host, and knowledge regarding manners in which these interactions promote the survival of both organisms are critical in developing strategies for the protection of these plants (Ruppel et al., 2013).

The nitrogen cycle is one of the main nutrient cycles in terrestrial ecosystems. This nutrient cycle is a globally vital biogeochemical process mediated almost entirely by microorganisms in the environment (Wang et al., 2015b). Nitrogen fixation is performed by microorganisms in the natural nitrogen cycle (Levy-Booth et al., 2014). The chemical form and content of nitrogen play a central role in regulating the composition of the soil microbial community (Fierer et al., 2011; Giagnoni et al., 2015). Nitrogen-fixing bacteria (NFB) is a PGPR with the unique ability to fix N_2 from the atmosphere into ammonium cations (NH_4^+), which are available for plant uptake (Alfaro-Espinoza and Ullrich, 2015). The global

quantity of biological nitrogen fixation is estimated at approximately 2×10^{13} g N/year globally (Falkowski, 1997). Therefore, further understanding of the community structure of NFB in the crop rhizosphere soil would be valuable. All nitrogen-fixing microorganisms contain the *nifH* gene. The *nifH* gene encodes for the nitrogen-fixing protein ferritin, and the phylogeny of the *nifH* gene is consistent with 16S rRNA (Levy-Booth et al., 2014). Therefore, the *nifH* gene is an ideal target for studying nitrogen-fixing microorganisms (Raymond et al., 2004). High-throughput sequencing technology can be used to compare the diversity and relative abundance of microbial species among different samples in order to investigate mechanisms underlying microorganisms soil plant interactions (DeLong, 2002). Despite an increase in the use of microbial inoculants and numerous studies investigating PGPR, the effects of microbial inoculants on nitrogen-fixing microbial communities in the crop rhizosphere soil under salt stress are poorly understood.

Wheat is one of the most important cereals worldwide for human diet. Although wheat is extensively cultivated in the area of Yellow River Delta, its area of cultivation is limited due the excess of salinity and alkalinity of soils (Liu et al., 2017). The “Bohai sea granary programme” that was recently launched in China has for objective to better exploit the salt-alkaline land for cereal cultivation. Therefore, the purpose of the present study was (i) screen strains with high salt tolerance and prominent nitrogen-fixing and other life-promoting characteristics from saline alkali soil of the Yellow River Delta, (ii) to test their effects on wheat growth under salt stress, and (iii) to analyze the effects of salt stress on the community structure of NFB in wheat rhizosphere soil. These results may provide new data and insights into future screening of PGPR in the rhizosphere of plants under salt stress.

MATERIALS AND METHODS

Isolation and Characterization of the Strain

In this study, the soil used to isolate strains was collected from wheat rhizosphere soil in the secondary salinization area of the Yellow River Delta, Shandong Province of China (118°49'15"E, 37°24'31"N). Ten grams of soil was mixed in 100 ml of sterile water and serially diluted to 10^{-4} , plated on NA plates (8% NaCl), and incubated at $30^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 3 to 4 days. The obtained salt-tolerant strains were inoculated into a nitrogen-free JNFB agar plate and continuously cultured at 30°C for 5 days to observe the bacterial growth as the evidence of nitrogen-fixing activity (Döbereiner, 1995).

The bacterium was subcultured twice. Finally, the isolates were streaked onto NA medium. A glycerol stock solution (30% v/v) of the isolate was prepared and stored at -80°C for later use. Based on colony morphology differences, 10 isolates were identified. Next, we tested the nitrogen fixation activity of the isolates (Marciano et al., 2012) and selected the strain with the highest nitrogen fixation activity for pot experiment.

Primary characterization of the test organism was done by fundamental microbiological and biochemical tests including Gram staining, Voges-Proskauer test, citrate utilization test, oxidase, aerobism, starch hydrolysis, catalase, and nitrate reductase following standard methods (Prescott and Harley, 1999). Using the BIOLOG Microstation™ system (BIOLOG Inc., Hayward, CA), the GEN III orifice 96 was tested to confirm the carbon source utilization and chemical sensitivity of bacteria to Rifamycin SV, Minocycline, etc.

Amplification and Sequencing of 16S rRNA Genes

To identify the bacteria at the molecular level, the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACT-3') were used to amplify the 16S rRNA bacterial gene sequences *via* PCR (Lane, 1991). The 16S rRNA sequences obtained from the strains by PCR were compared to those from the NCBI database. Pairwise evolutionary distances between the 16S rRNA sequence of the HG-1 test isolate and its related bacterial strains were calculated. A phylogenetic tree was constructed by the Neighbor-Joining method using MEGA v.5.0 (Tamura et al., 2011). Neighbor-Joining and bootstrap analyses were performed with 1,000 bootstrap replications. The evolutionary distances were computed by the Maximum Likelihood method (Tamura et al., 2011). The 16S rRNA gene sequence of HG-1 was deposited in the NCBI database under the accession No. SUB6437962 Seq1 MN582993.

Bioassays for the Promotion of Growth and Enhancement of Salinity Tolerance Traits

To investigate activity of phosphorus and potassium dissolution, we tested whether the bacterial colonies on NBRIP and silicate solid medium produce a clear circle, if formed, it is an indication of positive result showing a dissolved phosphorus and potassium (Mehta and Nautiyal, 2001). The dissolution efficiency of phosphorus and potassium in the strain was quantitatively analyzed by methods described in Bray and Kurtz (1945) and Lü and Huang (2010). The assay method was used to detect the nitrogenase activity of the strain (Berge et al., 2002). The Salkowski analysis was used to analyze the content of IAA produced by the strain in a liquid culture containing L-tryptophan (0.5 mg/ml) within 48 h (Moses et al., 2015). The activity of ACC deaminase was determined with the method described in Penrose and Glick (2003). Strain culture was inoculated on chrome azurole S (CAS) agar plate and incubated at 30°C for 4–5 days. After the experimental period, we observed if an orange formation would be visible around the bacterial colonies to test whether the strain produced iron carriers (Schwyn and Nielsens, 1987).

The activated strain sample was inoculated in 50 ml LB liquid medium, and cultured at 28°C for 14 to 16 h. The fermentation liquid was then extracted and inoculated into another medium (soluble starch 100 g/L, soybean cake powder 18 g/L, magnesium sulfate 0.75 g/L, potassium dihydrogen phosphate 1.0 g/L, pH

5.5) in a ratio of 2% (V/V) for 7 days. The fermentation liquid was then isolated after shaking the culture at 30°C, 200 r/min. The collected liquid was then centrifuged at 4°C and 10,000 r/min, for 2 min, to isolate and collect the supernatant. The C18 solid phase extraction column was initiated by adding 6 ml of methanol and 6 ml of 10% methanol, respectively, and the collected supernatant was slowly injected into the column at a rate of 0.8 ml/min. After the sample was added, it was rinsed twice with 6 ml of 10% methanol, and the effluent was discarded. Finally, it was eluted twice with 5 ml of 80% methanol and the eluate was collected. The eluate was air-dried at room temperature (23°C ± 2°C), dissolved in methanol and transferred to 2 ml centrifuge tube, and stored in a 4°C incubator. An Agilent ZORBAX Eclipse Plus C18 column (250 × 4.6 mm, 5 µm) was used. The mobile phase was a binary mixed solvent of methanol-water (containing 0.2% glacial acetic acid) in a volume ratio of 2:3. The column temperature was set to 35°C, with a flow rate of 0.8 ml/min, the injection volume was 10 µl, and the detection wavelength was 254 nm. Gibberellin Acid (GA₃) and Zeatin (ZT) standards (purity > 98%) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd. The contents of GA₃ and ZT were calculated from the standard curve.

The activated colonies were scribed into LB medium, cultured at 3°C for 24 h, and the samples were extracted at 40°C by PDMS/DVB65µm solid-phase microextraction head for 30 min. The extraction head was transferred to GCMS-TQ8050 (Shimadzu, Japan). Rtx-5MS capillary column (60 m × 0.25 µm ID × 0.25 µm thickness film) was used. The initial column temperature was maintained at 40°C, after 3 min, the temperature was increased to 160°C at the rate of 8°C/min, maintained for 2 min, and then increased to 240°C at the rate of 15°C/min, maintained for 3 min. Total operation time was 28.33 min. Helium was used as carrier gas and the flow rate is kept at 1.50 ml/min. The mass spectra of unknown compounds were compared with NIST17 and NIST17s (National Institute of Standards and Technology) standard mass spectrometry libraries to determine the structure of the substance corresponding to the peaks. The ratio of peak area to total peak area is the relative content of each volatile component.

Plant Materials and Treatments

Wheat cv jimai 21 seeds (provided by the College of Agriculture, Shandong Agricultural University) were rinsed with clean water, soaked in 75% ethanol for 10 min, then soaked in 30% sodium hypochlorite for 30 to 60 s, rinsed with sterile water five to six times, and dried. The soil used for potted plants was obtained at 0 to 20 cm of depth in wheat fields in the Yellow River delta (118° 41'07"E, 37°17'17"N) (Dongying City, Shandong, China) in October 2018. A 2-mm sieve was used to remove rubble, roots, and other debris. We filled a clay pot with an inner diameter of 30 cm with 3.0 kg of soil per pot. We selected uniform wheat seeds and planted them in a pot, (10 grains per pot) watered them abundantly, and managed by conventional methods (Reddy and Sudhakara, 2014). When the wheat seedlings grew to about 5 cm, we choose 20 pots with plants with the same

growth rate. Among them, ten pots were inoculated with HG-1 bacterial suspension. The bacterial inocula were prepared using sterile Milli-Q water by re-suspending cells (10⁸ CFU/ml) harvested from nutrient broth and a volume of 20 ml suspension was poured around the roots of the seedlings in each pot. Ten pots were treated with the same amount of sterile Milli-Q water (20 ml) and marked as the blank control group, CK (Wei et al., 2017). Randomized complete block design was used in this study (Wang et al., 2018). After 30 days of cultivation, wheat plants were taken out of the pots and plant biomass was measured. Ten soil cores were randomly selected from pots with different treatments and the rhizosphere soil was carefully collected, completely homogenized using a 2-mm sieve, and stored at -80°C for microbial community structure analysis (Wang et al., 2018).

Soil Sampling and Analysis

Soil sample used for plant growth studies was analyzed for its various chemical properties. Soil and water were mixed in 1:2.5 ratio; pH and electrical conductivity (EC) was measured with pH meter and conductivity meter (Mettler Toledo, Switzerland). The organic carbon content in the soil was determined with a method described in Aj et al. (1934). Soil available phosphorus (Olsen P) was determined using the method proposed by Olsen et al. (1954). Available nitrogen and exchangeable potassium values were obtained by a method described in Jackson (1979). For ion analysis, 0.2 g of wheat rhizosphere soil was treated with 1 ml deionized water and 5 ml concentrated sulfuric acid overnight, and then the cooked liquid was fixed to 50 ml. Measurements were carried out on 1 ml of the solution, which was extracted and diluted 10 times. Na⁺, K⁺ and Ca²⁺ content was measured *via* inductively coupled plasma optical emission spectroscopy (ICP, Thermo Scientific™ iCAP™ 7000 Plus, USA) (Singh and Jha, 2016).

Biomass and Antioxidant Activity of Wheat Plants

Plant growth was measured using root length, shoot length, fresh weight, and dry weight (Singh and Jha, 2016). Total soluble sugar content was determined using the method described by Irigoyen et al. (2006), and the proline content in wheat leaves was determined using the previously described method by Bates et al. (1973). Lipid peroxidation levels were determined by measuring malondialdehyde (MDA) from thiobarbital acid (TBA) reaction using the method described in Hodges et al. (1999). After the non-specific absorbance was removed, MDA concentration was determined according to its molar extinction coefficient (155 nm⁻¹.cm⁻¹), and the result was expressed as mmol.g⁻¹ FW.

Determination of Ion Content of Wheat Plants

According to the method described by Singh and Jha (2016), 1 g of bud tissue was acquired from plants of each treatment and mixed with a solution of perchloric acid, sulfuric acid, and distilled water in the ratio of 10:1:2. The contents of Na⁺, K⁺,

and Ca^{2+} were determined using ICP. Each sample analysis was repeated three times to ensure its accuracy.

PCR Amplification and Illumina MiSeq Sequencing

Three rhizosphere soil samples were combined into one DNA sample, and we prepared three replicate DNA samples per experiment (eighteen rhizosphere soil samples total). The soil samples were sequenced by the Majorbio company (China). Soil DNA Kit (Omega, Bio-Tek, USA) was used to extract total soil DNA. PCR was done using the extracted genomic DNA as a template, with *nifHF* (AAAGGYGGWATCGGYAARTCCA CCAC) and *nifHR* (TTGTTSGCSGCRATCATSGCCATCAT) as primers. The PCR reaction system included: 5 × FastPfu Buffer (4 µl), 2.5 mM dNTPs (2 µl), 0.8 µl of each for forward and reverse primers (5 µM), FastPfu Polymerase (0.4 µl), BSA (0.2 µl), template DNA (10 ng), and ddH₂O was added to 20 µl. The PCR procedure for the amplification of *nifH* gene was as follows: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 30 s, annealing at 55°C for 30 s, 37 cycles of extension at 72°C for 45 s, and a final extension at 72°C for 10 min. Purified PCR products were quantified by Qubit[®] 3.0 (Life Invitrogen) and every twenty-four amplicons whose adapters and barcodes were different were mixed equally. The pooled DNA product was used to construct Illumina Pair-End library following Illumina's genomic DNA library preparation procedure. Then the amplicon library was paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd) according to the standard protocols.

Processing of Illumina MiSeq Sequencing Data

The QIIME 1.17 software was used to analyze and screen the sequencing results. The sequences with poor sequencing quality were removed and the sequence length was screened. The sequences were determined to be the final sample sequences according to barcode. Data decontamination method and parameters were: 1. the bases were filtered with a mass value below 20 at the end of the read with a window of 50 bp. If the average mass value in the window was lower than 20, we cut off the back-end bases from the window and filtered the read below 50 bp after quality control; 2. According to overlap relation between PE reads, pairs of reads were merged into a sequence with the minimum overlap length of 10 bp; 3. The maximum allowed error matching ratio in the overlapping region of the splicing sequence was 0.2, and the non-conforming sequences were screened; 4. We differentiated the samples according to barcode and primer at both ends of the sequence and adjusted the direction of the sequence. The allowed barcode mismatches equaled 0 and the maximum primer mismatches equaled 2.

In the HG-1 group and the CK, MiSeq sequencing of *nifH* genes resulted in 109,119 and 152,540 high quality and chimera-free reads, respectively. The high-quality sequences were clustered into operational taxonomic unit (OTU) by the Usearch software (version 7.1). The non-repeating sequences (excluding single sequences) were clustered into OTUs according

to 97% similarity, and the representative sequences of OTU were obtained by removing chimeras in the clustering process. The classification information of each OTU was obtained by annotating 97% similar OTU representative sequences with RDP classifier Bayesian algorithm. The Mothur software (version 1.30.1) was used to evaluate Chao, Ace, and Shannon indices at around 97%. Principal component analysis (PCA) based on Bray-Curtis distances was used to analyze the overall structural changes of NFB communities in rhizosphere soil of wheat inoculated and uninoculated with HG-1 strain. Species abundance of each sample was calculated at different taxonomic levels. The community composition of control and HG-1 species was presented in the community pie chart. The species whose abundance accounted for less than 0.001 in all samples were classified as "others." All raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SUB6522639).

Statistical Analysis

All experiments were performed in triplicates. The SPSS 19.0 software (IBM, Chicago, USA) was used for one-way ANOVA of parameters of plants and soil. Significance was calculated by Student's t-test. Differences in mean values were considered significant when $P < 0.05$. Differences between NFB on the phylum level in CK and HG-1 samples were assessed using two-tailed Student's t-tests.

RESULTS

Isolation, Biochemical Characterization, and Identification of HG-1

Based on colony morphology, 10 strains of bacteria capable of growing on nitrogen free JNfb agar plate were isolated from the soil sample of the Yellow River delta. Nine strains were gram-positive and HG-1 was gram-negative. The nitrogen-fixing activity of HG-1 strain was measured quantitatively, and the nitrogen-fixing activity of HG-1 strain was the highest (13.105 ± 0.858 mg N/g glucose) (Figure 1). The measurements of the

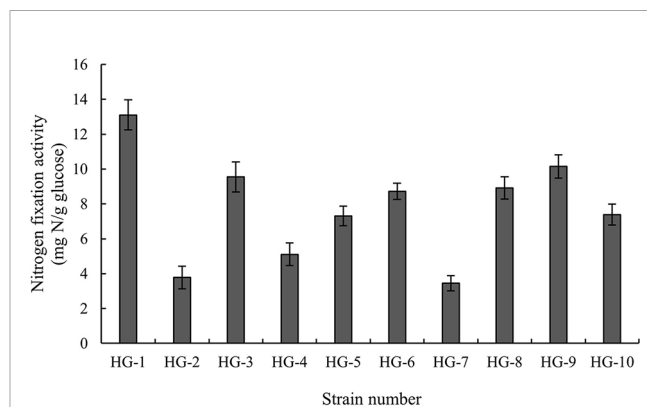


FIGURE 1 | The results of nitrogen fixation activity determination of the isolates.

physiological and biochemical indices of the isolated HG-1 strain revealed that the strain was a facultative anaerobe. The results of oxidase, starch hydrolysis, and urease tests were negative, whereas those of catalase, nitrate reduction, V-P, 5% NaCl, 10% NaCl, gelatin liquefaction, and citrate utilization tests were all positive. The isolated strain could utilize salicin, L-rhamnose, and inosine among other compounds. The complete list is presented in **Table 1**. Furthermore, the results of the antibiotic sensitivity analysis revealed that the HG-1 strain was resistant to rifamycin SV, troleandomycin, 1% sodium lactate, lincomycin, guanidine HCl, tetrazolium blue, tetrazolium violet, and vancomycin (**Table 1**). A 16S rDNA sequence alignment demonstrated that the strain belonged to the species *Enterobacter cloacae*. The phylogenetic tree constructed using MEGA software is presented in **Figure S1**. The salt-tolerant strains of saline soil were screened out using an 8% NaCl NA medium. The strains were then cultured in a JNFB-AGAR plate (no nitrogen source), silicate, or NBRIP media. We found that *Enterobacter cloacae* HG-1 grow normally in JNFB-AGAR plate (no nitrogen source). We further detected that the HG-1 strain formed transparent areas in both NBRIP and silicate media.

Plant Growth Promoting Features

This finding indicated that the strain could fix nitrogen and dissolve phosphorus and potassium. Among the plant growth-promoting (PGP) traits, the test organism produce IAA, GA₃, ZA, iron carrier and ACC deaminase (**Table 2**).

The metabolites of the HG-1 strain were obtained through gas chromatography, and volatile organic compounds (VOCs) with a peak area above 0.15% were analyzed. The results revealed the presence of PGP substances, including 2, 3-butanediol, [R-(R*.R*)] - (9.26%) and 1-hexanol (0.15%). Moreover, the HG-1 strain was determined to produce substances, such as phenethyl alcohol (2.19%), 2-undecanone (2.15%), 2-nonanone (1.20%), 2-nonanol (1.04%), 2-tridecanone (0.96%), 2-heptanone (0.38%), phenol (0.33%), and 2-pentadecanone (0.27%), that inhibit the growth of pathogens (**Table 3**).

Effect of HG-1 Inoculation on Soil Physicochemical Properties

The physical and chemical properties of soil are closely related to crop growth. After 30 days of planting the wheat, The pH, and K content were significantly lower than original soil ($P < 0.05$). The EC, organic C, Na and Ca content significantly higher than original soil ($P < 0.05$). After 30 days of inoculation with the HG-1 strain, the physical and chemical properties of soil as well as its nutrient contents were significantly altered. The pH (an increase of 2.40%) and EC (an increase of 7.92%) of uninoculated soil were significantly higher than those of inoculated soil ($P < 0.05$). The Olsen P (an increase of 29.39%), available N (an increase of 18.46%), exchangeable K (an increase of 10.96%), and organic C (an increase of 14.19%) were significantly higher in the inoculated soil than those in the uninoculated soil ($P < 0.05$) (**Table 4**).

Effect of HG-1 Inoculation on Plant Growth Under NaCl Stress

The effect of HG-1 inoculation on wheat biomass indices under salt stress was characterized by performing independent sample t-tests ($P < 0.05$). Results revealed that wheat growth under salt stress was significantly increased in plants inoculated with the HG-1 strain compared with control plants. In inoculated plants, the root length increased by 19.15% ($P < 0.05$), shoot length increased by 18.83% ($P < 0.05$), fresh weight (FW) increased by

TABLE 2 | Plant growth-promoting traits of HG-1.

Plant growth promoting properties	Activity
Nitrogen fixation	13.105 ± 0.858 mg N/g glucose
Phosphate solubilizing	74.298 ± 7.236 µg/L
Potassium solubilizing	9.514 ± 1.317 µg/ml
IAA production	21.652 ± 0.925 µg/ml
GA ₃	0.358 ± 0.009 µg/ml
ZA	1.364 ± 0.018 µg/ml
Siderophore production	+
ACC deaminase production	35.047 ± 2.317 µmol/(mg·h)

Data are means ± standard deviation (SD) (n = 3).

TABLE 1 | Biochemical and physiological characteristics of HG-1.

Characteristic	Result	Carbohydrate	Utilization	Carbohydrate	Utilization	Chemical Sensitivity Assays	Reaction
Grams stain	–	Dextrin	○	Salicin	+	Rifamycin SV	+
Oxidase	–	D-Maltose	+	N-Acetyl-D-Glucosamine	+	Minocycline	–
Aerobism test	Facultative anaerobic	D-Trehalose	+	N-Acetyl-β-D-Mannosamine	+	D-Serine	○
Catalase test	+	D-Cellobiose	+	N-Acetyl-D-Galactosamine	+	Troleandomycin	+
Nitrate reductase	+	Gentiobiose	+	α-D-Glucose	+	1% Sodium Lactate	+
Starch hydrolysis	–	Sucrose	+	D-Mannose	+	Lincomycin	+
V-P test	+	Turanose	–	D-Fructose	+	Guanidine HCl	+
5% NaCl	+	Stachyose	+	D-Galactose	+	Tetrazolium Blue	+
10% NaCl	+	D-Raffinose	+	3-Methyl-D-Glucose	–	Tetrazolium Violet	+
Gelatin liquefaction test	+	α-D-Lactose	○	L-Fucose	○	Nalidixic acid	–
Citrate utilization test	+	D-Melibiose	+	L-Rhamnose	+	Vancomycin	+
Urease	–	β-Methyl-D-Glucoside	+	Inosine	+	Lithium Chloride	○

+, positive; – negative; ○, critical.

TABLE 3 | Main volatile substances of HG-1 strain and their functions.

Peak Number	VOCs	Function	Peak area %	References
11	2,3-Butanediol, [R-(R*,R*)]-	Promote plant growth	9.26	(Ryu et al., 2003; Farag et al., 2006)
38	Phenylethyl Alcohol	Inhibit microbial growth	2.19	(Mo and Sung, 2007)
45	2-Undecanone	Inhibit microbial growth	2.15	(Yuan et al., 2012; Melkina et al., 2017)
36	2-Nonanone	Inhibit microbial growth	1.20	(Yuan et al., 2012)
37	2-Nonanol	Inhibit microbial growth	1.04	(Abarca et al., 2017)
55	2-Tridecanone	Inhibit microbial growth	0.96	(López-Lara et al., 2018)
23	2-Heptanone	Inhibit microbial growth	0.38	(Melkina et al., 2017)
26	Phenol	Inhibition of fungal growth	0.33	(Yuan et al., 2012)
64	2-Pentadecanone	Inhibit bacteria growth	0.27	(Giorgio et al., 2015)
20	1-Hexanol	Promote plant growth	0.15	(Blom et al., 2011)

TABLE 4 | Effects of *Enterobacter cloacae* HG-1 on chemical properties of rhizosphere soil.

Treatment	pH	EC ($\mu\text{S}/\text{cm}^{-1}$)	Olsen P (mg/kg)	Available N (mg/kg)	Exchangeable K (mg/kg)	Organic C (g/kg)	Na(g/kg)	K(g/kg)	Ca(g/kg)
Original soil	8.62 \pm 0.02a	404 \pm 8.04c	8.59 \pm 0.28b	62.57 \pm 1.69b	337.07 \pm 6.33b	19.07 \pm 0.38c	1.08 \pm 0.05b	0.61 \pm 0.02a	0.24 \pm 0.03c
CK	8.55 \pm 0.03b	504 \pm 8.04a	8.95 \pm 0.47b	68.41 \pm 4.59b	347.02 \pm 6.13b	22.70 \pm 0.31b	2.09 \pm 0.04a	0.16 \pm 0.01c	0.41 \pm 0.03b
HG-1	8.35 \pm 0.01c	467 \pm 3.56b	11.58 \pm 1.16a	81.04 \pm 3.90a	385.04 \pm 6.94a	25.92 \pm 0.58a	2.02 \pm 0.05a	0.24 \pm 0.02b	0.55 \pm 0.02a

Values are means \pm SD. Different lowercase letters in the table represent the test results of significance of difference between uninoculated and inoculated HG-1 soil indicators, $P < 0.05$.

TABLE 5 | Effects of *Enterobacter cloacae* HG-1 on wheat biomass, antioxidant and ion absorption.

	Root length (cm)	Shoot length (cm)	FW(g)	DW(g)	Soluble sugar ($\mu\text{g g}^{-1}$ FW)	Total protein (mg g ⁻¹ FW)	Proline ($\mu\text{mol g}^{-1}$ FW)	MDA(mmol g ⁻¹ FW)	Na ⁺ (mg g ⁻¹ DW)	K ⁺ (mg g ⁻¹ DW)	Ca ²⁺ (mg g ⁻¹ DW)
CK	12.74 \pm 0.51b	16.25 \pm 0.48b	1.44 \pm 0.05b	0.167 \pm 0.004b	2.88 \pm 0.06a	3.35 \pm 0.17b	7.24 \pm 0.26b	10.97 \pm 0.58a	1.75 \pm 0.08a	1.45 \pm 0.08b	0.73 \pm 0.05b
HG-1	15.18 \pm 0.45a	19.31 \pm 0.43a	1.68 \pm 0.03a	0.197 \pm 0.008a	3.01 \pm 0.08a	4.35 \pm 0.16a	8.14 \pm 0.21a	7.98 \pm 0.21b	1.20 \pm 0.11b	1.75 \pm 0.09a	1.15 \pm 0.10a

Values are means \pm SD. The small letters in the table represent the significant difference between the indexes of uninoculated and inoculated HG-1 wheat, $P < 0.05$.

16.67% ($P < 0.05$), and dry weight (DW) increased by 17.96% ($P < 0.05$) compared with control plants (Table 5).

Factors that affect plant osmotic regulation, such as the total contents of protein, proline, soluble sugar, and malondialdehyde (MDA), were measured to assess the effects of HG-1 inoculation on wheat resistance to salt stress. In inoculated plants, the soluble sugar content of wheat leaves increased by 4.51% ($P < 0.05$), the total protein content increased by 29.85% ($P < 0.05$), and the proline content increased by 12.43% ($P < 0.05$) compared with those in uninoculated plants. We then compared the MDA level in investigated plants because MDA levels reflect the level of lipid oxidative damage induced by salt stress. The MDA levels in inoculated plants decreased by 27.26% ($P < 0.05$) when compared with those in the uninoculated plants (Table 5).

To investigate the role of the HG-1 strain in alleviating wheat ion stress, we measured differences in Na⁺, K⁺, and Ca²⁺ concentrations in inoculated and uninoculated wheat seedlings. The ion analysis results revealed a difference in the ion concentrations of inoculated and uninoculated plants. The Na⁺

concentration in inoculated wheat seedlings was significantly lower than that in uninoculated seedlings (31.43%, $P < 0.05$), whereas the concentrations of K⁺ (20.69%) and Ca²⁺ (57.53%) were significantly higher in the inoculated plants than those in the uninoculated plants ($P < 0.05$) (Table 5).

Microbial Diversity Response to HG-1 Inoculation in the Wheat Rhizosphere Soil

Miseq sequencing technology was used to sequence the *nifH* gene of the nitrogen-fixing organism, and a total of 261,659 original sequences with an average length of 398.31 bp were identified from six samples. After the screening, 136,578 valid sequences were obtained. A 97% similarity check revealed 799 operational taxonomic units (OTUs), which were obtained using an OTU analysis of nonrepeating sequences. We determined that CK and HG-1 shared 319 OTUs, 277 OTUs were identified in CK alone, and 203 OTUs were identified in HG-1 alone (Figure 2A). The microbial diversity index gradually increased with the number of sequencing bars, and the dilution curve

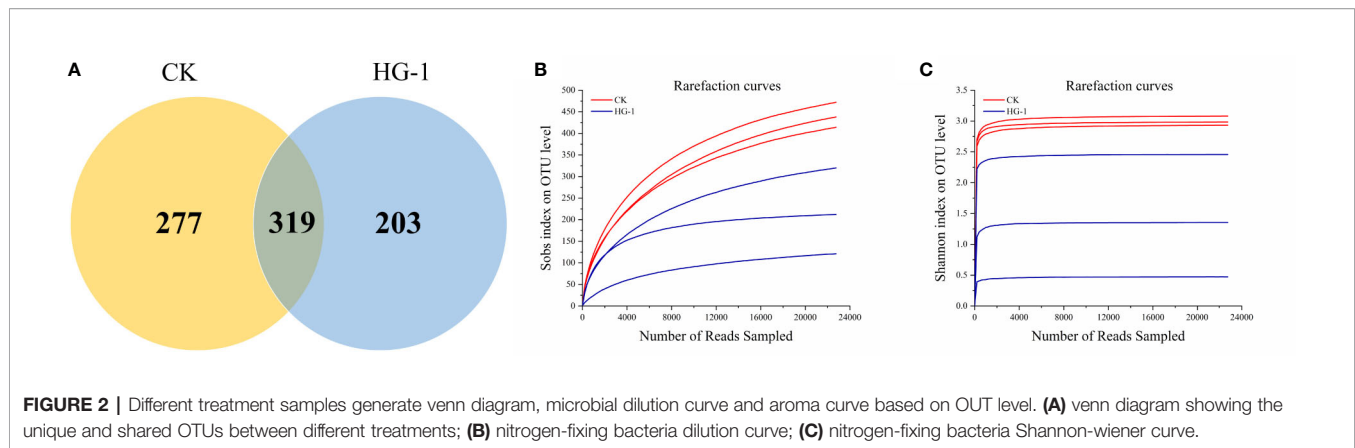


FIGURE 2 | Different treatment samples generate venn diagram, microbial dilution curve and alpha curve based on OTU level. **(A)** venn diagram showing the unique and shared OTUs between different treatments; **(B)** nitrogen-fixing bacteria dilution curve; **(C)** nitrogen-fixing bacteria Shannon-wiener curve.

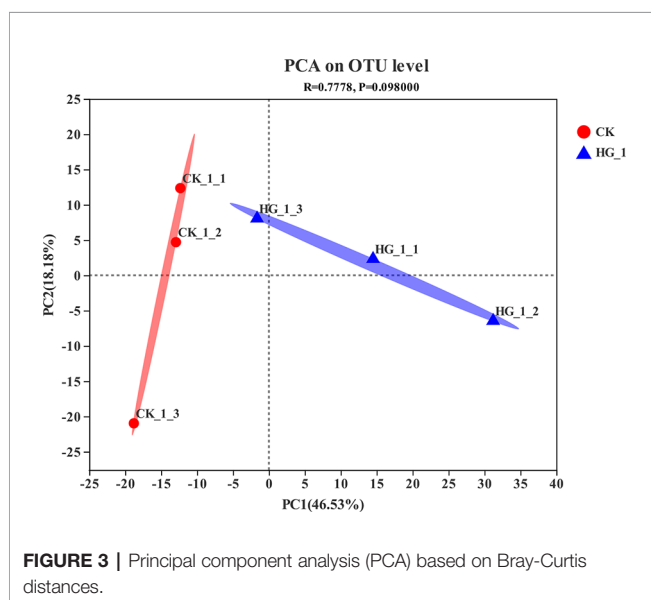


FIGURE 3 | Principal component analysis (PCA) based on Bray-Curtis distances.

became smooth at the last stage, which indicated that sequencing data reached saturation (Figures 2B, C), and thus that this study could cover most species of the nitrogen-fixing bacterial community in wheat rhizosphere soil.

The richness, diversity and coverage of NFB communities in wheat rhizosphere soil were analyzed by the diversity index. The Sobs, Chao and Ace indices reflect the richness of the community in the sample. The results in Figures 2A, B and Table 6 showed that compared with the control, the inoculation of HG-1 strain significantly reduced the richness of NFB community in wheat rhizosphere soil ($P < 0.05$) (Table 6). Simpson, Shannon index

reflects the community diversity in the sample. The results of diversity analysis showed that compared with the control, the inoculation of HG-1 strain did not significantly affect the diversity of NFB community in rhizosphere soil ($P < 0.05$) (Table 6).

The results of PCA showed that the NFB communities in the wheat rhizosphere soil were significantly separated under HG-1 and CK treatment, and ANOSIM analysis further confirmed the significant structural reorganization of the NFB communities in the soil (Figure 3).

Microbial Communities Response to HG-1 Inoculation in the Wheat Rhizosphere Soil

Figure 4 illustrates differences in the composition of NFB communities in inoculated and uninoculated samples at the phylum and genus levels. No differences in the community composition were observed at the phylum level among the investigated samples. However, after inoculation, the abundance of *Proteobacteria* increased from 37.69% to 75.86% and the abundance of *Verrucomicrobia* decreased from 13.34% to 1.98% (Figures 4A, B). At the genus level, in the inoculated samples, the abundance of *Geobacter* and *Azoarcus* decreased from 1.64% to 0.34% and from 8.87% to 2.26%, respectively. The R language software vegan package was employed. Figure 4 illustrates differences identified in each sample for 6 phyla (Figure 4A) and 32 genera (Figure 4B). The dominant phylum and genus of NFB differed between inoculated and uninoculated plants. The abundance of *Azoarcus*, *Rhodopseudomonas*, *Bradyrhizobium*, *Cyanothece*, *Sinorhizobium*, *Azotobacter*, *Anaeromyxobacter*, and *Vibrio* was lower in inoculated soil than in CK samples, whereas *Azospirillum*, *Anabaena*, and five other genera were only recorded in CK samples.

TABLE 6 | Diversity index of nitrogen fixing bacteria in wheat rhizosphere soil samples under different treatments.

Sample	sobs	Shannon	Simpson	ace	chao	coverage
CK	441.33 ± 23.80a	3.00 ± 0.06a	0.18 ± 0.01a	519.82 ± 22.29a	502.69 ± 24.93a	0.9955 ± 0.000170a
HG-1	217.67 ± 81.34b	1.43 ± 0.81a	0.57 ± 0.27a	256.99 ± 99.57b	256.69 ± 100.41b	0.9979 ± 0.001207a

Sobs, chao and ace value are indicators of community richness. Shannon, Simpson and coverage are indicators of community diversity. Values are mean ± SD ($n = 3$). The small letters in the table represent the significant difference between the indexes of uninoculated and inoculated HG-1 wheat, $P < 0.05$.

Results revealed that the average relative abundance of Proteobacteria in the inoculated samples increased significantly ($P \leq 0.05$), and the average relative abundance of Verrucomiobacteria was significantly decreased ($P \leq 0.01$) compared with that in CK samples (Figure 5A). Moreover, the differences in the abundance of 27 common bacterial genera were compared. The mean relative abundance of *Azoarcus*, *Geobacter*, *Sinorhizobium*, *Skermanella*, *Azohydromonas*, and *Mesorhizobium* was significantly lower in inoculated samples than in CK samples ($P \leq 0.05$) (Figure 5B).

DISCUSSION

PGPR can induce plant systemic tolerance (IST) by increasing the concentrations of dissolved phosphorus, ACC deaminase activity, volatile substances, iron carriers, and plant hormones (Frag et al., 2013; Kumari S. et al., 2015). Some studies have shown the potential of *E. cloacae* to be a plant growth promoter and its characteristic of salt tolerant (Bhise et al., 2016; Macedo-Raygoza et al., 2019). In the present study, ten strains were isolated from alkali-saline soil samples and screened to obtain *E. cloacae* HG-1 that had high salt tolerance. The physiological and biochemical and PGP characteristics were measured. We determined that the strain was resistant to antibiotics, including rifamycin SV, troleandomycin, 1% sodium lactate, and lincomycin. Furthermore, the analysis revealed that the strain had characteristics related to processes of nitrogen fixation and the dissolving of phosphorus and potassium and could produce IAA, GA₃, ZA, iron carriers, and ACC deaminases. Therefore, the strain HG-1 was determined to have the potential to promote plant growth. *E. cloacae* has

high nitrogen fixation activity. Numerous studies have shown that *E. cloacae* seems to be not virulent. Based on analyses of *in vitro* hemolysis of red blood cells and antibiotic resistance, *E. cloacae* has no hemolytic activity. At the same time, this strain not only exists widely in rhizosphere soil, but also can be detected and isolated in plants (Hinton and Bacon, 1995; Wang et al., 2012; Macedo-Raygoza et al., 2019). Under both biotic and abiotic stress, it has a growth promotion effect on cowpea, wheat, pea, citrus, corn, banana and other crops and thus is considered a promoter for plant growth (Hinton and Bacon, 1995; Araújo et al., 2002; Kumaran et al., 2010; Ramesh et al., 2014; Khalifa et al., 2016; Macedo-Raygoza et al., 2019). Moreover, *Enterobacter* strains were determined to have various other PGP properties. For example, Liu isolated 11 strains of nitrogen-fixing bacteria from the rhizosphere of the salt-tolerant plant sunflower (*Helianthus tuberosus*) in the Yellow River area, China, and tested the strains for nitrogen-fixing, phosphorus-dissolving, and IAA-producing activities. Among them, *Enterobacter* sp.N10 can significantly increase the root length and plant height of sunflower and wheat, and is the best rhizobia to increase wheat yield (Liu et al., 2017). Also Li et al., have shown that among the 8 strains of salt-tolerant bacteria, *E. cloacae* HSNJ4 can promote the germination of rape seeds, balance the relative content of IAA and ethylene in seedlings, increase root length, stem length, lateral root content and chlorophyll content, and improve its salt tolerance (Li et al., 2017). *E. cloacae* MDSR9, isolated from soybean rhizosphere, was reportedly capable of producing IAA, iron carriers, ammonia, ACC deaminase, soluble phosphorus, potassium, and zinc. Wheat inoculated with this strain displayed an increase in seedling and seed weight of 39.13% and 49.14%,

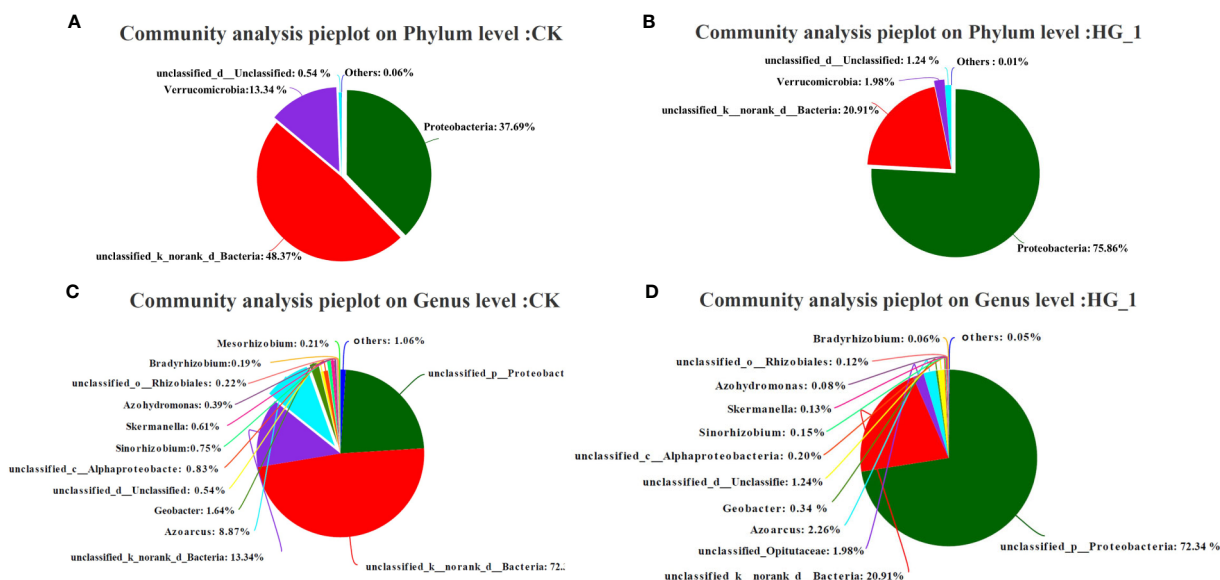


FIGURE 4 | Relative abundance of nitrogen fixing bacteria in wheat rhizosphere soil at phylum and genus level. (A, B) relative abundance of nitrogen-fixing bacteria in wheat rhizosphere soil of control group at phylum level, and (C, D) relative abundance of nitrogen-fixing bacteria in wheat rhizosphere soil inoculated with HG-1 strain at genus level. Different colors represent different species, and pie area represents the percentage of the species.

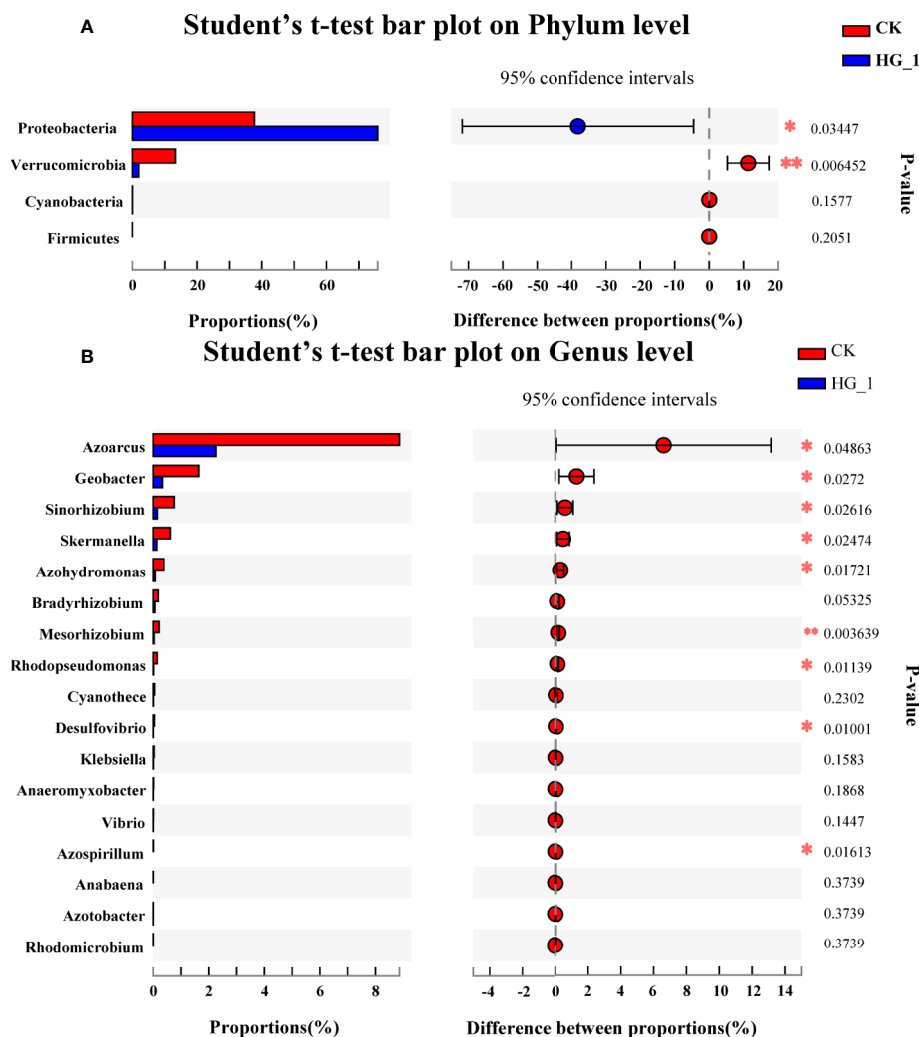


FIGURE 5 | Difference test on (A) phylum and (B) genus level of nitrogen-fixing bacteria in wheat rhizosphere soil under different treatments. The vertical axis represents the species names at a certain level of classification, and each column corresponding to the species represents the average relative abundance of the species in various groups, with different colors representing different groups. The intermediate region is the difference of the percentage of species abundance between the two groups within the confidence interval set. The color of the dot is the group color with a relatively large proportion of species abundance. The I-type interval on the dot is the upper and lower limit of the difference. Error bars indicate standard errors ($n=3$). The rightmost is P value, $*0.01 < P \leq 0.05$, $**0.001 < P \leq 0.01$.

respectively (Ramesh et al., 2014). Furthermore, after inoculation with *Enterobacter*, the expression of salt stress response genes related to proline biosynthesis in *Arabidopsis* was upregulated (Kim et al., 2014).

The HG-1 strain can improve the physical and chemical properties of saline soil. PGPR are principal biological factors controlling plant growth through the adsorption and desorption of ions and the transformation of nutrients and their availability to crops (Schloss and Jo, 2006). Although the present study is only a preliminary analysis of the effect of PGPR on the rhizosphere soil, numerous studies have determined that the application of microbial agents is also an effective method to significantly reducing soil pH and salinity, accelerate the management of saline-alkali soil, and improve the survival rate

of plants in saline-alkali soil (Mbarki et al., 2017). This type of biological management method has a quick effect on the plant growth and is sustainable, low-cost, and does not generate any pollution. Therefore, for the use of microbial agents has great potential to grow plants in saline-alkali soil (Dodd and Pérez-Alfocea, 2012; Paul and Lade, 2014; Nabti et al., 2015; Shrivastava and Kumar, 2015). A high concentration of salt in soil can inhibit plant growth because large numbers of sodium ions affect the nutrient utilization rate of plants and inhibit the activity of various enzymes. Moreover, salt stress affects plant growth by disrupting water balance, causing oxidative stress and ethylene production (Nakbanpote et al., 2014). In this study, soil pH and EC were significantly decreased after the inoculation of soil with HG-1, whereas the available phosphorus, available N,

exchangeable K, and organic C in soil increased, which improved the nutritional environment of wheat and reduced the effects of salt stress. The improvement of the soil environment may also increase the adaptability and activity of microorganisms. Biological regulation is an effective measure to improve soil quality and crop yield by promoting the circulation and transformation of soil nutrients and enhancing plants' absorption of nutrients (Mayak et al., 2004; Egamberdieva et al., 2017). Inoculating plants with these microorganisms can improve soil micro-ecological environments (Lerner et al., 2006; Fließbach et al., 2009).

The HG-1 strain can reduce ion toxicity, osmotic stress, and oxidative damage to plants under salt stress. A study found that *E. cloacae* ZNP-3 can produce ACC deaminase along with several other properties namely IAA production, mineral phosphate solubilization, hydrogen cyanide (HCN) and ammonia production. Its inoculation to wheat plant resulted in a considerable increase in growth parameters, biomass, and chlorophyll content under salinity stress. The inoculation also decreased the accumulation of Na^+ and increased K^+ uptake in shoots and roots, leading to maintenance of favorable K^+/Na^+ ratios in bacterial-treated plants for alleviating the toxic effect of salt stress (Singh et al., 2017). IAA plays a crucial role in the differentiation of plant cells, tissues, and new organs. The IAA concentration in crops was significantly reduced after salt stress; for instance, the IAA concentration was decreased by approximately 75% in tomato plants (Dunlap and Binzel, 1996). Furthermore, when the isolates of salt-tolerant IAA strains *B. endophyticus*, *B. tequilensis*, and *Planococcus* were re-inoculated into salt-tolerant grass, the germination rate of grass increased by 7%–11%, the shoot length increased by 13%–22%, the root length increased by 44%–57%, and the FW increased by 21%–54% compared with uninoculated plants (Zhao et al., 2016). Gibberellin (GA) is involved in several stages of plant growth and development, including seed germination, leaf growth, photolithogenesis, shoot elongation, flower organ development, and fruit maturation. GA also plays an active role in cell division and elongation and in the regulation of hypocotyl, root, and leaf meristem size (Sun and Gubler, 2004; Daviã-Re and Achard, 2013; Wang G.L. et al., 2015). GA_3 is a bioactive GA, which can significantly improve the germination rate of seeds and the water utilization rate of plants under low salt stress (Maggio et al., 2010), as well as can significantly improve the growth condition of plants grown under high salt stress (Javid et al., 2011). The inoculation of rice with *Bacillus amyloliquefaciens*, which produces GA_3 , can increase the salicylic acid concentration in rice, upregulate the expression of endogenous GA-related genes, and significantly promote plant growth (Shahzad et al., 2016). GA produced by *Bacillus* sp. and *Azospirillum* sp. was determined to increase plant nitrogen uptake (Kucey, 2010). CKs mainly regulate plant cell division, apical dominance, chloroplast biogenesis, nutrient regulation, leaf senescence, tube tissue differentiation, light signal conduction development, bud differentiation, and anthocyanin production. CKs also participate in the formation of plant resistance to biotic and abiotic stress (Hwang and Sheen, 2001;

O'Brien and Eva, 2013). For instance, when *Platycladus orientalis* was inoculated with *B. subtilis*, thereby producing CKs, the CK concentration in the bud increased and the resistance to osmotic stress was stronger in inoculated plants than in uninoculated plants (Liu et al., 2013). In this study, the HG-1 strain produced the plant hormones IAA, GA_3 , and CKs. Our results indicated that the investigated strain could promote the growth and development of wheat seedlings; improve the accumulation of wheat biomass; and positively promote plant height, root length, FW, and DW.

Potassium ions, one of the three main elements in crop nutrition, are ubiquitous in crops. They are involved in almost all physiological and biochemical processes of plants and extensively affect the growth and metabolism of crops. Potassium and sodium have competing regulatory actions on osmotic potential (Ashraf et al., 2005). K^+/Na^+ in plants is a valuable indicator of their salt tolerance. Studies have indicated that K^+ can enhance the salt tolerance of crops (Ruppel et al., 2013). As essential mineral elements, calcium ions are crucial regulators of plant growth and development and are vital components of the plant cell wall (Hepler, 2005). Calcium ions are a protective permeable substance in the vacuole that maintain cell membrane stability and intracellular ion balance. Maintaining the balance of calcium ions in plant cells is crucial in the normal growth of plants (Al-Whaibi et al., 2010; Dayod et al., 2010; Dodd et al., 2010; Gilliam et al., 2011). Proline may play a role in osmoregulation as a crucial component of antioxidant defense during water scarcity, which protects macromolecules and participates in the pentose phosphate pathway (Hare and Cress, 1997; Miller et al., 2010). To date, no accurate conclusion has been drawn regarding the role of PGPR in plant nutrient absorption, transport, accumulation, and calcium ion removal under salt stress. Therefore, studying the mechanisms of K^+ , Ca^{2+} , and proline under salt stress from multiple perspectives can help improve plant growth and development. In this study, the inoculation of wheat with the HG-1 strain significantly reduced the accumulation of Na^+ in the leaves, which might have reduced Na^+ toxicity in wheat and increased K^+ and Ca^{2+} , thereby maintaining the ion balance. Furthermore, a significant increase in the proline content was detected, indicating that inoculation with HG-1 enhanced the osmotic regulation ability of wheat. Furthermore, K^+ and Ca^{2+} in wheat leaves were significantly higher in inoculated plants than in uninoculated (control) plants ($P < 0.05$), whereas Na^+ accumulation was significantly lower ($P < 0.05$). These factors are critical for preserving the function of biological macromolecules, preventing enzyme inactivation, maintaining physiological activity such as photosynthesis, and increasing the tolerance of crops to salt stress (Furusho et al., 2005).

High salinity also leads to increased production of reactive oxygen species (ROS), which can damage the integrity of plant cell membrane systems and affect fatty acids, amino acids, pigments, and other biological molecules in plants (Miller et al., 2010). The MDA level can be used to characterize the degree of oxidative damage caused by ROS (Jain et al., 2001). In this study, the MDA level was significantly lower in plants inoculated with HG-1 than in uninoculated plants, indicating that inoculation with the HG-1 strain could reduce the oxidation

of membrane lipids, proteins, and DNA, thereby reducing oxidative damage in plants.

Excessive ethylene production under salt stress can inhibit root growth, and higher ethylene levels in the nodules can reduce the quantity of fixed N (Ma et al., 2002). Reducing stress-induced increases in ethylene levels is beneficial to plant growth (Glick, 2004). Producing ACC deaminase and catalyzing the conversion of ACC (a precursor in ethylene biosynthesis) into ammonia and alpha-ketobutyrate are crucial functions of PGPR, which reduce ethylene levels in plants under salt stress (Senthilkumar et al., 2009; Etesami and Beattie, 2017). Penrose and Glick (2003) reported that ACC deaminase activity was higher than 20 nmol α -KB mg^{-1} protein h^{-1} , which could improve the salt tolerance of plants. The ACC deaminase activity of HG-1 was up to 35.047 ± 2.317 $\mu\text{mol } \alpha\text{-KB } \text{mg}^{-1}$, which indicated that HG-1 could improve the salt tolerance of plants.

VOCs have relatively low molecular weight and are hydrophobic and volatile at room temperature and pressure, which allows them to be readily dispersed in the atmosphere and soil (Hung et al., 2015). Several studies have investigated the volatile metabolites of specific strains. Salme et al. (2014) proposed an effective new method to test VOC strains to promote plant growth and improve plant stress tolerance. Improving our understanding of the metabolites of bacterial strains could solve the problem of food security caused by climate change. For example, *Alcaligenes faecalis* JBCS1294 can reprogram auxin and GA to increase plant salt tolerance by producing adipic acid, butyric acid, and other volatiles (Bhattacharyya et al., 2015). In the present study, the VOCs of salt-resistant *E. cloacae* HG-1 were detected. We determined that various substances could inhibit the growth of pathogenic bacteria, common fungi, and bacteria caused by the soil-borne disease control effect. However, multiple VOCs exhibit synergistic effects, and determining components involved in IST is difficult before isolating each volatile compound for analysis.

The expression “microbiome” can describe the complex and dynamic genetic content of a microorganism living in a specific habitat (Bulgarelli et al., 2013; Reinhold et al., 2015). The role of the rhizosphere soil microbial community in maintaining plant health and improving plant adaptability is being increasingly recognized as the knowledge of this field increases. The related microbial community is considered the second genome of plants (Berendsen et al., 2012; Turner et al., 2013). Therefore, the study of the microbial community structure and diversity in rhizosphere soil is valuable in understanding the mechanism of PGPR and in evaluating the optimal measures for increasing plant salt tolerance. The diversity of soil microbial community is closely related to crop growth and the prevention and control of soil-borne diseases (Miransari, 2013; Sui et al., 2019).

The NFB community in crop rhizosphere soil is a functional bacterial community, which has a critical effect on plant growth (Xun et al., 2019). The number of studies on the characteristics and changes in the nitrogen-fixing microbial community structure in the rhizosphere of major food crops, especially under salt stress, remains insufficient. The HG-1 strain investigated in this study was determined to have high nitrogen fixation activity. The results

of this study may provide references for future screening, evaluation, and use of NFB. Evidence indicates that plant roots can rapidly select specific microorganisms and maintain relatively stable communities during any cycle of plant growth, suggesting that the structural basis of the rhizosphere microbial community in the early stages of plant growth is essential (Edwards et al., 2015; Reinhold et al., 2015). Studies have indicated that plants may benefit from the synergy with various interacting microbial communities rather than from the individual members of the community. The results of the present study provide new insights into the future use of culturable beneficial microbiota in enhancing plant salt tolerance and improving agricultural production under saline-alkali conditions. In this type of symbiosis, plants maintain and protect microorganisms using rhizosphere metabolites (Jones et al., 2009), provide carbon sources for their growth, and influence the activity and composition of microbial communities (Mendes et al., 2013). This microbiology-based plant biotechnological research has proven to be more effective than plant breeding and genetic modification methods (Smith, 2014). With the increasing use of microbial fertilizers, the effect of microbial agents on the community structure of nitrogen-fixing microorganisms in the rhizosphere soil of crops has considerable research value and could be used as a new standard for evaluating the effects of soil improvement on plant growth promotion.

Nitrogen is closely correlated with the community structure of nitrogen-fixing microorganisms in the soil. The form and content of nitrogen have significant effects on the composition and diversity of the soil bacterial community (Enwall et al., 2007; Campbell et al., 2010). (Li et al., 2014) reported that the diversity and richness of the soil bacterial community decreased with an increase in nitrogen application. (Fierer et al., 2011) determined that nitrogen application had a significant effect on the bacterial composition but no significant effect on bacterial diversity. In this study, we investigated the effects of inoculation with HG-1 strain on wheat rhizosphere microbial diversity and richness. The Sobs, Ace, and Chao indices demonstrated that after the inoculation of roots with HG-1, the community richness of wheat NFB in rhizosphere soil was significantly lower than in the CK group ($P < 0.05$). We determined that the abundance of *Proteobacteria* in the HG-1 sample increased significantly ($P \leq 0.05$), whereas the abundance of *Verrucomicrobia* decreased significantly ($P \leq 0.01$) compared with control samples. At the genus level, we determined that the abundances of *Azospirillum*, *Rhodomicrobium*, *Anabaena*, and two other unclassified genera were lower in HG-1 samples than in CK samples. Moreover, the abundance of *Azoarcus*, *Rhodopseudomonas*, *Bradyrhizobium*, *Cyanothece*, *Sinorhizobium*, *Azotobacter*, *Anaeromyxobacter* and *Vibrio* was significantly lower ($P \leq 0.05$) in HG-1 samples than in CK samples. The relative abundance of some of these strains was low; however, they played a crucial role in the community function (Shi et al., 2016). The phylum *Proteobacteria* includes numerous bacteria responsible for nitrogen fixation (Wen-Ming et al., 2003; Wang et al., 2015a). Increasing the abundance of *Proteobacteria* in the rhizosphere soil may be of positive significance to the growth of plants. This may be because the HG-1 strain increases the nitrogen content in the soil.

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OsPAL2-1 Mediates Allelopathic Interactions Between Rice and Specific Microorganisms in the Rhizosphere Ecosystem

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The use of plant allelopathy to control weeds in the field has been generally recognized as a win-win strategy because it is an environmentally friendly and resource-saving method. The mechanism of this natural weed-control method relies on allelochemicals, the rhizosphere microbiome, and their bio-interaction, and exploring the link between allelochemicals and specific microbes helps accelerate the application of allelopathic characteristics in farming. In this study, we used allelopathic rice PI312777 (PI), its genetically modified OsPAL2-1 repression (PR) or overexpression (PO) lines, and non-allelopathic rice Lemont (Le) as donor plants to reveal the bio-interaction between rice allelochemicals and rhizosphere specific microorganisms. The results showed a higher content of phenolic acid exudation from the roots of PI than those of Le, which resulted in a significantly increased population of *Myxococcus* in the rhizosphere soil. Transgenic PO lines exhibited increasing exudation of phenolic acid, which led to the population of *Myxococcus xanthus* in the rhizosphere soil of PO to be significantly increased, while PR showed the opposite result in comparison with wild type PI. Exogenous application of phenolic acid induced the growth of *M. xanthus*, and the expressions of chemotaxis-related genes were up-regulated in *M. xanthus*. In addition, quercetin was identified in the culture medium; according to the bioassay determination, a quercetin concentration of 0.53 mM inhibited the root length by 60.59%. Our study indicates that OsPAL2-1 is among the efficient genes that regulate rice allelopathy by controlling the synthesis of phenolic acid allelochemicals, and phenolic acid (ferulic acid, FA) induces the chemotactic aggregation of *M. xanthus*, which promoted the proliferation and aggregation of this microbe. The potential allelochemical, quercetin was generated from the FA-induced *M. xanthus* cultured medium.

Keywords: allelochemicals, allelopathic interaction, *Myxococcus xanthus*, rice, phenolic acid

INTRODUCTION

Plant allelopathy is an ecological phenomenon involving the interaction of multiple factors including plants, microorganisms, allelochemicals, and the environment. There are two main types of allelochemicals in rice. Phenolic acids are considered to be one group of allelochemicals, and the other group contains terpenoids and flavonoids. Chou et al. (1981); Rice (1984) and Chou, 1989 believed that phenolic acids, such as *p*-coumaric acid, ferulic acid, *p*-hydroxybenzoic acid, and oxalic acid, produced during the decomposition of rice residues, could be fixed by soil aggregate structural substances or humic acid and stored in the rhizosphere soil to inhibit the growth of rice seedlings and weeds (Chou et al., 1981; Rice, 1984; Chou, 1989; Rimando et al., 2001; Seal et al., 2004). However, Olofsdotter et al. (2002) had a skeptical view of this issue, suggesting that phenolic acid compounds might not be the allelochemicals that inhibit target weeds, since she considered that 4-aminoantipyrine spectrophotometry, often used for the determination of the action dosages, was unsuitable. Moreover, there are documents that indicate that it is not effective unless 4-aminoantipyrine spectrophotometry is used to determine the contents of non-volatile phenolic substances in root exudates from rice (Li et al., 2017). He et al. (2004) used an orthogonal regression design to determine the allelopathic action under laboratory conditions and found that the optimal mixture of five phenolic acids had a higher inhibition rate than each of the individual phenolic acids, which aided in the suppression of barnyard grass. This result suggested that the allelopathic potential of rice might not be related to the concentration of a single allelochemical but rather to the synergistic effect of multiple phenolic acid allelochemicals in an optimal mixture, which explained in part why each of the phenolic acids had a higher inhibitory effect on the target weeds in the bioassay under laboratory conditions but not in the field, simply because the action dosage was much higher in the laboratory than in the field. Terpenoids, such as momilactone B, are often considered promising allelochemicals of rice, since they have a higher inhibitory effect on target weeds at low dosages of 3–30 μ M compared with phenolic acids (Kato-Noguchi, 2004, 2011; Kato-Noguchi and Ino, 2005; Wang et al., 2010; He et al., 2012).

However, all of the aforementioned results were obtained under laboratory conditions. Moreover, the effective concentrations of phenolic acids and terpenoids used for laboratory bioassays were all higher than those detected in allelopathic rice under field conditions, which therefore has frequently been questioned by some scholars. With the development of research work, scientists have gradually understood that allelopathy is a quantitative trait, which is involved in an extremely complex chemobiological process in the rhizosphere ecosystem. Shin et al. (2000) found that the genes coding the two key enzymes, participating in the pathway of phenolic acid synthesis, cinnamate 4-hydroxylase (CA4H) and phenylalanine ammonia-lyase (PAL), were upregulated earlier and to a higher level. Consequently, the encoded enzymic activities and the products of phenolic acid synthesis in the pathway were also higher in allelopathic rice than in its counterpart non-allelopathic rice when the two

rice accessions were exposed to U-VB treatment (Shin et al., 2000). It was also found that the allelopathic potential of rice was significantly enhanced with the increase in barnyard grass density in a coculture treatment under hydroponic conditions (He et al., 2012). Further studies by our research group also showed that the expression of all genes coding for nine enzymes associated with the phenolic metabolism pathway was up-regulated in allelopathic rice PI312227 under low nitrogen stressful conditions compared with a normal nitrogen supply using a qPCR (real-time quantitative PCR detecting system) approach (Xiong et al., 2007). The reverse was true in the case of a non-allelopathic rice accession under the same nitrogen condition, except that the two genes coding for cinnamate CoA ligase and phenylalanine ammonia-lyase showed a slight up-regulation in non-allelopathic rice than in allelopathic rice under the nitrogen deficiency condition (Xiong et al., 2007). Therefore, it was suggested that the enhanced weed inhibition ability of allelopathic rice was mainly related to the significant up-regulation of some gene members of the PAL family. Based on our previous research work on the differential expression patterns of 11 members of the PAL family genes in rice roots with different allelopathic potential under different types of environmental stress, we found that most of the PAL family genes in allelopathic rice accessions were up-regulated in response to nitrogen deficiency or a high accompanying density of barnyard grass, and the enhanced expression of the PAL-2-1 gene was the most obvious among the gene members of the PAL family. However, the differential expression abundance of the PAL family genes, especially PAL-2-1 genes in non-allelopathic rice, was not significant under the same treatment, implying that the PAL-2-1 gene might be related to the regulation of the enhanced chemical defense ability of allelopathic rice in early response to environmental stress (Fang et al., 2011). Further studies confirmed that the phenolic acid synthesis was blocked in the PAL-2-1- inhibited transgenic line of allelopathic rice PI312777, and subsequently, the ability to inhibit barnyard grass was significantly decreased, indicating that the PAL-2-1 gene is one of the functional genes closely related to the ability of allelopathic rice to inhibit grass (Fang et al., 2013). More recently, in order to further explain why a lower action dosage of phenolic acids detected in rhizosphere soil is able to produce a higher allelopathic effect on the target weeds under field conditions, further studies have been conducted, and the results suggested that plant allelopathy includes direct allelopathic effects caused by allelochemicals, and indirect allelopathic effects mediated by allelochemicals through microbial utilization, transformation, and resynthesis. Therefore, we speculated that allelopathy in rice might result from the interaction of allelochemicals with specific microorganisms, such as myxobacteria in rhizosphere soil (Vivanco et al., 2004; Lin et al., 2007; Kong et al., 2008; Qu and Wang, 2008), which needs further investigation (Xiong et al., 2012; Fang et al., 2013, 2015; Lin, 2013).

Our previous research comparatively studied the composition difference of microbial communities in the rhizosphere soil of allelopathic and non-allelopathic rice accessions and found 31 unique microbial genera in the rhizosphere soil of allelopathic rice PI312777, seven of which belongs to myxobacteria at the

rice seedling stage (3–7 leaf stage) (Xiong et al., 2012). Fang et al. (2015) documented that the inhibitory effect of allelopathic rice accessions on target barnyard grass under field conditions might be related to miRNA expression, which is associated with nucleotide excision repair, auxin signal and its transduction pathway in target barnyard grass, thereby reducing its ability to repair DNA damage, subsequently resulting in blocked synthesis of auxin and ultimately repressing the growth of barnyard grass. However, what is involved in the rhizosphere biological process still remains unknown. We speculated that the underlying mechanism might be associated with the process through the chemotaxis and mediation of specific myxobacteria by phenolic acid secretion from the roots of allelopathic rice accessions in the rhizosphere ecosystem. Recent research work has suggested that plant root exudates, such as low-molecular-weight organic compounds, amino acids, and plant secondary metabolites, could effectively shift the structure and function of soil microbial community and thus affect the interaction between plants and microorganisms (Bressan et al., 2009; Shi et al., 2011), implying that rice allelopathy might result from the interaction between the specific myxobacteria and allelochemicals in rhizosphere soil ecosystems.

Myxobacteria have the largest genome among prokaryotes, giving them a large number of luxury genes (Weissman and Müller, 2009). These not only make myxobacteria have complex multicellular behavior but also allow them to produce rich secondary metabolites that have a wide range of anti-eukaryotic biological activities (Weissman and Müller, 2009). The multicellular population behavior of myxobacteria is regulated by complex signal networks, and group life is realized through unique sliding movement, namely the social motility system (S-motility system) and adventure motility system (A-motility system) (Zusman et al., 2007). The completion of the S movement requires cell-to-cell cooperation (Mauriello et al., 2010), which is controlled by the Frz allelopathic system (Sun et al., 2000). Early research found that the chemotactic gene family coding for Frz of myxobacteria contains seven gene members: *FrzA*, *B*, *CD*, *E*, *G*, *F*, and *Z* (Shi and Zusman, 1995), which are homologous with the chemotactic gene of *Escherichia coli*. It was documented that *FrzA* and *CheW* have functional similarity. The same was true in the case of *FrzE*, *CheA*, and *CheY* (Zusman and McBride, 1991). In addition, in the presence of ATP, *FrzE* is able to be autophosphorylate (McBride et al., 1993); *FrzF* and *FrzG* play the same role of *CheR* and *CheB* in chemotaxis. *FrzCD* is a methyl-accepting chemotaxis protein (MCP). Methylated *FrzCD* is related to how cells perceive nutrients or substances in their environment (Dworkin, 1996). Only the *FrzB* protein is unique to myxobacteria. Further studies revealed the chemotactic mechanism of *Myxococcus xanthus*, such as *CheC* and *CheD*, which act similarly to a chemotactic two-component signal transduction system (Kirby and Zusman, 2003). Further studies found that the Frz system consists of eight genes, namely, *FrzA*, *FrzB*, *FrzCD*, *FrzE*, *FrzF*, *FrzG*, *FrzS*, and *FrzZ*, which are related to differentiation and movement, which are involved in the predation behavior of myxobacteria (Pham et al., 2005). *M. xanthus* is often used as a model strain for the study of multicellular behavior (Hans, 1999), and this

specific microbe strongly relies on a signal transduction pathway to regulate its own cellular behavior and complete the quorum sensing reaction in some specific bacteria, and at least five signal factors, such as A (Asg), B (Bsg), C (Csg), D (Dsg), and E (Esg), have been determined (Downard et al., 1993), of which C signal encoded by *csgA*, works at the last time of all signals, and plays an important role in cell aggregation and spore formation during the aerobic growth of *M. xanthus*. The signal is transduced only when cells are in contact with each other, and this in turn results in the expression of genes related to cell aggregation and movement (Søgaard-Andersen et al., 1996; Kaiser, 2003). The expressed C signal further activates the FruA protein through post-transcriptional modification, and the FruA protein is necessary for the processes of cell aggregation and sporulation, which requires both the A signal and E signal for its synthesis. *FruA* (Ellehauge et al., 1998) has a target, the *Frz* gene, which is equivalent to a phosphate repeater. The transcribed activated FruA protein transmits the signal along the frz phosphate repeater, methylating the FrzCD protein; as a result, the fruiting body is formed in this process. Therefore, rice allelopathy is a very complex process of rhizosphere biology, and we need to further uncover the underlying mechanism involved in the specific microbe and allelochemicals in soil ecosystems.

In order to further investigate the correlation between rice *OsPAL2-1* gene expression abundance and myxobacteria populations in its rhizosphere the possible link between them needs to be explored. In this study, allelopathic rice PI312777 (PI), its genetically modified *OsPAL2-1* repression (PR) or overexpression (PO) lines, and non-allelopathic rice Lemont (Le) were taken as research materials; these genetically modified lines were developed in our previous studies (Fang et al., 2015; Li et al., 2020). The population number of *Myxococcus* sp. from the rhizosphere of these rice and the phenolics contents in the rice were detected. *Myxococcus xanthus* from *Myxococcus* sp. was isolated from rice rhizosphere soil to investigate the bio-interaction with phenolics, and to evaluate the inhibitory effect of *M. xanthus* and the metabolites on the growth of barnyard grass. The subsequent studies attempted to further elucidate the mechanism and mode of action of allelochemicals on specific microorganisms colonizing in the rhizosphere soil of allelopathic rice so as to provide a theoretical basis for further understanding the biochemical process and mechanism of rice allelopathy in the suppression of target weeds in rhizosphere soil ecosystems.

MATERIALS AND METHODS

Preparation of Strain and Plant Materials Rice Accessions Used in the Experiment

The following materials were used: allelopathic rice accession PI312777 (abbreviated as PI), and its genetic derivatives, of which the gene expression of *OsPAL2-1* was inhibited or overexpressed, according to the technique of RNA interference (PAL-RNAi, PR) or overexpression (PAL-overexpression, PO), respectively, and non-allelopathic rice Lemont (Le) developed and provided by the Institute of Agroecology, Fujian Agriculture and Forestry University, Fuzhou, China (Fang et al., 2013; Li et al., 2020)

were used as the test materials. *OsPAL2-1* is known as the key gene involved in the regulation of the phenylalanine metabolism pathway, and the expression level of *OsPAL2-1* determines the contents of the phenolic acids in rice. CK refers to the blank control. The seeds of all the test rice were sterilized using sodium hypochlorite for 30 min, and soaked in sterilized ddH₂O for 24 h, and then soaked in hygromycin (50 mg/mL) for 24 h. After that, the hygromycin was completely removed, and the seeds were pre-germinated in petri dishes in a 30°C incubator for 2–3 days. The experiment was conducted under field conditions and in the indoor laboratory at the Institute of Agroecology, Fujian Agriculture and Forestry University, from March 2017 to September 2018.

During the experiment, the annual average temperature was 20–25°C, the annual average maximum temperature in the daytime was 26°C, and the annual average minimum temperature at night was 16°C. The average monthly temperature during the early rice planting month from March to April was between 20°C and 26°C, and the average temperature of the late rice planting month from July to September ranged from 33 to 37°C. The annual relative humidity was about 77%.

Field Experimental Design and Soil Sample Collection

The test seedlings of all rice entries were cultured in a dry-raised nursery followed by the method described by Li et al. (2018). The field soil contained 1.94 g·kg⁻¹ total nitrogen (potassium dichromate-sulfuric acid digestion method), 61.37 mg·kg⁻¹ alkali hydrolyzable nitrogen (alkali hydrolysis diffusion method), 0.76 g·kg⁻¹ total phosphorus (sulfuric acid-perchloric acid digestion method), 30.7 mg·kg⁻¹ available phosphorus (phosphomolybdate blue colorimetry NH₄F-HCl extraction), 1.68 g·kg⁻¹ total potassium (NaOH melting-flame photometer method), 208.64 mg·kg⁻¹ available potassium (flame photometry after extraction with 1M ammonium acetate solution buffered at pH 7), and 22.5 g·kg⁻¹ organic matter (potassium dichromate volumetric method), with a pH value of 6.21 (a portable pH meter, IQ 150, Spectrum technologies Inc., Aurora, IL, United States) in the tillage layer (Bao, 2000; Pansu and Gauthier, 2007). Rice seedlings were transplanted on 25 March 2017 and 25 July 2018, with appropriate basal fertilizers (70% of total N as basal dressing and the rest as top dressing, with total N = 225.0 kg/ha, P = 29.5 kg/ha, K = 149.4 kg/ha). The three nutrient elements N, P, and K were applied in the form of urea (N), calcium superphosphate (P), and potassium chloride (K), respectively, to each plot according to the proportion of the three elements as mentioned above. Each variety was planted separately in different plots (3 m × 1 m), and this area was evenly divided into three parts (1 m × 1 m); the seeding rate was 150 g/m². In addition, CK was set as the blank control soil in the same area without rice. After the emergence of rice seedlings, field management followed recommended methods. Random sampling was conducted in each treatment at the 3-, 5-, and 7-leaf stages of rice seedlings.

Pot Trials

Pot trials were carried out in a plastic barrel (outer diameter of 33 cm and inner diameter of 31 cm), 12.0–12.5 kg,

per barrel of sun-dried soil, and an appropriate amount of fertilizer per bucket (converted according to N = 225 kg/ha, P = 29.5 kg/ha, K = 149.4 kg/ha, applied in the form of urea, calcium superphosphate and potassium chloride, conversion according to barrel area, that is 0.64, 2.81, 0.45 grams of urea, calcium superphosphate and potassium chloride basal dressing, and 0.183 and 0.091 grams of urea for topdressing per bucket, respectively), and irrigated with 3 liters of water. Pre-germinated seeds were seeded on 21 April 2017, at 15 seeds per barrel. CK (blank control without rice), and the PI, Le, PR, and PO lines were directly seeded in 10 barrels. When the rice grew to the 1-leaf stage, five rice uniform plants were retained per barrel. The same amount (1 L) of tap water was applied every day, and topdressing was applied twice during the seedling stage at 3- and 5-leaf stages. Potted plants were placed in a growth chamber and were sheltered from rain with white plastic. Temperature varied from 25 to 30°C, while humidity varied from 65 to 78% during the experiment. Random soil sampling was conducted at the 3-, 5-, and 7-leaf stages of the rice seedlings.

The Collection of Blank Soil and Rhizosphere Soil Samples

From the plots without rice, surface soil was removed along with vegetation to collect the 0–5 cm deep soil layer. The uniform rice plants grown in pots were carefully uprooted preventing any damage to the roots. The soil sticking to the rhizoplane was brushed off and collected as rhizosphere soil samples. All of the soil samples were stored in a refrigerator until needed for tests.

Isolation and Identification of the Key Microorganism, *Myxococcus xanthus*, in the Rhizosphere Soil of Allelopathic Rice

The rabbit feces induction method (Zhang et al., 2003) was used in this experiment. The procedure is as follows: First, 10 g of the rhizosphere soil of PI was air-dried and placed in a tissue culture bottle with 20 mL of sterilized ddH₂O. Then, a filtered and sterilized mixed solution of antibiotics (gentamicin 40 mg/mL, kanamycin 10 mg/mL, and ampicillin 40 mg/mL, total 1 mL, and actinomycin 40 mg/mL dissolved in 1 mL dimethyl sulfoxide solution) was added to the tissue culture bottle. The culture bottle was placed in a water bath at 55°C for 10 min and then stored overnight at room temperature. Second, rabbit feces were collected and sterilized and then stored in tissue culture bottles. Third, fruiting bodies were induced by the specific microbe: The excess liquid from the treated soil sample was poured out, and the wet soil (approximately 3 mm thick) was placed on a sterile plate. Four or five pieces of sterilized rabbit feces were half-buried in the treated soil sample with tweezers and cultured at 28°C. After 48 h, the formation of fruiting bodies on the rabbit feces could be observed. Then, strains were screened and purified: the induced fruiting bodies were picked out on sterilized VY/4 medium (0.5% yeast powder; CaCl₂·2H₂O 0.2%, agar 2%, pH 7.2) with an inoculation ring for subgeneration and subcultured at 28°C. A large number of vegetative cells

could be observed within 24 h, and fruiting bodies of the microbe were formed within 48 h. After repeated subculture until there were no other miscellaneous bacteria, the expected *Myxococcus* strains were screened, proliferated and preserved. Finally, the strain was preserved at normal culture temperature in the following way: The bacteria were cultured on VY/4 medium and were stored at 28°C after colony growth. The new colonies were marked, purified, and preserved at regular intervals. The bacterial cryopreservation for subsequent studies was as follows: 600 mL bacteria liquid was placed in a 1.5 mL EP tube; 60% glycerol of the same volume was added followed by storage at −80°C.

DNA Extraction of the Isolated Strain

Due to the influence of the unique biological characteristics of myxobacteria, the traditional method for the extraction of bacterial DNA was not ideal, the amount of extracted DNA was low and impurities were obtained. After considerable exploration, we referred to the extraction method that used a small amount of DNA (Kutchma et al., 1998) and the CTAB/NaCl precipitation method of bacteria, and in a short time, we achieved large amounts of highly pure extracted DNA, through the combination and simplification of the two methods.

The specific methods are as follows. First, the isolated and purified strain was picked up in VY/4 liquid medium and cultured for 72 h at 28°C, 150 rpm. Second, the mature bacterial liquid was centrifuged and suspended in 750-μL DNA extract liquid (0.25 M EDTA (pH = 8.0), 0.1 M Tris-HCl (pH = 8.0), 2% CTAB, 2% PVP, 2 M NaCl₂, 2 M CaCl₂, 0.4 M LiCl), and then placed in a water bath at 55°C for 10 min. Subsequently, the same volume of Tris equilibrium phenol-chloroform-isoamyl alcohol (25:2:1) was added, mixed well (shaken), and then centrifuged to obtain the supernatant. Depending on the purity of the bacterial solution, the DNA was repeatedly extracted two to three times until there was no protein layer. Then, the same volume of isopropanol was added to the supernatant and passed through the DNA recovery column followed by a 70% ethanol washing column; this step was repeated followed by blow drying. Finally, DNA was eluted from the column with a suitable amount of sterile water.

PCR Verification and Identification of the Strain

After the total DNA extracted by the above method was recovered by a DNA Gel Extraction Kit (Omega, United States), the 16S rRNA gene sequence was amplified by bacterial universal primers (P1:5'-AGAGTTTGATCCTTGGCTCAG-3' and P2:5'-AGAAAGGAGGTGATCCAGCC-3'). The PCR system consisted of 25 μL as follows: template 1 μL, primer 1 1 μL, primer 2 1 μL, 2 × EcoTaq PCR Supermix 12.5 μL, H₂O 9.5 μL. The amplification conditions were as follows: 95°C for 5 min, 95°C for 1 min, 55°C for 50 s, 72°C for 90 s, and 72°C for 10 min. The PCR products were purified using a UNIQ-10 spin column DNA Gel Extraction Kit (Shanghai Sangon Biotech), and the samples were sent to the Shanghai Sangon Biotech Sequencing Center for DNA sequencing. The sequence was then alignment in GenBank using Blastn.

The Interaction of *Myxococcus xanthus* With Allelopathic Rice Accessions

qPCR Analysis of *M. xanthus* in the Rhizosphere Soil of Allelopathic Rice Accessions

The total DNA of the CK, PI, PR, and PO rhizosphere soil sampled from the pot trials was extracted in four replicates according to Peršoh et al. (2008). The absolute fluorescence quantitative PCR (qRT-PCR) of *Myxococcus* spp. in rhizosphere soil was performed following Martin and Bull (2006) using nested PCR. The first round of amplification was a common PCR. The primers were Myxo70F, 5'-CGCGAATAGG-GGCAAC-3', and Myxo1346R, 5'-GCAGCGTGCTGATCTG-3'. The PCR procedure was as follows: pre-denaturation at 95°C for 5 min, 95°C for 30 min, 58°C for 1 min, 72°C for 1 min, for 25 cycles, and extension of 5 min at 72°C. The PCR products were performed by electrophoresis and stored in a refrigerator at 4°C for the second round of PCR. The second round of quantitative real-time PCR fluorescence analysis was performed on an Eppendorf realplex4 quantitative PCR instrument. The primers were Myxo184F, 5'-CACG-GTTTCTTCGGAGACT-3', and Myxo1128R, 5'-CTCTAGA-GATCCACTTGCGTG-3'. The configuration was based on the SYBR Premix Ex Taq™ (TaKaRa, China) instruction manual, and the reaction system was 15 μL. The reaction parameters were as follows: pre-denaturation at 95°C for 15 min, denaturation at 95°C for 20 s, annealing at 61°C for 20 s, extension at 72°C for 40 s, for 41 cycles, and then 95°C for 15 s, 60°C for 15 s, heating for 10 min to 95°C (reading fluorescence once every 0.2 degrees), and then 95°C for 15 s to form a dissolution curve. According to the threshold (CT value) generated by the software, the 2-ΔΔct method was used to calculate the relative gene expression. Each DNA sample of five rice accessions in rhizosphere soil was replicated four times.

Induction Effects of *M. xanthus* by Different Concentrations of Phenolic Acid

Optimal concentrations of ferulic acid, cinnamic acid, vanillic acid, and *p*-hydroxybenzoic acid were prepared following the method described by He et al. (2004), and these phenolic acids were, respectively, added to 1/2 CTT medium, to investigate the effect on the *M. xanthus*. Then, dynamic sampling was carried out, and the growth curve was drawn by the weighing method. Six replicates were used for each treatment, and the treated concentration of each phenolic acid was prepared and used as shown in Table 1.

TABLE 1 | The types and treated concentration levels of each phenolic acid inducing the growth of *Myxococcus xanthus*.

Factor	Concentration levels (mmol·L ⁻¹)
<i>p</i> -hydroxybenzoic acid	0.40 0.50 0.60
Cinnamic acid	0.12 0.20 0.28
Vanillic acid	0.02 0.10 0.18
Ferulic acid	0.05 0.1 0.15

Inoculation Effect of Mixed Phenolic Acids on *M. xanthus* in Soil Culture

Soil was sampled from the experimental field of Fujian Agriculture and Forestry University in which no rice plants had been grown before. The soil was sterilized at 121°C for 0.5 h, stored at room temperature for 8 h, and then sterilized at 121°C for 0.5 h. This process was repeated three times. The sterilized soil was placed in a sterilized plastic culture box, and each box was filled with 0.8 kg aseptic soil.

Myxococcus xanthus and phenolic acid solution, which contained 3, 4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, vanillin, ferulic acid, coumarin, benzoic acid, salicylic acid, and cinnamic acid mixed in equal concentrations were added to aseptic soil as treatment groups. Different quantities of phenolic acid mixture (10 µg/kg soil, 20 µg/kg soil, 50 µg/kg soil, and 100 µg/kg soil) were used for the test. Aseptic soil with *M. xanthus* but without any phenolic acid mixture was used as a control. The above treatments were cultured in an artificial climate chamber, and sampling was performed on the 4th and 7th day after treatment initiation. qRT-PCR was used to study the effects of different concentrations of mixed phenolic acids on the population growth of *M. xanthus* added to the soil. Three repeats were set for each test concentration. Each treatment was inoculated with *M. xanthus* (2 mL).

Differential Expression Analysis of the Chemotaxis-Related Genes of *M. xanthus* in Response to Phenolic Acid

The *M. xanthus* strain stored at -80°C, transferred to 1/2 CTT solid medium for 5 days, and then transferred to 1/2 CTT liquid medium for logarithmic culture. The above bacterial solution was inoculated into 1/2 CTT liquid medium at 1% (v/v), while ferulic acid (0.05 mM and 0.10 mM) and mixed phenolic acids (3, 4-dihydroxybenzoic acid 0.1 µM, *p*-hydroxybenzoic acid 0.4 µM, cinnamic acid 0.2 µM, vanillic acid 0.1 µM, and ferulic acid 0.2 µM) were added to the liquid medium to induce growth. The treatment and control, which contained three repeats, were placed on a shaker and cultured at 250 rpm and 30°C. The bacterial culture samples were collected after 58 h of shaking culture in the ferulic acid treatment group, and 3, 6, 9, 12, 24, and 48 h after treatment initiation in the mixed phenolic acid treatment group. The bacterial total RNA was extracted according to the instructions accompanying Trizol reagent (Takara, China). Subsequently, cDNA was synthesized according to the instructions of the TIANScript RT Kit (Tiangen Biochemical Technology Co., Ltd., Beijing, China). The expression of important differential genes in *M. xanthus* induced by phenolic acid was studied by qRT-PCR with the primers shown in Table 2. The 16S rRNA of myxobacteria was used as the internal standard gene (forward primer sequence: 5'-GACGGTAACTGACGCTGAGAC-3'; reverse primer sequence: 5'-CCCAGGCGGAGAACTTAATGC-3'). Real-time fluorescence analysis was carried out in an Eppendorf realplex4 quantitative PCR instrument. The reaction system was 15 µL, and the configuration followed the operating instructions of

TABLE 2 | Primers of the chemotactic genes of *Myxococcus xanthus* induced by phenolic acid used in qPCR.

Gene	Forward primer 5' to 3'	Reverse primer 5' to 3'
<i>frzA</i>	GTGAGAACGTGCTCGAAGTG	AAGTTGAGGAGGTTGATGGC
<i>Frz B</i>	GTGGACCTCCTCTTCTTC	CAGTGCCCTTCCCTGAGT
<i>frzCD</i>	CTGCTGGAGGGCTTTGGC	GCGGTGCTCTCGTGGATG
<i>frzE</i>	GGCGGCAATCGCTTCGACAA	AACCCGTCCAGCTTGGGCATCT
<i>frzG</i>	TTCAGCGGGTGAGTCGTTCCG	GCTTCGCTCGGGCAATGGT
<i>frzS</i>	AAAATCCTGATCGTCGAAAG	TTGCCGCAGATGAGGTAG
<i>csgA</i>	TGGCAGGTGTTGTTCTCA	TAGATGCAGTCTGGTCAACCG
<i>csgB</i>	AGCCGCAGCAGGTTATGATTT	TGTACGCGAATAGCCATTT
<i>csgG</i>	GAATCTTTCAATGCCGTGACC	TCTTTTGTTGCCGTGATGT
<i>fruA</i>	TGCAACATTAGCAACAGCATT	TTGGACCTTCTGTTTCACGTT

RealMasterMix (SYBR Green) (Tiangen Biochemical Technology Co., Ltd., Beijing, China).

Allelopathic Effect of Rhizosphere Special *M. xanthus* on the Targeted Barnyard Grass

Evaluation of the Inhibition Effect of *M. xanthus* on Barnyard Grass

Seed disinfection

Rice and barnyard grass seeds were separately placed in a tissue culture bottle, sterilized with 70% ethanol for 1 min, and soaked and shaken in ethanol again to achieve full sterilization. The ethanol was then discarded, and the seeds were rinsed with water 3 to 4 times until the smell of ethanol disappeared. Sterilized mercury (1%) was added to the tissue culture bottle, shaken, and seeds were soaked for 15 min. Seeds were rinsed again with sterile water seven to eight times. The sterilized seeds were soaked in sterile water for germination.

Inhibitory effect on the grass

We divided this study into three groups with three repeats. The following treatments were established, A: *M. xanthus* with barnyard grass seeds; B: culture medium not inoculated with *M. xanthus* but with barnyard grass seeds; C: *M. xanthus* with rice seeds. The purified strains were coated on plates according to the experimental setting. In the experimental groups, the seeds of allelopathic or non-allelopathic rice accessions and barnyard grass were planted in VY/4 medium, 30 seeds per plate, and were cultured at 28°C for 48 h. Then, the germination rate was calculated.

Analysis of the myxobacterial activity to suppress the barnyard grass

Phenolic acids at optimal concentrations (3,4-dihydroxybenzoic acid 0.1 µM, *p*-hydroxybenzoic acid 0.4 µM, cinnamic acid 0.2 µM, vanillic acid 0.1 µM, and ferulic acid 0.2 µM), which were determined in a pre-experiment, were added to the modified 1/2 CTT solid medium, in which the inorganic salt component was reduced to 1/6. The allelochemical group was inoculated with 50 µL of the activated *M. xanthus* solution, and its

control consisted of only 50 μ L sterile water. The group without allelochemicals was also inoculated with 50 μ L of the activated *M. xanthus* solution. The treatment with only 50 μ L water was used as the positive control and that with 50 μ L *E. coli* was used as the negative control. Each treatment and control contained three replicates and were incubated at 30°C for 5 days. Under sterilized conditions, the pre-germinated disinfected barnyard grass seeds were evenly sown, 30 seeds per bottle, and the tissue culture bottles were placed in a growth chamber at 30°C for 7 days under 12 h (6:00-18:00) of light and 12 h of dark. Barnyard grass germination and barnyard grass stem length were calculated.

Data analysis and Software

The barnyard grass germination and root length were determined on the 7th day after sowing. The inhibitory rate was calculated according to formula (1):

$$\text{Inhibitory rate (IR)} = (1 - T/C) \times 100\%,$$

where C is the measured value of the control group and T is the measured value of the treatment group. $IR > 0$ indicates inhibition, and $IR < 0$ indicates a promotion effect. The measured data were statistically analyzed using DPS v 7.05 and the Excel 2003 program.

Inhibitory effect of *M. xanthus* culture inoculation on the targeted barnyard grass in soil culture

The sterilized soil was placed in the sterilized plastic culture box; each box was filled with 0.8 kg aseptic soil, then 30 presoaked seeds of the barnyard grass were pre-seeded in the aseptic soil of each box with three replicates. Then, 2 mL, 5 mL, 10 mL, or 20 mL of *M. xanthus* solution and 2 mL of its fermentation broth were added; the broth was the supernatant obtained by centrifuging *M. xanthus* solution for 10 min at 12000 rpm as a treatment. Barnyard grass seeds were planted in aseptic soil without any addition of *M. xanthus* solution and its fermentation broth as a control and were then cultured in an artificial climate box at 37°C for 4 and 7 days. After 4 and 7 days, the germination rates of barnyard grass were calculated, and the effects of different populations of *M. xanthus* on the germination of barnyard grass and rice seeds were determined.

The Identification and Evaluation of Allelochemicals in the Fermentation Broth With the Induction of Different Phenolic Acids and Their Mixtures Based on GC-QQQMS Analysis

The activation of XAD-16 resin: We weighed the XAD-16 resin (1% w/v) and soak it in double distilled water for 30 min. The resin was collected by filtration and immersed in methanol for 30 min. Then, ultrapure water (50% w/v) was added to soak the resin for further use.

Sample Preparation

The activated *M. xanthus* was transferred to 1/2 CTT liquid medium and cultured at 200 rpm, at 30°C for 1 week. Then, the supernatant was collected by centrifugation and passed through a 0.22- μ m filter membrane.

The activated XAD-16 resin was added to the supernatant (10%, w/v) and absorbed at 20°C, 200 rpm overnight (12 h), after which the resin was collected by filtration. Methanol was added for extraction at 20°C, 200 rpm overnight, and collected by filtration, followed by extraction with acetone. Then, the methanol extract phase and the acetone extract phase were combined and concentrated by rotary evaporation. The injection bottle for GC-QQQMS was prepared by passing diluted (2 mL) acetone, containing the extract, through a 0.22- μ m filter.

The control and other treatments were then measured. The control group included the blank culture medium (CK₀); PA₀ refers to the blank culture medium to which the combination of phenolic acids was added. The test group was inoculated with *M. xanthus* on the basis of the corresponding control group: CK₁ was inoculated with *M. xanthus* in the blank medium; PA₁ refers to the addition of the combination of phenolic acids to the blank medium followed with *M. xanthus* inoculation.

Exogenous Addition of Quercetin and Its Effect on Barnyard Grass

Determination of the Quercetin Content in the Myxobacterial Fermentation Broth

A quercetin standard (Solarbio Biotechnology Co., Ltd., Beijing, China) was prepared with standard solution concentrations of 3.31×10^{-4} mM; 6.62×10^{-4} mM; 0.17×10^{-2} mM; 0.33×10^{-2} mM, and 0.66×10^{-2} mM in acetone. The standard solution series and the fermentation liquid sample of *M. xanthus* were injected into a high-performance liquid chromatograph. The chromatograms were recorded, and the peak area was measured. The standard curve was plotted with concentration X (mM) as the abscissa and the chromatographic peak area as Y on the vertical axis.

Chromatographic conditions

Column: Shimadzu ODS column (4.6 mm \times 150 mm, 5 μ m), mobile phase of acetonitrile-water (containing 2% acetic acid), flow rate: 0.8 mL/min, injection volume: 20 μ L, column temperature: 25°C, detection wavelength: 360 nm. Eluting procedure: 0 min, acetonitrile 17%, water (containing 2% glacial acetic acid) 83%; 18 min, acetonitrile 40%, water (containing 2% glacial acetic acid) 60%.

Effect of Exogenously Added Quercetin on the Growth of Barnyard Grass

According to the content of quercetin detected in the fermentation broth sample determined by HPLC, the linear equation coefficient of determination (R^2) of the quercetin standard concentration was established: $Y = 11709X - 1118.1$ ($R^2 = 0.9989$). The result indicated that the linear relationship of the quercetin standard concentration was in the range of 3.31×10^{-4} mM– 0.66×10^{-2} mM. The chromatogram of the quercetin standard and PA₁ sample is shown in **Supplementary Figure S1**. According to the chromatographic peak area and regression equation, the quercetin content in the sample was 1.06×10^{-3} mM.

Macias (1995) proposed that the approximate biological activity range of phenolic acid in higher plants is 10^{-6} – 10^{-5}

M based on the unified biological test standard. Accordingly, a series of quercetin concentrations were set in the 10^{-7} – 10^{-4} M concentration range, i.e., 1.06×10^{-4} mM, 1.06×10^{-3} mM, 1.06×10^{-2} mM, 0.053 mM, 0.106 mM, and 0.53 mM dosages of quercetin standard were prepared and used for the allelopathic bioassay. The control did not contain quercetin. Each treatment had three replicates and consisted of 30 pre-germinated barnyard grass seeds that were sown in Petri dishes. The tissue culture bottles were placed in a growth chamber at 30°C under light conditions for 12 h (6:00–18:00). After culture for 7 days, the root length and stem length of the barnyard grass were determined, and the inhibitory rate was calculated.

Dynamics of Phenolic Acids in Root Exudates of Rice Measured by Solid-Phase Extraction and High-Performance Liquid Chromatography Method

The experiment was conducted in a greenhouse at the Agroecological Institute of Fujian Agriculture and Forestry University in Fuzhou, China. The air temperature ranged from 25°C to 35°C, averaging 30°C during the trials.

Plant materials rice (*Oryza sativa*) accession PI312777 (PI, allelopathic rice) and Lemont (Le, non-allelopathic rice) introduced from the United States were selected as donor plants. Barnyard grass (BYG, *Echinochloa crus-galli* L.) was used as the receiver plant.

Seeds of the two rice accessions and BYG were germinated on seedling trays. Then uniform rice seedlings (2-leaf stage) and BYG seedlings (2-leaf stage) were transplanted into Styrofoam plates with 30 perforated holes (5 × 6 holes with 5 cm × 5 cm row space). The seedlings were stabilized with cotton plugs. The Styrofoam plate was floated on a pot (45 cm × 35 cm × 15 cm) filled with 10 L Hoagland hydroponic solution (served as normal nutrient condition). After 3 days of recovery in the Hoagland hydroponic solution, 30 rice seedlings were transplanted to the Styrofoam plate. In rice/weed mixed culture system, allelopathic rice PI and non-allelopathic rice Le were mixed with BYG at 2:1 ratios with the BYG seedlings in the center surrounded by the rice seedlings in the hydroponic culture system. When rice seedlings reached 3-leaf, 5-leaf, and 7-leaf stages, additional Hoagland nutrient solution was added to each pot to maintain a 10 L solution volume, and the remaining concentration of N, P, and K in hydroponic solutions were detected, then adjusted to the initial concentration levels using NH_4NO_3 , K_2SO_4 , and KH_2PO_4 (the initial concentration of N, P, and K were 20 mg/L, 5.65 mg/L, and 42.72 mg/L, respectively), furthermore, pH was adjusted to 5.5. A monoculture of BYG under the same condition was used as the control (CK) for each treatment. 250 mL of hydroponic culture was collected for the determination of phenolic acids in rice root exudates. The treatments were conducted in triplicate (He et al., 2012; Li et al., 2017).

SPE-HPLC procedure

Reagents and Apparatus: Standards of 3,4-dihydroxybenzoic acid, *p*-hydroxybenzoic acid, vanillic acid, syringic acid, vanilla, ferulic acid, coumarin, benzoic acid, salicylic acid, and cinnamic acid were obtained from the Chinese Institute of Biological

Products Control (Beijing, China). Methanol was of HPLC grade from Merck. Ultrapure water from Milli-Q system (Millipore, Bedford, MA, United States) with conductivity of 18.3 MQ was used in all experiments. All solutions were filtered through 0.45 μm membranes (Millipore) and degassed prior to use (Li et al., 2017).

An Agilent 1200 liquid chromatography system, equipped with a quaternary solvent delivery system, an autosampler, and DAD detector, was used. A Zorbax SB-C18 column (150 mm × 4.6 mm, 5 μm) connected with a Zorbax SB-C18 guard column (20 mm × 4 mm, 5 μm) at a temperature of 30°C was applied for all analyses.

Solid-phase extraction

Before SPE, hydroponic culture was filtered through a 0.45 μm microporous filter under a weak vacuum. All samples were adjusted pH to 4.0 with 1.0 M HCl, after that, sodium chloride was added to the aqueous solution to obtain an 8% salt solution. The aqueous solution was subjected to SPE using cleanert PEP-SPE cartridges (500 mg; Agela Technologies Inc., Bellefonte, PA, United States) previously activated with ultrapure water (8 mL), methanol (8 mL) and ultra-pure water (4 mL). The phenolic acids were finally eluted with 4 mL of methanol at a rate of 1–2 mL/min. The eluate was dried by nitrogen spraying. The residues were dissolved in 1 mL methanol and a 5 μL aliquot was injected into the liquid chromatography column for HPLC analysis (Li et al., 2017).

Chromatographic conditions

Detection wavelength was set at 280 nm. The mobile phase consisted of (A) methanol and (B) 0.1% phosphoric acid (v/v) using a gradient elution of 27% A at 0–6 min, 27–50% A at 6–15 min. Re-equilibration duration was 5 min between individual runs. The flow rate was 1.6 mL/min and aliquots of 5 μL were injected (Li et al., 2017).

The Total Concentration of Phenolic Compounds in Root and Leaf Tissues Measured Using the Folin–Ciocalteu Colorimetry Method

Root tissue and leaf tissue were sampled from the recovered transgenic lines and WT plants as described. In a precooled mortar, 1 g of root tissue or leaf tissue frozen with nitrogen liquid was grinded and immersed in 10 mL methanol or sterilized water 10 mL for 24 h at 4°C, and the procedure was repeated twice. These methanolic or water extracts were centrifuged at 13,000 r/min for 15 min, and the supernatants were pooled and stored at 4°C (Shen et al., 2009).

The total content of phenolics was determined using a minimally modified Folin–Ciocalteu method (Singleton et al., 1999). Root tissue or leaf tissue extracts by methanol or water (3.25 mL) were added to test tubes with 0.5 mL of Folin–Ciocalteu's reagent and 1.25 mL of sodium carbonate (2 M). After mixing, the tubes were allowed to stand for 40 min in dark. Then OD was measured at 760 nm. The total phenolic compounds were expressed in tannic acid equivalents in micromole per liter of root or leaf extracts (Fang et al., 2013; Li et al., 2017).

RESULTS

The Population Number of *M. xanthus* in the Rhizosphere Soil of the Allelopathic Rice Accession

As shown in **Figure 1**, qPCR analysis indicated the same changing trends of the *Myxococcus* sp. population number in the rhizosphere soil of allelopathic rice (PI) and its counterparts (PO, PR) at the 3–7-leaf stages. The population numbers of rhizosphere *Myxococcus* sp. were 1047, 3424, and 378 cells/g soil in the rhizosphere soil of the allelopathic rice accession PI at the 3–7-leaf stages, a significant difference compared with the blank soil (CK). The *OsPAL2-1*-inhibited transgenic line (PR) had 512, 78, and 17 cells/g soil population numbers of *Myxococcus* sp. in rhizosphere soil, significantly lower than the allelopathic rice accession (PI). The reverse was true in the case of the *OsPAL2-1*-overexpressed transgenic line (PO), 974, and 3209 cells/g soil *Myxococcus* sp. population numbers in rhizosphere soil at the 3- and 5-leaf stages, had no significant difference compared with the of PI312777, and contained the largest population number of *Myxococcus* sp., 10899 cells/g soil, in rhizosphere soil at the 7-leaf stage, which resulted in the highest population numbers of *M. xanthus* in rhizosphere soil at the 3–7-leaf stages.

Myxococcus xanthus Isolated and Identified From the Rhizosphere Soil of Allelopathic Rice

The biological characteristics of the strains were as follows: the fruiting body induced from rabbit feces was yellow and spherical, and the secondary colony obtained by line culture was milky white, raised in the middle with an irregular edge (**Supplementary Figure S2A**). In the early stage, the colony

showed a transparent or translucent milky white irregular shape, and the edge was irregular. After about 48 h of culture, the fruiting body began to appear in the colony; the fruiting body was yellow and granular, and the colony also changed to yellow. In addition, the viscosity increased, becoming transparent or translucent, and the edge of the colony was irregular (**Supplementary Figures S2B,C**). The 16s rRNA fragment that amplified from the genomic DNA of the above strains was purified and sequenced. Based on Blast sequence alignment combined with its colony morphology, this strain was identified as *M. xanthus* with the highest similarity.

In vitro Interactions of *M. xanthus* With Phenolic Acid Allelochemicals in Rice

Figure 2 shows that different phenolic acid allelochemicals showed various effects on the growth of the myxobacteria, indicating that cinnamic acid at 0.12 mM and ferulic acid at 0.05–0.1 mM significantly promoted the proliferation of the myxobacteria. However, no significant difference was found for the effect of hydroxybenzoic acid and vanillic acid on the bacteria at 0.4 mM and at 0.02–0.18 mM, respectively, in the *in vitro* interaction test. The influence of the phenolic acid mixture at different concentrations on the population growth of *M. xanthus* showed a tendency of an increasing and then a decreasing pattern under soil culture conditions (**Figure 3**). There was no significant difference in the population of *M. xanthus* in the coculture with different concentrations of phenolic acid mixture for 4 days. However, after 7 days of coculture, the population of *M. xanthus* was significantly different and was the largest in the soil with the 20 $\mu\text{g/kg}$ of phenolic acid mixture. These findings suggested that the population growth of myxobacteria was closely related to the concentration of the mixed phenolic acids in soil. However, there was an optimal concentration range in terms of the action effect, i.e., too low or too high a concentration of the phenolic acid mixture, added by external sources or secreted by the root system of rice accessions, was unfavorable for the growth of the special microbial flora in the rice rhizosphere.

Differential Expression Analysis of the Related Chemotaxis Genes of *M. xanthus* in Response to Phenolic Acids

Further analysis demonstrated that a single phenolic acid or mixture of phenolic acids could effectively induce the up-regulated expression of the related chemotaxis genes in *M. xanthus* at different levels (**Figures 4A,B**). Gene expression changes in each member of the *Frz* family were detected in *M. xanthus* cocultured with different concentrations of ferulic acid for 58 h, as shown in **Figure 4A**. The result showed that the changes in *FrzS* gene expression were non-significant between the coculture treatment with 0.05 mM ferulic acid and the control without the addition of any phenolic acids. However, the up-regulated rate of *FrzS* in *M. xanthus* in the cocultured treatment with 0.10 mM ferulic acid was 2.56-fold higher than that of the control. Furthermore, the same situation was observed for gene expression levels of four other *Frz* family members, showing that *FrzA*, *FrzB*, *FrzCD*, and *FrzG* in *M. xanthus* were significantly

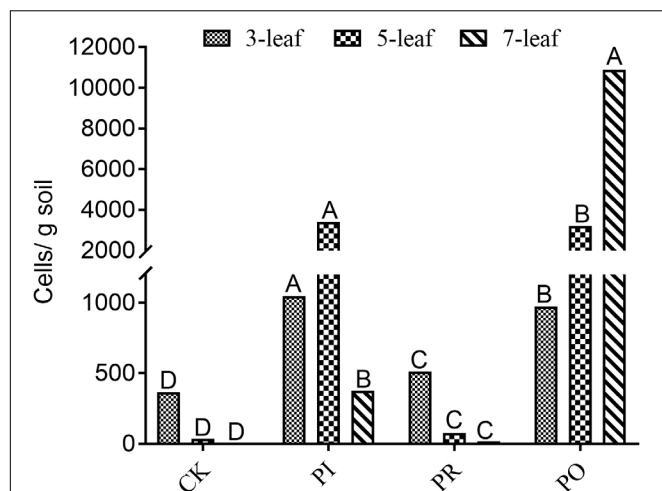
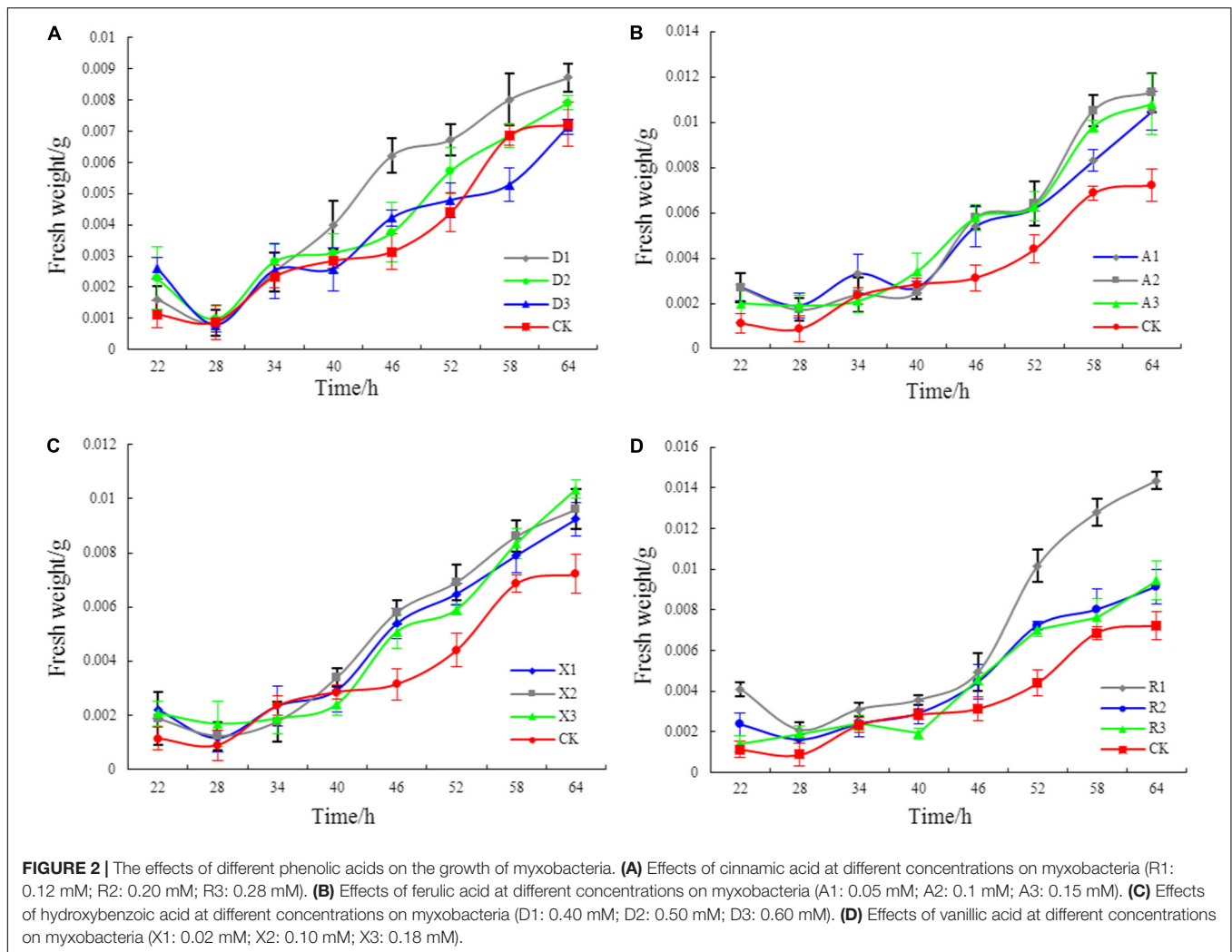


FIGURE 1 | qRT-PCR analysis of soil *Myxococcus* spp. in rhizosphere soil at different leaf stages of the different rice accessions. PI, allelopathic rice PI 312227; PR, *OsPAL2-1*-inhibited allelopathic rice; PO, *OsPAL2-1*-overexpressed allelopathic rice; CK, blank soil. Different letters indicate significant difference (LSD, $p < 0.05$).



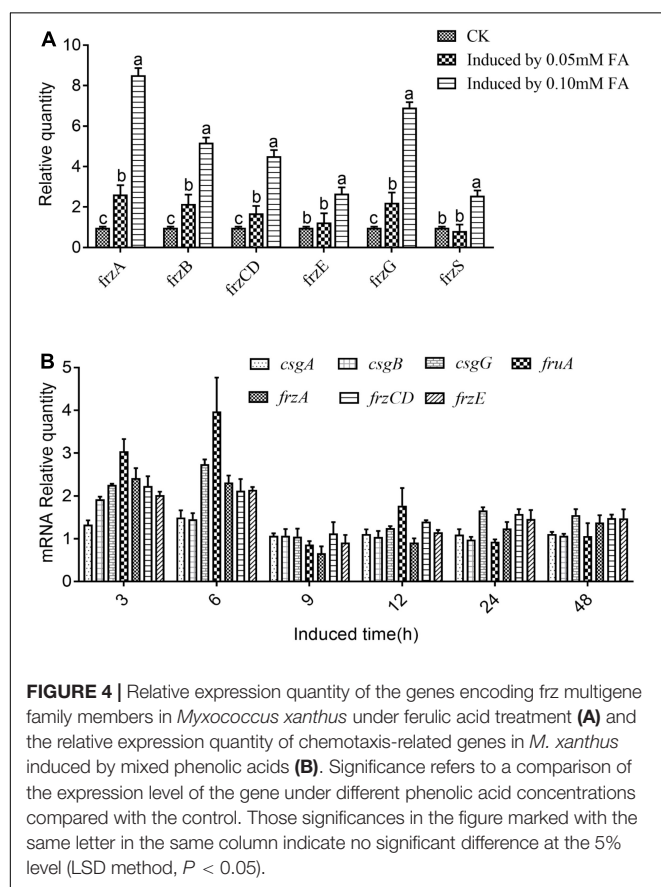
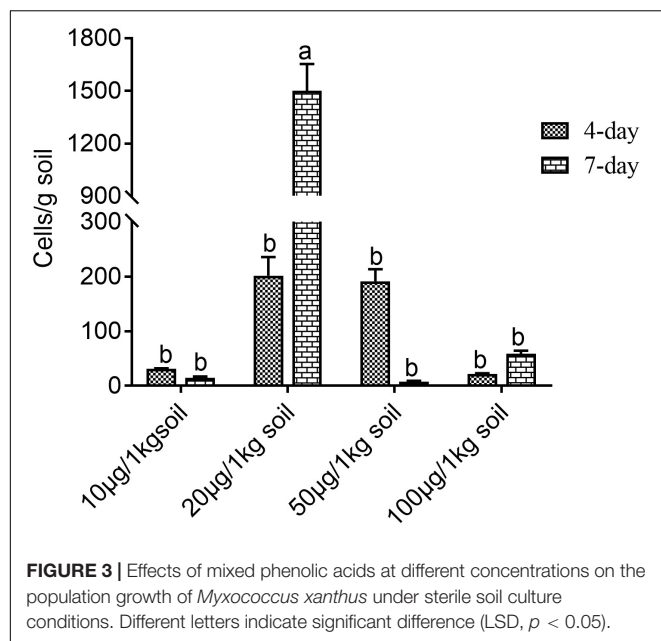
higher under the coculture with ferulic acid at 0.05 mM compared with the control, indicating that the up-regulated rate was 2.60-fold, 2.16-fold, 1.75-fold, and 2.23-fold higher. However, the change in the *FrzE* gene was not significant between the coculture treatment and the control. The up-regulated expressions of the *FrzA*, *FrzB*, *FrzCD*, *FrzE*, and *FrzG* genes in *M. xanthus* under the cocultured treatment with 0.10 mM ferulic acid were also increased by 8.53-fold, 5.16-fold, 4.52-fold, 2.70-fold, and 6.91-fold, respectively, compared with the control without coculture with 0.10 mM ferulic acid (Figure 4A).

qPCR analysis of the related chemotactic genes was conducted on *M. xanthus* cocultured with the mixtures of phenolic acids, as shown in Figure 4B. It was found that the up-regulated rate of each gene was higher than that of the control, and the highest gene expression level was detected in *M. xanthus* cocultured with phenolic acid for 3–6 h. *CsgA* was up-regulated in *M. xanthus* cocultured with the phenolic acid, by 2.09-fold and 2.76-fold compared with the control at 3 h and 6 h, respectively, and then the expression level dropped. *FruA* was also up-regulated in the coculture treatment, by 3.17-fold and 3.19-fold, compared with the control at 3 h and 6 h, respectively, and then almost returned

to its normal expression level. The expression level of *FrzCD* in *M. xanthus* cocultured with the phenolic acid at 3 h and 6 h was also increased, by 1.95-fold and 1.34-fold, respectively, compared with the control. However, in the coculture treatment for 9 h, the *FrzCD* expression level in *M. xanthus* decreased and was then up-regulated 1.99-fold compared with the control at 48 h. *CsgB*, *CsgG*, *FrzA*, and *FrzE* in *M. xanthus* showed a similar expression trend in response to the coculture treatment, indicating the highest up-regulated rate of those genes in *M. xanthus* cocultured with the phenolic acid at 3 h was 2.99-fold, 5.61-fold, 2.48-fold, and 3.48-fold, respectively, compared with the control without the addition of the phenolic acid. Subsequently, the expression levels of these genes were decreased gradually in the coculture treatment as shown in Figure 4B.

Allelopathic Effect of Rhizosphere Special *M. xanthus* on the Targeted Barnyard Grass

The result of the bioassay showed that for the targeted barnyard grass seeds, which were cocultured with the screened



myxobacterial strain in the medium, the germination rate was 18.89%, i.e., a significant decrease of 61.08% compared with the control, as shown in **Figure 5A**. We also observed a significant allelopathic interaction between rhizosphere special

myxobacteria and phenolic acids in coculture with the targeted barnyard grass (**Figure 5B**). The inhibition rate of barnyard grass seed germination and the stem length of the target plant was 8.25 and 21.89%, respectively, in the plate (LM) containing *M. xanthus*. The inhibition rate of barnyard grass seed germination and the target stem length increased to 19.70 and 43.59%, respectively, on the plate (LM + phenolic acid) containing *M. xanthus* and the phenolic acid mixture; these values were much higher than those (2.34%, 10.98%) for the plate containing only the phenolic acid mixture. *Escherichia coli* was used as control bacteria in this study (negative control, NC). The results showed that barnyard grass germination on the plate containing only *E. coli* was not significantly different from that of the control, and the target stem length was lower than that of the control but significantly higher than that in the other treatments (**Figure 5B**). The findings suggested that the combination of the phenolic acid mixture with the myxobacterial strain had the most significant inhibitory effect on the germination and seedling growth of the targeted barnyard grass, showing that the interaction between phenolic acid allelochemicals and *M. xanthus* had a significant impact on the allelopathic potential of rice.

To investigate the allelopathic inhibitory effect of *M. xanthus* on barnyard grass in soil, the effects of the treatments with *M. xanthus* and its fermentation broth at different added amounts on barnyard grass seed germination were determined under sterile soil conditions. The result showed that the inhibitory effect of added inoculant (5 mL) on the germination of barnyard grass seeds was significantly higher than in any other treatments, indicating a 53.4% inhibition rate in the suppression of the target weed at 4 and 7 days. It was also found that when we added 2 mL of the fermentation broth to the soil, the inhibition rate significantly reached 78.80 and 75.80% at 4 and 7 days, respectively, i.e., significantly higher than that in the addition treatment with 5 mL of the myxobacterial inoculant solution added to the sterile soil pre-seeded with the target barnyard grass (**Table 3**). These findings suggested that the effect of the myxobacteria on the growth of barnyard grass was based on the population density of the target plants in the soil, and the fermentation broth of *M. xanthus* showed the highest allelopathic potential for suppression of the target weeds, but this needs further study.

The Mechanism of Suppression of Barnyard Grass Under Coculture Treatment With Myxobacteria and the Optimal Mixture of Different Phenolic Acids

The analyses and identification of allelochemicals in different coculture samples were conducted by GC-QQQMS, and the results showed that the quantity and composition of the secondary metabolites in the myxobacterial fermentation broth were quite different under different conditions, such as in the medium with the mixture of the phenolic acids (PA₀) or with *M. xanthus* and the optimal mixture of phenolic acid (PA₁). Numerous compounds, such as ketones, esters, and aromatic and sulfur compounds, significantly increased in the coculture

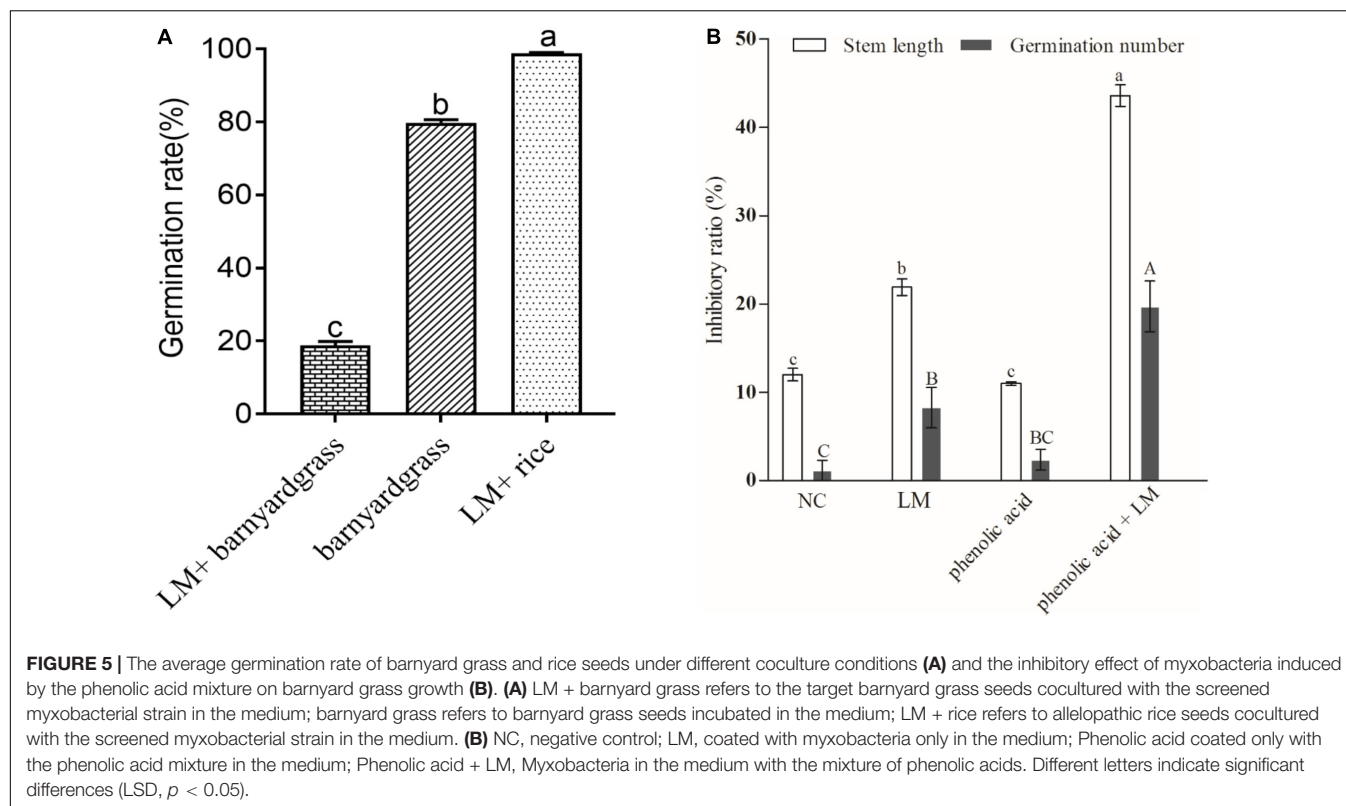


TABLE 3 | Effects of rhizosphere special myxobacteria and its fermentation broth at different dosages on the germination of barnyard grass added to sterile soil.

Co-culture days	Treatment	Germination rate (%)	Standard deviation	Inhibition rate (%)	Standard deviation
4D	CK	97.80	0.1361 A	2.30	0.1361 C
	2 mL M	71.00	0.0346 AB	29.00	0.0346 BC
	5 mL M	46.60	0.135 BC	53.40	0.135 AB
	10 mL M	51.20	0.1015 BC	48.80	0.1015 AB
	20 mL M	60.00	0.2000 ABC	40.00	0.2000 BC
7D	2 mL F	22.20	0.1015 C	78.80	0.1015 A
	CK	100.00	0.1155 A	0.00	0.1155 C
	2 mL M	73.40	0.0651 AB	26.70	0.0651 BC
	5 mL M	46.60	0.135 BC	53.40	0.1350 AB
	10 mL M	51.20	0.1015 BC	48.80	0.1015 AB
	20 mL M	73.40	0.2000 B	26.60	0.2000 BC
	2 mL F	24.20	0.0751 C	75.80	0.0751 A

CK, blank barnyard grass. M refers to the myxobacterial (*M. xanthus*) solution with barnyard grass. F refers to the fermentation broth of *M. xanthus* after 3 days, at a 2-mL dosage with barnyard grass. Different capital letters A, B, and C indicate significant differences (LSD, $p < 0.01$).

sample from the treatment of *M. xanthus* with the mixture of the different phenolic acids (PA₁) compared with the sample treated only with the phenolic acid (PA₀), for the determination and comparison of allelopathic inhibitory effects on the target weeds. Compared with those in the CK₀ and CK₁ treatments, the ketones and esters increased and alkanes/alkenes increased significantly under the treatment of PA₁, which suggested that the optimal mixture of different phenolic acids was able to promote the production of secondary metabolites in the specific myxobacteria under the coculture condition (Supplementary Tables S1–S5),

which increased its allelopathic potential of the suppression of the target weeds.

Further analysis showed that quercetin, a well-known allelochemical, was detected in the sample under PA₁ treatment, but not in the other treatment (PA₀), suggesting that it might interact with other toxic ketones and quinones to influence the herbicidal potential of the myxobacteria, as shown in Table 4.

Further results, shown in Figure 6, indicated that the inhibitory effect of the added quercetin at a concentration of 1.06×10^{-3} mM on barnyard grass stem length and root

TABLE 4 | Identification of the secondary metabolites in the fermentation broth of the rhizosphere special *Myxobacteria* in the treatment with the phenolic acid (PA₁).

Treatments	Molecular Formula	Name
PA ₁	C ₁₀ H ₁₆ O	2 – decalin ketone
	C ₄ H ₂ BrN ₅ O	5- brompyrazole - [3,4-d] -s- triazine -4(3H)- ketone
	C ₁₆ H ₂₄ O	Allyl ionone
	C ₁₃ H ₁₂ O ₅ S	2-(2, 5-dioxo tetrahydrofuran-3-group) thio-3,5, 6-trimethyl-p-quinone
	C ₁₉ H ₁₈ O ₆	6, 7-dimethoxy -3- [2-(2-methoxy phenyl)- 2-oxyethyl] -1(3H) – benzofuranone
	C ₃₀ H ₅₂ O	cycloxylenol
	C ₁₅ H ₁₂ O ₂	1-phenyl-3-m-hydroxyphenyl-allenone
	C ₁₆ H ₁₃ NO ₄	<i>Trans</i> -4'- methoxy -4- nitrochalcone
	C ₂₂ H ₂₁ N ₃ O	2, 3-dihydro-3 -(4-dimethylaminophenyl)- 2-phenyl-quinazoline -4(1H)- ketone
	C ₁₈ H ₂₂ N ₂ O	4- [[4-(diethyl amino)-2, 6-dimethyl-phenyl] imino] -2, 5-cyclohexadiene -1- ketone
	C ₆ H ₄ C ₁₂ O ₃	3, 4-dichloro-5-methoxy -2(5H) – furanone
	C ₁₅ H ₁₀ O ₇	Quercetin
	C ₁₄ H ₂₂ O ₂	4-(3-hydroxy-2,6, 6-trimethyl-cyclohexan-1-alkene) pente-3-alkene-2-ketone

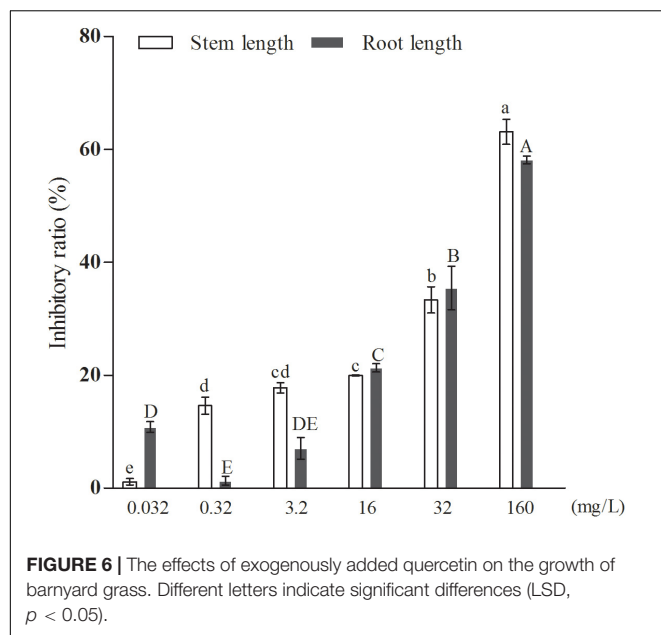
length was 12.68 and 14.17%, respectively, and all of them were significantly different compared with the control. When the concentration of the added quercetin was reduced 10 times, i.e., 1.06×10^{-4} mM, it had no inhibitory effect on barnyard grass stem length, while the inhibitory effect on the root length of the target plant was significant, with an inhibition rate of 32.91% in comparison with the control. When the dosage was increased by 10, 50, 100, and 500 times, i.e., in the coculture with the added quercetin at 1.06×10^{-2} mM, 0.053 mM, 0.106 mM, and 0.53 mM, respectively, the inhibition rate increased with the increase of the treated concentrations of quercetin in the suppression of the target weeds, indicating that the inhibition rates were 16.59, 17.80, 29.82, and 47.06% with respect to the suppression of the stem length of the target plant and 30.55, 34.59, 36.30, and 60.59% for the root length of the target plan. Based on these findings, it can be concluded that the effect of quercetin on the root length of the target plant was greater than that on the stem length of the target plant (barnyard grass).

DISCUSSION

Allelochemicals from root exudates have a significant influence on the biodiversity of soil microorganisms, and specific microbes interact with allelochemicals increasing the allelopathic inhibition on target weeds (Lin et al., 2007, 2011; Kong et al., 2008; Qu and Wang, 2008; Shi et al., 2011). The allelopathic rice accession PI attracted higher numbers of microbial populations in the rhizosphere soil than that of non-allelopathic rice accession Le, of which seven were identified as myxobacteria (Xiong et al., 2012). The higher contents of phenolic acids in the PI than in the Le was determinate in this study (Supplementary Table S6) and these compounds played roles in promoting the proliferation of specific microbe, i.e., *M. xanthus*. The indoor experiment showed that the exogenous ferulic acid (FA) increased the population of *M. xanthus*, and the combined activity of FA and *M. xanthus* exhibited the highest allelopathic inhibition of the barnyard grass, which was higher than that under the individual application of FA or *M. xanthus* (Fang et al., 2015).

Phenolic acid is mainly synthesized in the phenylalanine metabolism pathway (Ferrer et al., 2008), and this is one of the dominant pathways for the synthesis of rice allelochemicals; *OsPAL* is the first key catalyticase in the pathway. The activity of *OsPAL* contributes to the intensity of metabolism. Overexpression of *OsPAL2-1* in PI resulted in an enhanced rhizosphere microbial population and allelopathic inhibition of barnyard grass, whereas silencing of *OsPAL2-1* in PI led to a decrease of allelopathic inhibition and microbial population and diversity (Fang et al., 2015; 2013), indicating the vital role of *OsPAL2-1* in the regulation of rice allelopathic potential. *OsPAL2-1* of allelopathic rice was reported to be highly sensitive to environmental stress (such as low nitrogen and high weed density) compared with non-allelopathic rice (Wang et al., 2010; Lin, 2013; Zhang et al., 2018). An increase in the *OsPAL2-1* expression level resulted in a significant increase in the secretion of secondary metabolites, especially phenolic acids (Supplementary Table S7), which contributed to increased microbial populations in the rhizosphere.

Our comparative study on the difference in the rhizosphere microbial population of PI, PR, and PO, shows that *Myxococcus* spp. was significantly decreased in the rhizosphere soil of the *OsPAL2-1* gene-silenced rice line PR compared with its wild-type of PI (Figure 1). The *OsPAL2-1*-overexpressed transgenic line (PO) had significantly greater abundance of *M. xanthus* compared with PI (Figure 1), which was related to the process involved in the influence of the *OsPAL2-1* gene expression abundance on the synthesis and secretion of allelopathic rice rhizospheric phenolic acids, leading to proliferation of the myxobacteria in rhizospheric soil. The results *in vitro* interaction and sterile soil tests confirmed that the proliferation of *M. xanthus* could be promoted by some phenolic acids such as ferulic acid or a phenolic acid mixture in a given range of active dosages. Previous studies reported that myxococcales are known as metabolic factories and can produce a large number of secondary metabolites and can inhibit the germination of weeds and the proliferation of various harmful bacteria and fungi (Weissman and Müller, 2009). Ye et al. (2020) also documented that myxobacterium *Coralloccoccus* sp. strain EGB modified the



soil microbial community structure and reduced the quantities of *Fusarium oxysporum* f. sp. *cucumerinum* in the soil by predation, which makes the beneficial microbial community stable and reduces the occurrence of diseases.

Myxobacteria occur in a complex soil environment and rely on signaling pathways to regulate their cellular behavior to adapt to changing environments (such as physical, chemical, and biological stimuli). This behavior is called quorum sensing (QS). Some of the bacteria produce chemical signaling substances, whereas other cells use the chemical signals to determine population density and changes in the surrounding environment and respond accordingly by multiplying or by producing toxins. However, myxobacteria release signals that are not acyl-homoserine lactones (AHLs) or autoinducer-2; however, they produce and use the A-signal and C-signal (Whitworth, 2008) in response to environmental stress. Mcvittie first identified intercellular signals during myxobacterial growth using different mutants in 1962 (Mcvittie et al., 1962). So far, at least five kinds of intercellular signals have been identified: A (Asg), B (Bsg), C (Csg), D (Dsg), and E (Esg) (Downard et al., 1993; Kaiser and Warrick, 2011). The chemical properties of the A-signal and C-signal have been determined; both play important roles in the aggregation of vegetative cells and the early development of fruiting bodies.

In the present study, the chemotactic motility of the rhizosphere special myxobacterial strain could be directly observed under the induction of the phenolic acids using the slide method (data not shown). Further analysis demonstrated whether a single phenolic acid or mixture of phenolic acids could effectively induce the unregulated expression of chemotaxis-related genes in *M. xanthus* (Figures 4A,B). The results showed that ferulic acid at an appropriate concentration effectively stimulated the *Frz* family member automatic chemotaxis system of *M. xanthus*, which is conducive to the completion of the

chemotactic behavior of bacteria, thus promoting the aggregation of the specific bacteria in the rhizosphere soil of the allelopathic rice accession. The findings suggest that the regulation of the *OsPAL2-1* gene could significantly mediate the interaction of allelopathic rice with *M. xanthus* in rhizosphere soil, together with increased phenolic acid exudation in this process, which might play an important role in the chemotactic aggregation of the special microbe in the rhizosphere soil of allelopathic rice. In cucumber, the myxobacterium *Coralloccoccus* sp. was found migrating toward the roots and root exudates of the cucumber plants via chemotaxis (Ye et al., 2020). In this study, the phenolic acid mixture secreted from the allelopathic rice could promote the production of secondary metabolites of the myxobacteria (Supplementary Table S1), of which quercetin was determined as the main allelochemical, suggesting that it might interact with other toxic ketones and quinones to influence the herbicidal potential of the myxobacteria (Table 4).

CONCLUSION

In conclusion, this study suggested that allelopathic rice could affect the metabolism of phenolic acid allelochemicals via the regulation of the key gene *OsPAL2-1*. The phenolic acids secreted by allelopathic rice into soil could mediate the myxobacteria to gather themselves together in the rhizosphere, through which the production of a large number of secondary metabolites with allelopathic activity begins. One such example is quercetin, which is a potential allelochemical, generating from the FA-induced *M. xanthus* cultured medium and playing role in suppression of weed germination and growth. This could be considered an underlying reaction of rhizosphere biochemistry in rice allelopathy. However, the manner in which myxobacteria regulate their own physiological and biochemical reactions to achieve a high aggregation effect (quorum sensing effect) in a short time remains unknown. Therefore, in-depth answers to these questions are of great theoretical and practical significance for the ultimate understanding of the rhizosphere biological process and molecular ecological mechanism of rice allelopathy, which can also provide a scientific basis for the research and development of safer and more efficient regulation methods to improve the allelopathic inhibition of weeds in paddy fields.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

WL and YiL conceived the study and wrote the manuscript. YiL, XJ, YuL, and XZ performed the experiments. YiL and LX performed the statistical analyses. MK revised the manuscript. All of the authors discussed the results and commented on the manuscript, and they have approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.01411/full#supplementary-material>

FIGURE S1 | HPLC of quercetin and the sample.

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FIGURE S2 | The morphology of fruiting bodies (A) of the strain induced by rabbit feces and the morphology of fruiting bodies (B) and the colony (C) of the purified strain.

TABLE S1 | The secondary metabolites of *Myxobacteria* induced by different allelochemicals.

TABLE S2 | The secondary metabolites in the blank culture medium (CK₀).

TABLE S3 | The secondary metabolites of *M. xanthus* in the blank culture medium (CK₁).

TABLE S4 | The secondary metabolites in the blank culture medium with the mixture of phenolic acids (PAO).

TABLE S5 | The secondary metabolites of *M. xanthus* in the coculture with different phenolic acid mixtures (PA₁).

TABLE S6 | Concentrations of phenolic acids in root washings of Le and PI in a normal hydroponic culture system and under BYG stress (μg/L).

TABLE S7 | Concentration of total phenolics in root and leaf tissues measured using the Folin-Ciocalteu method (mg/g Root/Leaf fresh weight).

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Climatic Aridity Gradient Modulates the Diversity of the Rhizosphere and Endosphere Bacterial Microbiomes of *Opuntia ficus-indica*

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Recent microbiome research has shown that soil fertility, plant-associated microbiome, and crop production can be affected by abiotic environmental parameters. The effect of aridity gradient on rhizosphere-soil (rhizosphere) and endosphere-root (endosphere) prokaryotic structure and diversity associated with cacti remain poorly investigated and understood. In the current study, next-generation sequencing approaches were used to characterize the diversity and composition of bacteria and archaea associated with the rhizosphere and endosphere of *Opuntia ficus-indica* spineless cacti in four bioclimatic zones (humid, semi-arid, upper-arid, and lower-arid) in Tunisia. Our findings showed that bacterial and archaeal cactus microbiomes changed in inside and outside roots and along the aridity gradient. Plant compartment and aridity gradient were the influencing factors on the differentiation of microbial communities in rhizosphere and endosphere samples. The co-occurrence correlations between increased and decreased OTUs in rhizosphere and endosphere samples and soil parameters were determined according to the aridity gradient. *Blastococcus*, *Geodermatophilus*, *Pseudonocardia*, *Promicromonospora*, and *Sphingomonas* were identified as prevailing hubs and were considered as specific biomarkers taxa, which could play a crucial role on the aridity stress. Overall, our findings highlighted the prominence of the climatic aridity gradient on the equilibrium and diversity of microbial community composition in the rhizosphere and endosphere of cactus.

Keywords: *Opuntia ficus-indica*, bacterial and archaeal microbiome, aridity gradient, rhizosphere soil, endosphere root, co-occurrence network

INTRODUCTION

Semi-arid and arid zones occupy nearly 40% of the world's terrestrial surface and are the most vulnerable to climate changes (Schlaepfer et al., 2017; Canter, 2018). These zones are characterized by high temperatures and low precipitations, and they are probably the most threatened by desertification. Abiotic stress, including high and low temperature, water deficit (drought) and flooding, salinity, UV radiation, light, and poor nutrient soils, have a significant impact on soil

fertility and subsequently on agricultural production in semi-arid and arid regions (Shahid and Al-Shankiti, 2013; Cuevas et al., 2019; Hussain et al., 2019).

Cacti (*Cactaceae*) represent one of the most xerophyte plants that have their origin in arid and semi-arid ecosystems of central Mexico and the American continent (Hernandez-Hernandez et al., 2014). *Cactaceae* are an extremely diverse family of plants among which the genus *Opuntia* is the most numerous (Hernandez-Hernandez et al., 2014). Several morphological and physiological adaptations have been developed by the genus *Opuntia* in arid environments where water is the main factor limiting the development of most plant species. Prominent among these adaptations is the Crassulacean acid metabolism (CAM), a water-efficient type of photosynthesis, which allows plants to fix carbon dioxide through the night and prevent water loss during the day (Nobel, 2010). Besides, their morphologies are characterized by succulent bodies, thick epidermis of cladodes covered with spines, and shallow and extensive root systems that enable cacti to exploit scarce rainfall and high temperatures. These physiological and biochemical strategies developed by cacti have permitted them to resist and spread under conditions of high temperatures and scarce and erratic rainfall (Nefzaoui et al., 2010, 2014). They play a significant role in the protection of local fauna in arid ecosystems. Moreover, plantations of cactus for fruit and forage production have been developed in all continents (Nefzaoui et al., 2010). The increasing interest in cactus cultivation, particularly *Opuntia ficus-indica*, is due to its implication in the future achievement of sustainable agriculture in arid and semi-arid zones. It is widely documented that a significant diversity of microbes associated with plants form a biological unit named “holobiont” (Bordenstein and Theis, 2015; Vandenkoornhuysen et al., 2015). These microorganisms can affect host fitness, development, adaptation, survival, and distribution of plants. Several previous studies, based on culture-dependent and culture-independent approaches, reported that bacteria and fungi live in different plant compartments, and some of them are involved in promoting plant growth and/or tolerance to abiotic stress in arid ecosystems. Numerous factors influencing the plant microbiome, such as the host genotype, the physical and chemical properties of soil, the seasonal variations, the agricultural practices, the plant compartment, the biogeography of the plant species, and the plant development, have been studied using high-throughput sequencing technologies (Coleman-Derr et al., 2016; Fonseca-García et al., 2016; Eida et al., 2018; Lee et al., 2019; Zheng and Gong, 2019). However, despite many studies carried out using culture and uncultured-based methods to characterize cactus-associated microbiome in arid zones, the effect of aridity on microbiome assembly has not been fully explored. Several studies have cataloged the endophytic bacteria and fungi colonizing intercellular spaces in various internal plant tissues of cactus species in different countries (Bezerra et al., 2017). Besides fungal endophytes, endophytic bacteria and bacteria on the rhizoplane of cacti from the desert areas in Mexico have been described by other studies, revealing the effect of the association of bacteria, archaea, and fungi on seed germination and cactus persistence in their natural environment (Puente et al., 2004a,b, 2009a,b; Fonseca-García et al., 2016;

Bezerra et al., 2017). Cactus endophytic plant growth-promoting bacteria (PGPR) are known to facilitate plant growth by promoting production of phytohormones (auxins, gibberelins, cytokinins, and ethylene), diazotrophic fixation of nitrogen, and solubilization of phosphate. Moreover, they enhance the plant's tolerance to various stresses, such as high salinity, drought, metal toxicity, pesticide load, and activity against a broad spectrum of phytopathogens (Puente et al., 2009a; Lopez et al., 2011; Kavamura et al., 2013). Two rhizobacteria related to the genus *Bacillus* were isolated from Brazilian cacti exhibiting plant growth-promotion abilities under drought conditions (Kavamura et al., 2013).

In North Africa, particularly in Tunisia, wild and cultivated populations of *Opuntia ficus-indica* (*Cactaceae*) exist from north to south in all bioclimatic zones, from the humid to the semi-arid zones (Nefzaoui and Salem, 2001). It is mainly cultivated for fruit production and forage, particularly in upper-arid regions (Nefzaoui et al., 2010). The investigation on microbial communities and the mechanisms by which particular bacteria associated with cactus plants induce abiotic stress tolerance in plants is still poorly understood. The main focus of the present research was to characterize the prokaryotic (bacteria and archaea) community's diversity and composition in *Opuntia ficus-indica* using DGGE and high-throughput sequencing (Illumina MiSeq) approaches. The impact of the aridity gradient and soil physical and chemical properties on the diversity and the composition of the prokaryotic communities associated with spineless cacti were evaluated.

MATERIALS AND METHODS

Sampling

Prickly pear trees (*Opuntia ficus-indica* f. *inermis*) growing in wild areas protected by the forest administration in Tunisia were selected for this study: rhizosphere soil (5 mm of soil adhering to the root surface) and root endosphere (the interior of the root). The studied locations were chosen to represent an increased aridity gradient characterized by a decreased total annual precipitation and mean temperature ranging from 1200 to 100 mm (Verner et al., 2018) and from 18 to 20.2°C (meteorological stations in Tunisia), respectively, and covering four bioclimatic zones (humid, semi-arid, upper-arid, lower-arid) (Table 1). The latitude and longitude for each site were noted using a GPS device during the field work (Table 1). Roots collected from cactus plants aged between 3 and 4 years were considered as young wild plantations. The height of cactus plants is about 3–3.5 m. After digging until the first primary root appeared, the lateral fine roots were collected. Both rhizosphere soil samples and approx. 30 g of roots were collected early March 2015 from healthy plants. All samples were collected in sterile bottles, transported in cold conditions to the laboratory, and kept aseptically at –80°C until analysis. At each sampling site, three to five distinct replicates were collected. To avoid any contamination with left root fragments, rhizospheric samples were sifted by a 2 mm sieve to remove all rocks, roots, and large debris. Collected root samples were washed by shaking during

TABLE 1 | Geographic positions and annual precipitation of selected *Opuntia ficus-indica* plantation fields.

Bioclimatic stage	Annual precipitation (mm)	Annual mean temperature (°C)	Altitude (m)	Sample name	Sample location name	GPS coordinates	
						Latitude	Longitude
Humid	800–1200	18	<10	BZ	Bizerte	37.1227	9.54539
				BA1		36.5056	10.5222
Semi-arid	400–600	19.5	90–150	BA2	Bou Argoub	36.5073	10.4164
				BA3		36.5056	10.4322
				KA1		35.3806	9.3004
		20	400–600	KA2	Kairouan	35.3942	9.2859
				KA3		35.4008	10.005
				KS1		35.2112	8.4806
				KS2		35.2111	8.4750
Upper-arid	200–400	19	900–1000	KS3	Kasserin	35.2223	8.4631
				KS4		35.2234	8.4632
				KS5		35.2244	8.4632
				KS6		35.2921	8.6621
				SB1		35.3123	9.2129
		20	200–400	SB2	Sidi Bouzid	35.1719	9.2701
				SB3		35.3123	9.2701
				GF1		34.1504	9.1716
				GF2		34.1502	9.1713
Lower arid	100–200	20.2	100–150	GF3	Gafsa	35.5547	10.1754

Rhizosphere and endosphere samples were collected along an increased aridity and passed through four bioclimatic stages ranging from humid to lower-arid sites.

30 min in sterilized water after a 2 min wash in 1% sodium azide to eliminate epiphytic microorganisms.

Soil Measurements

Soil properties were measured as previously described (Bao, 2000), including total organic carbon (TOC) following the method of Walkley (1947), total nitrogen (TN) using the modified Kjeldahl procedure (Liao, 1981), and total soil phosphorus (TP) using the Mehlich method (Föhse et al., 1991), and the ionic contents of potassium (K^+), calcium (Ca^{2+}), and iron (Fe^{3+}) were determined using the atomic absorption method (Lindsay and Norvell, 1978). Soil pH was measured at a water-to-soil mass ratio of 1:1 using a pH meter with an adjusted combined glass electrode (FE20, Mettler-Toledo Instruments, China). Soil moisture (MO) was determined gravimetrically by weighing after drying in an oven at 105°C for 10 h (Craze, 1990).

DNA Extraction

MO BIO PowerSoil DNA Isolation Kit (MO BIO Laboratories) was used to extract total community DNA from 250 mg pellet of rhizosphere and powdered endosphere roots according to the manufacturer's protocol. Subsequently, all DNAs were quantified using NanoDrop System (2000; Thermo Fisher Scientific).

PCR-DGGE and Sequence Analysis

The V3–V5 hypervariable regions of the 16S rRNA gene were amplified using a bacteria-specific primer set (341FGC/907rR, Muyzer et al., 1993, 1995). The PCR products were analyzed by denaturing gradient gel electrophoresis (DGGE) using the DCode multiple system (Bio-Rad). DGGE was carried out as

previously described using a denaturing gradient of 35–65% denaturants in 6% (w/v) polyacrylamide gel (BenAbdallah et al., 2016). Predominant DGGE bands were excised, reamplified, and sequenced. Sequences and phylogenetic analysis of the rRNA 16S sequences were performed as previously reported (BenAbdallah et al., 2018).

Illumina Sequencing and Data Processing

Sequencing of PCR amplicons of 16S rDNA was conducted with the Illumina MiSeq platform (CBS, Sfax, Tunisia) targeting the V3–V4 hypervariable regions using prokaryotic universal (Pro341/Pro805R) primer sets (Takahashi et al., 2014) for 2 × 300 bp paired-end sequencing (Illumina). The amplification of PCR products and Illumina library preparations were achieved as previously reported (BenAbdallah et al., 2018). Raw data acquired from the Illumina MiSeq sequencing platform were analyzed using the QIIME software package 1.9.1 (Caporaso et al., 2010) as previously described (BenAbdallah et al., 2018). Plant chloroplast and mitochondrial OTUs were removed from all sequences samples.

Statistical Analysis

Significant differences between physicochemical parameters of soil across the different bioclimatic zones were calculated using the HSD–Tukey test. The relative abundance of each phylum, class, and order in different rhizosphere and endosphere samples was summarized in histogram graphs using R software environment (v.3.2.5) (R Core Team, 2017), and the significant

differences were determined using HSD Tukey statistical test. A Venn diagram was constructed using the package Venn Diagram in R (v 3.2.5). α -diversity indices (Simpson and number of observed OTUs) were determined after normalizing to a depth of 1000 reads and were calculated by QIIME (Caporaso et al., 2010) using ANOVA test analysis. Differences in β -diversity between the samples were tested through permutational multivariate analysis of variance (PERMANOVA, 5000 permutations) and visualized using non-metric multidimensional scaling (Langfelder and Horvath, 2012). All heat maps were drawn by the “aheatmap” function in the “NMF” package of R¹. Network analysis was performed on sample OTUs and soil properties. Prior to analysis, OTUs were classified based on their abundances whether increased or decreased, following strictly ($>$ or $<$ 0.9) the aridity gradient in the endosphere and rhizosphere samples, using the humid relative abundance data as a reference. Co-occurrence of OTUs was defined based on their Pearson/Spearman correlations using the WGCNA package (Oksanen et al., 2013). The node shapes represent increased or decreased OTUs in the endosphere or rhizosphere; the node was colored by taxonomy, and the edges connecting the nodes represent correlations between OTU pairs and soil properties. Negative and positive co-occurrence relationships based on strength of correlation at $r \leq -0.9$ and ≥ 0.9 and $p \leq 0.05$. All P -values were adjusted for multiple testing using the Benjamini and Hochberg FDR controlling procedure (Benjamini et al., 2006). Networks were created and visualized with the open source platform Cytoscape 3.7.1.

Data Access

Bacterial sequence data of the DGGE bands has been submitted to GenBank, and the assigned accession numbers were from MK208464 to MK208475. 16S raw reads have been deposited in the Short Read Archive of NCBI under project no. PRJNA511384.

RESULTS

Soil Physicochemical Analysis

The pH, soil water content, humidity%, organic matter%, nitrogen%, calcium, potassium, iron, phosphate, and capacity exchange cation (CEC) parameters were measured using standard soil analytic methods. Soil characteristics associated with spineless *Opuntia ficus-indica* are presented in **Table 2** and **Supplementary Figure S1**. A significant difference in the parameter values or percentages between bioclimatic zones (humid, semi-arid, upper-arid, and lower-arid) was observed. For instance, Fe^{3+} , K^+ , TP, and CEC concentrations and the% of humidity and TOC decreased while the Ca^{2+} concentration increased concomitantly with the aridity gradient (HSD Tukey test $p < 0.05$; **Supplementary Table S1**). pH and the total nitrogen were comparable in all bioclimatic zones (HSD Tukey test $p < 0.05$; **Supplementary Table S1**).

Prokaryotic Diversity Profiles in the Endosphere and Rhizosphere of *Opuntia ficus-indica*

The composition and diversity of rhizosphere and endosphere prokaryotic communities were assessed by next-generation sequencing of 16S ribosomal RNA gene amplicons. After quality filtering and removal of potential chimera's sequences, approximately 1,709,770 high-quality sequences with an average read length of 450 bp were obtained for further analyses. All sequences were classified at phylum level, except for proteobacterial groups, which were further divided by class (**Figure 1A**). Sequences obtained were assigned to thirteen bacterial phyla and one archaea. The global structure of the prokaryotic communities found from each sample is shown in **Figure 1A**. In rhizosphere samples, the bacteria domain was mainly represented by Proteobacteria (6.96–40.43%) followed by Actinobacteria (19.42–60.69%), Firmicutes (7.26–41.43%), Chloroflexi (0.3–16.22%), Acidobacteria (0.16–5.67%), Bacteroidetes (0.28–4.82%), Cyanobacteria (0.01–2.92%), Gemmatimonadetes (0.04–2.23%), and Verrucomicrobia (0.06–1.28%). In the Proteobacteria phylum, Alphaproteobacteria (5.09–32.15%) and Betaproteobacteria (0.43–21.86%) are clearly dominating in all samples, followed by Gammaproteobacteria (0.18–2.08%) and Deltaproteobacteria (0.16–1.56%) classes. Other phyla with low abundances ($<1\%$ of all sequences) were Nitrospirae (0–0.41%), TM7 (0.006–0.41%), Armatimonadetes (0–0.06%), and Tenericutes (0–0.04%) (**Figure 1A**). Proteobacteria (26.29–82.48%), Cyanobacteria (1.22–49.46%), Actinobacteria (9.40–40.97%), Bacteroidetes (0.55–7.28%), Acidobacteria (0.006–5.63%), Firmicutes (0.43–5.22%), TM7 (0.42–4.25), Chloroflexi (0.30–3.91%), and Verrucomicrobia (0.07–2.05%) represent the major phyla in endosphere samples. Proteobacteria, the dominant phylum, was divided into four classes: Gammaproteobacteria (0.69–42.10%) followed by Betaproteobacteria (2.28–41.10%), Alphaproteobacteria (7.70–35.89%), and Deltaproteobacteria (0.08–3.24%). Tenericutes (0–0.11%), Armatimonadetes (0–0.09%), and Gemmatimonadetes (0–0.05%) were detected in lower abundance ($<1\%$ of all sequences, **Figure 1A**). Within the domain Archaea, a low relative abundance in the rhizosphere (0–7.22%) and endosphere (0–0.56%) were retrieved. Only six OTUs, belonging to the classes Halobacteria and Methanomicrobia within the Euryarchaeota phylum were detected. Among Halobacteria, 1 and 2 OTUs were affiliated with the genera *Haloarcula* and *Halorubrum*, respectively.

Our results revealed that Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, Bacteroidetes, Cyanobacteria, and Verrucomicrobia dominated in rhizosphere and endosphere samples, whereas Gemmatimonadetes and TM7 were only abundant in the rhizosphere and endosphere, respectively. The comparison of the relative abundance between the rhizosphere and endosphere exhibited that Actinobacteria, Firmicutes, and Chloroflexi phyla were more abundant in rhizosphere samples than in endosphere samples. However, Cyanobacteria, Proteobacteria (especially Gammaproteobacteria, and Betaproteobacteria classes) and TM7 phyla were more

¹<http://nmf.r-forge.r-project.org/aheatmap.html>

TABLE 2 | Principal characteristics of soil samples of spineless *Opuntia ficus-indica* collected across the four bioclimatic zones (humid, semi-arid, upper-arid, and lower-arid).

Bioclimatic stage	Sample name	pH	Humidity (%)	TP (mg/l)	TOC (%)	TN (%)	Fe ³⁺ (mg/kg)	Ca ²⁺ (mg/kg)	K ⁺ (mg/kg)	CEC (mol/kg)
Humid	BZ	74.3	3.91	13.01	4.44	0.45	20.86	4775.63	78.88	19.80
	BA1	7.88	4.06	22.70	1.72	0.39	18.58	1080.46	86.19	8.80
Semi-arid	BA2	6.85	0.08	5.14	0.26	0.70	17.85	1400.71	103.63	0.95
	BA3	7.36	2.07	13.92	0.99	0.545	18.21	1240.58	94.91	4.87
	KA1	6.68	4.31	1.30	0.52	0.46	11.58	2227.83	88.29	2.35
	KA2	6.65	6.05	13.33	1.34	0.56	12.21	2293.08	98.48	7.70
	KA3	6.61	2.56	3.48	0.47	0.52	11.58	2227.83	88.29	1.75
	KS1	5.74	2.08	1.49	0.60	1.41	13.26	2599.17	91.52	5.10
	KS2	7.82	6.52	1.14	1.69	1.56	12.93	3142.08	79.09	13.00
	KS3	7.61	1.52	1.21	0.54	0.70	16.01	1298.42	50.75	4.30
Upper-arid	KS4	6.42	1.81	1.00	0.74	0.88	13.95	1452.50	51.84	4.45
	KS5	6.46	2.13	1.10	1.39	0.72	12.93	3142.08	79.09	5.90
	KS6	6.74	1.47	0.72	0.59	0.40	16.01	1298.42	50.75	5.00
	SB1	8.19	6.81	2.15	1.21	0.51	14.53	4812.92	170.45	6.75
	SB2	7.99	3.82	1.88	0.95	0.65	14.34	6004.17	163.68	7.45
	SB3	8.00	8.87	0.92	1.44	0.46	14.53	4812.92	170.45	20.55
	GF1	8.22	4.24	3.16	2.24	0.61	17.36	4923.75	131.03	8.00
	GF2	7.77	6.04	18.39	4.70	0.46	17.45	5740.42	274.63	11.50
Lower arid	GF3	8.34	3.63	6.64	1.96	0.57	17.36	4923.75	131.03	7.75

K⁺, Potassium ion; Ca²⁺, calcium ion; Fe³⁺, iron ion; TOC, pH, total organic carbon; TN, total nitrogen; TP, total phosphorus; H, humidity; CEC, cation exchange capacity.

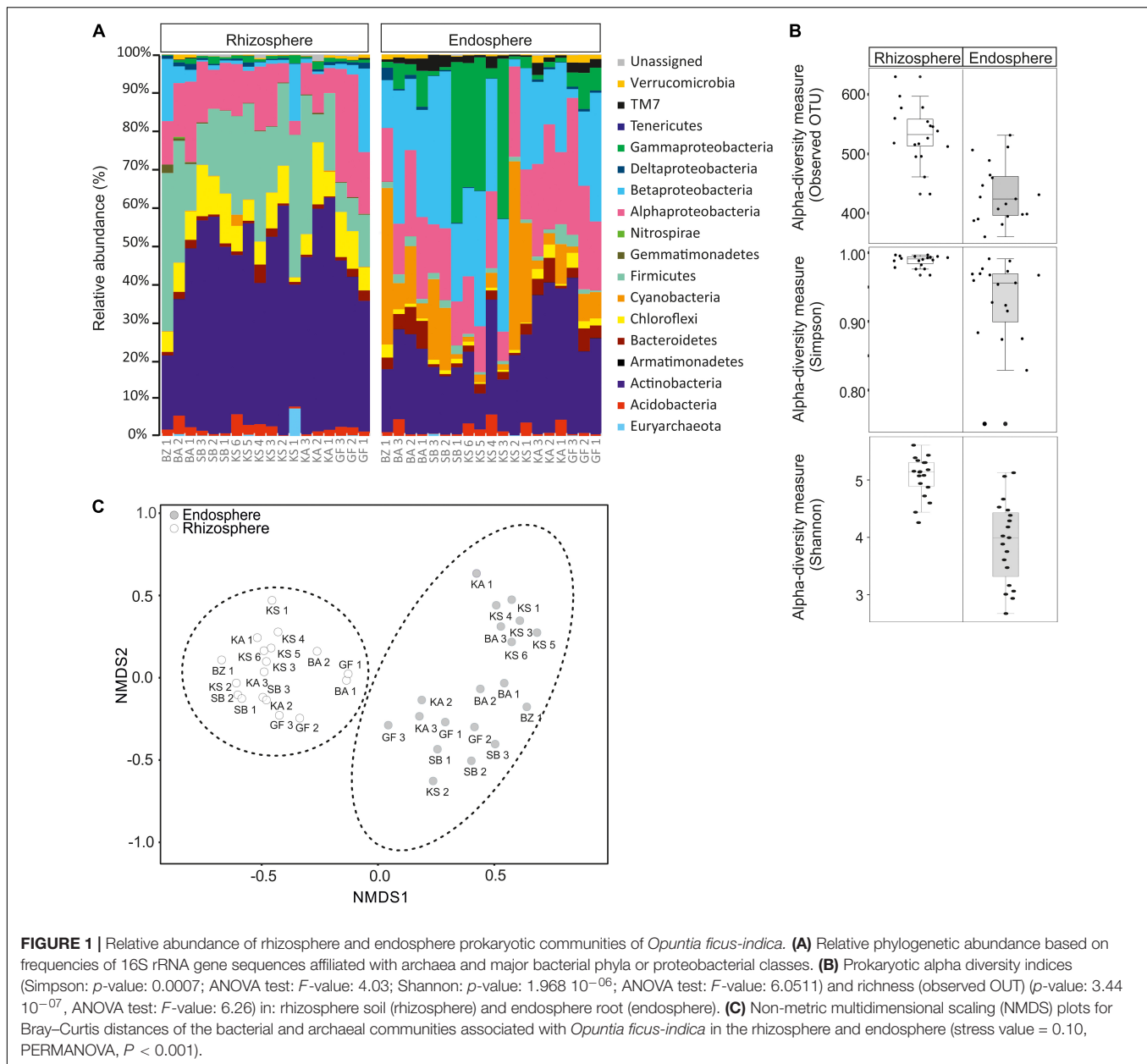
dominant in endosphere than rhizosphere samples. Furthermore, rhizosphere samples displayed a higher prokaryotic richness (i.e., observed OUT, **Figure 1B**) and diversity (Simpson and Shannon indices, **Figure 1B**) than endosphere samples. All of these results suggest that the community composition was highly related to sample type (rhizosphere or endosphere). In order to identify community structure and co-occurrence patterns between different samples, a non-metric multidimensional scaling (NMDS) analysis based on the distribution of prokaryotic structure at OTU level was performed (**Figure 1C**). The NMDS analysis showed two distinct groups associated with rhizosphere and endosphere samples.

The Prokaryotic Microbiome Diversity Structure of the Cactus Endosphere and Rhizosphere in the Different Bioclimatic Zones

In order to explore the effect of the aridity degree on the prokaryotic microbiome community structure and composition, DGGE and high-throughput sequencing of 16S rRNA genes analysis were investigated. DGGE profiles from rhizosphere and endosphere samples are presented in **Supplementary Figure S2**. Eight and four bands from rhizosphere and endosphere samples were excised, reamplified, and sequenced. The results of band DGGE sequences obtained from rhizosphere samples within the bacterial domain show that sequences were regrouped in the Firmicutes, Actinobacteria, and Proteobacteria (Betaproteobacteria class) phyla (**Supplementary Table S2**). All bands were closely related to the genera *Bacillus*, *Arthrobacter*, *Rubrobacter*, *Geodermatophilus*, and *Frankia*

(95–99% similarity). In endosphere samples, all band DGGE sequences were clustered within Betaproteobacteria class and Actinobacteria phylum (**Supplementary Table S2**). *Burkholderia*, *Massilia*, *Streptomyces*, and *Amycolatopsis* genera were identified in endosphere samples.

Using the high-throughput sequencing approach (MiSeq Illumina), the rRNA 16S sequences acquired from all samples were clustered according to their bioclimatic zones and analyzed at the phylum level (**Figure 2A**). The relative abundance of populations related to Alphaproteobacteria and Actinobacteria were significantly enriched along the aridity gradients in both rhizosphere and endosphere (HSD Tukey test $p < 0.05$; **Supplementary Table S3**). However, Betaproteobacteria and Verrucomicrobia were enriched in the endosphere and rhizosphere, respectively (HSD Tukey test $p < 0.05$; **Supplementary Table S3**). Firmicutes, Gemmatimonadetes, and Betaproteobacteria decreased along the aridity degree in the rhizosphere, and Cyanobacteria and Deltaproteobacteria decreased in the endosphere (HSD Tukey test $p < 0.05$; **Supplementary Table S3**). Our findings revealed that rhizosphere and endosphere prokaryotic microbiomes change across the climatic aridity gradient, which is supported by NMDS analysis based on the distribution of prokaryotic composition at the OTU level in each compartment (**Figure 2B**). Distinct groups associated with different bioclimatic zones were observed in the rhizosphere and endosphere. Alpha diversity (richness and diversity) of prokaryotic communities in all bioclimatic zones in both rhizosphere and endosphere samples was analyzed (**Figure 2C**). Our results showed that the semi-arid bioclimatic zone presents the highest richness in the rhizosphere and endosphere. In the rhizosphere, the diversity



(Simpson and Shannon indices) of prokaryotic communities was similar among all bioclimatic zones. In the endosphere sample, the highest diversity was observed in the lower-arid zone, the most arid zone. The highest diversity was observed in the humid zone whatever the sample (rhizosphere or endosphere). Prokaryotic assembled analysis along the aridity gradient at the OTU level was investigated. Samples from humid, semi-arid, upper-arid, and lower-arid bioclimatic zones shared a high-degree overlap (551 and 433 OTUs) in rhizosphere and endosphere samples, respectively (Figure 2D). The Venn diagram showed specific number of OTUs for each aridity zone, and their taxonomic affiliation changed following the aridity gradient as presented in Figure 2D. The higher numbers of OTUs were observed in the upper-arid bioclimatic zone in

rhizosphere and endosphere samples, respectively (153 and 249 OTUs). Their representative OTU sequences were mainly related to the Actinobacteria phylum. To get better insight into the differences of the dominant prokaryotic community across the five different bioclimatic zones in the rhizosphere and endosphere, we applied heat map analyses of the most abundant OTUs ($>1\%$ of all sequences), which highlighted their relative distributions and abundances (Figure 3). Interestingly, among the abundant OTUs ($>1\%$), the rhizosphere was dominated by twenty-five abundant OTUs, affiliated to Alphaproteobacteria, Betaproteobacteria, Actinobacteria, Firmicutes, and Gemmatimonadetes phyla (Figure 3). Furthermore, twenty-eight representative OTUs related to Proteobacteria (Alpha, Beta, Gamma, and Delta), Actinobacteria,

Cyanobacteria, and Chloroflexi phyla (**Figure 3**) were detected in endosphere samples. These dominant OTUs belonging to genera *Sphingomonas*, *Ralstonia*, *Burkholderia*, *Pseudarthrobacter*, *Actinoplanes*, *Pseudonocardia*, and *Streptomyces* were detected in both rhizosphere and endosphere. Specific species associated with the rhizosphere were related to *Skermanella*, *Microvirga*, *Bacillus*, *Tumebacillus*, *Longimicrobium*, *Rubrobacter*, *Geodermatophilus*, and *Blastococcus* genera while *Amycolatopsis*, *Promicromonospora*, *Humibacter*, *Ktedonobacter*, *Loriellopsis*, *Sinorhizobium*, *Rhizobium*, *Agrobacterium*, *Rhodoligotrophos*, *Massilia*, *Phaselicystis*, *Pseudomonas*, *Escherichia*, and *Erwinia*, genera, which were only present in endosphere samples. Additionally, among these dominant OTUs, some rhizosphere or endosphere OTUs increased, in inside and outside roots, across the aridity gradient and became more and more abundant in the lower-arid, the most arid zone (**Figure 3**). Within Actinobacteria, three rhizosphere OTUs (966091, 453616, and 731014) and two endosphere OTUs (4465539 and 509487) were related to *Blastococcus*, *Geodermatophilus*, *Actinoplanes*, *Promicromonospora*, and *Pseudonocardia* genera, respectively. Moreover, two other endosphere OTUs (965129 and 696234) belonged to *Sphingomonas* and *Agrobacterium* within the Alphaproteobacteria class.

Bacterial Correlation Networks of the Cactus Rhizospheric Under Increased and Decreased Aridity Degree

Bacterial OTUs, which could involve the increased and decreased OTUs following the aridity degree in both rhizosphere and endosphere samples, were classified and determined based on their relative abundance ($>0.1\%$ of all sequences) (**Supplementary Table S4**). Heat map analysis was applied to display all increased and decreased bacterial OTUs in rhizosphere and endosphere samples following the aridity gradient (**Supplementary Figure S3**). Interestingly, our results demonstrated that rhizosphere and endosphere samples exhibited forty-eight and fifty-two increased OTUs, respectively, among which seven OTUs were shared by both rhizosphere and endosphere samples. The increased OTUs were closely related to Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Alphaproteobacteria, and Betaproteobacteria. Furthermore, increased OTUs affiliated with Cyanobacteria and TM7 groups were identified in endosphere samples while one OTU related to unidentified prokaryotic microbe was recognized in the rhizosphere (**Supplementary Figure S3** and **Supplementary Table S4**). Eight and seventeen decreased OTUs were revealed in the rhizosphere and endosphere, respectively. These OTUs were affiliated with Acidobacteria, Actinobacteria, Chloroflexi, Firmicutes, and Alphaproteobacteria. Moreover, the TM7 group was detected in rhizosphere samples while Bacteroidetes, Verrucomicrobia, and Cyanobacteria were observed in endosphere samples (**Supplementary Figure S3** and **Supplementary Table S4**). In inside and outside roots (rhizosphere and endosphere), specific increased and decreased OTUs were found displaying the effect of aridity on the

composition of rhizosphere and endosphere communities associated with spineless cacti.

These findings allowed constructing co-occurrence patterns of potential interactions that could firstly occur between bacterial taxa related to increased and decreased OTUs following the aridity degree present in the rhizosphere and endosphere in spineless cactus and secondly with soil environmental parameters. Clearly, non-random co-occurrence patterns of bacterial communities were identified. Two subnetworks (the increased OTUs following aridity degree and the decreased OTUs following aridity degree subnetworks) constructed based on correlation were visualized on the network (**Figure 4**). The increased OTUs following the aridity degree subnetwork were characterized by a strong co-occurrence between increased OTU in rhizosphere and endosphere samples (**Supplementary Figure S4** and **Supplementary Table S5**). These were later negatively correlated with potassium. The first subnetwork was negatively correlated with the second subnetwork (the decreased OTUs following the aridity degree subnetwork). The decreased OTU following the aridity degree subnetwork in rhizosphere and endosphere samples displayed a positive and robust interaction with iron, humidity, and phosphate. Positive correlation was also revealed between environmental parameters: nitrogen, phosphate, organic matter, and the cation exchange capacity of the soil. Iron and humidity were positively correlated whereas potassium was negatively correlated with them. The increased OTUs following the aridity degree subnetwork exhibited strong co-occurrence bacterial patterns mostly related to Actinobacteria and Alphaproteobacteria, followed by Firmicutes, Chloroflexi, Betaproteobacteria, Bacteroidetes, TM7, Cyanobacteria, and unidentified prokaryotes. The top twenty-two rhizosphere and endosphere OTUs with a high degree of connectivity (≥ 56) were designated (**Table 3**). Among them, five increased OTUs, previously identified as dominant OTUs and possessing a positive correlation and a strong interaction between them, were selected. Two rhizosphere-increased OTUs (966091 and 453616) were related to bacterial species *Blastococcus* and *Geodermatophilus*, and three endosphere-increased OTUs (731014, 4465539, and 965129) were affiliated with *Pseudonocardia*, *Promicromonospora*, and *Sphingomonas* genera (**Table 3**). Our results suggest that bacterial species related to these five hubs could play a major role on aridity stress.

DISCUSSION

Composition and Diversity of Prokaryotic Communities Associated With Spineless Cacti: Differences Between Rhizosphere and Endosphere

Plant-associated microbes are recognized to have several beneficial effects on host plants, but they are affected by a wide variety of environmental and host-related factors, including compartment, host genotype and phenotype, geographic site, season, and soil chemistry. Our work consists in evaluating the composition of prokaryotic communities associated

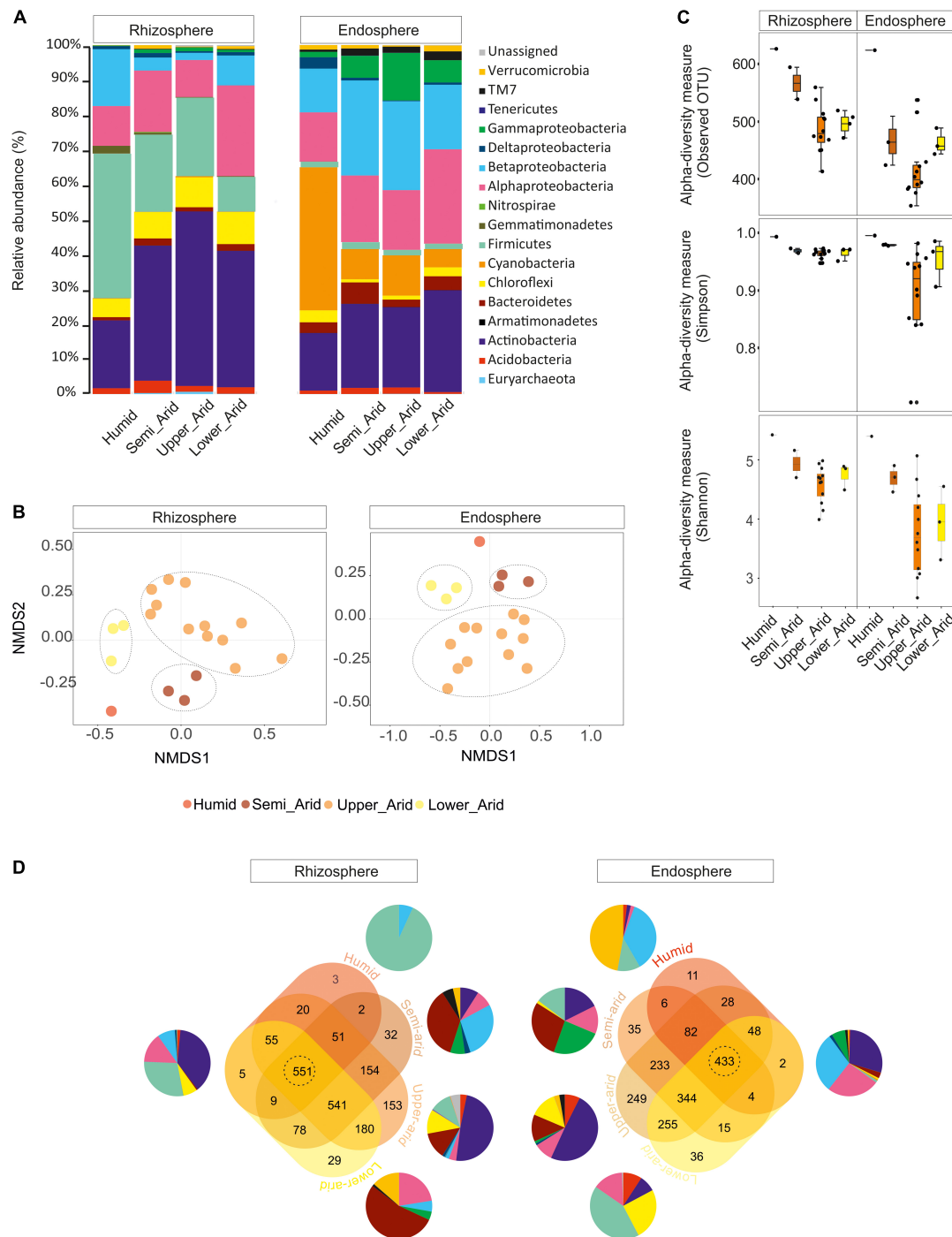
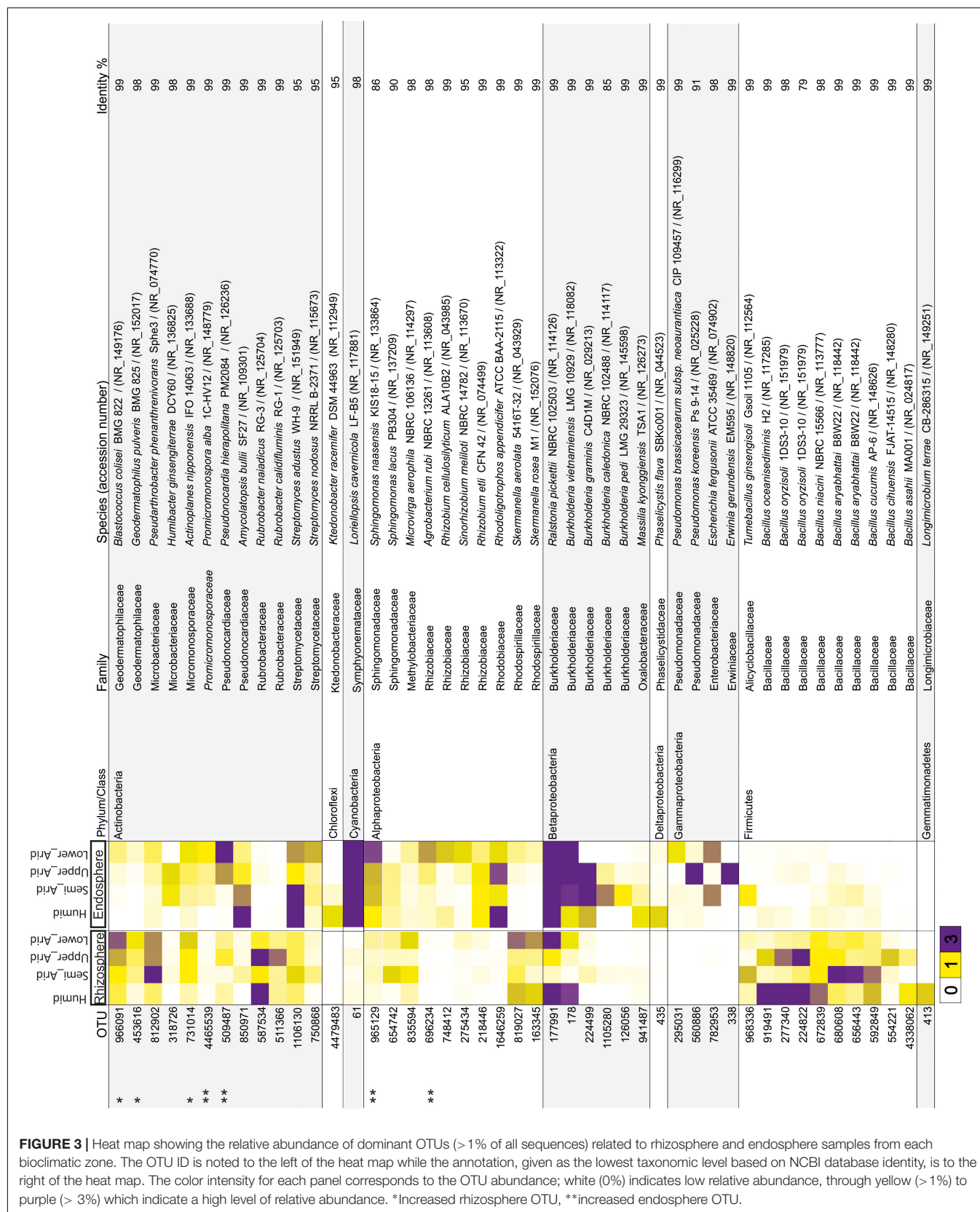


FIGURE 2 | Relative abundance of rhizosphere and endosphere prokaryotic communities of *Opuntia ficus-indica* across the different bioclimatic zones (humid, semi-arid, upper-arid, lower-arid). **(A)** Relative phylogenetic abundance based on frequencies of 16S rRNA gene sequences affiliated with archaea and major bacterial phyla or proteobacterial classes. **(B)** Non-metric multidimensional scaling (NMDS) plots for Bray-Curtis distances of the prokaryotic communities associated with *Opuntia ficus-indica* across bioclimatic zones in the rhizosphere (stress value = 0.122, PERMANOVA, $P < 0.001$) and endosphere (stress value = 0.093, PERMANOVA, $P < 0.03$). **(C)** Prokaryotic alpha diversity indices (Simpson: p -value: 0.0024; ANOVA test: F -value: 4.24; Shannon: p -value: 1.9713×10^{-05} , ANOVA test: F -value: 8.0451) and richness (observed OTU) (p -value: 3.13×10^{-06} , ANOVA test: F -value: 9.83) in the rhizosphere and endosphere from each bioclimatic zone. **(D)** Venn diagrams showing the distribution of prokaryotic measurable OTUs associated with *Opuntia ficus-indica* in the rhizosphere and endosphere from each bioclimatic zone and shared OTUs as described for **(A)**. Samples from the humid, semi-arid, upper-arid, and lower-arid bioclimatic zones shared a high-degree overlap (551 and 433 OTUs) in the rhizosphere and endosphere, respectively. Specific number of OTUs for each bioclimatic zone and their taxonomic affiliation were changed across the aridity gradient. The higher numbers of OTUs (153 and 249 OTUs) were observed in the upper-arid bioclimatic zone in rhizosphere and endosphere samples, respectively.



Increased OTUs following aridity degree subnetwork

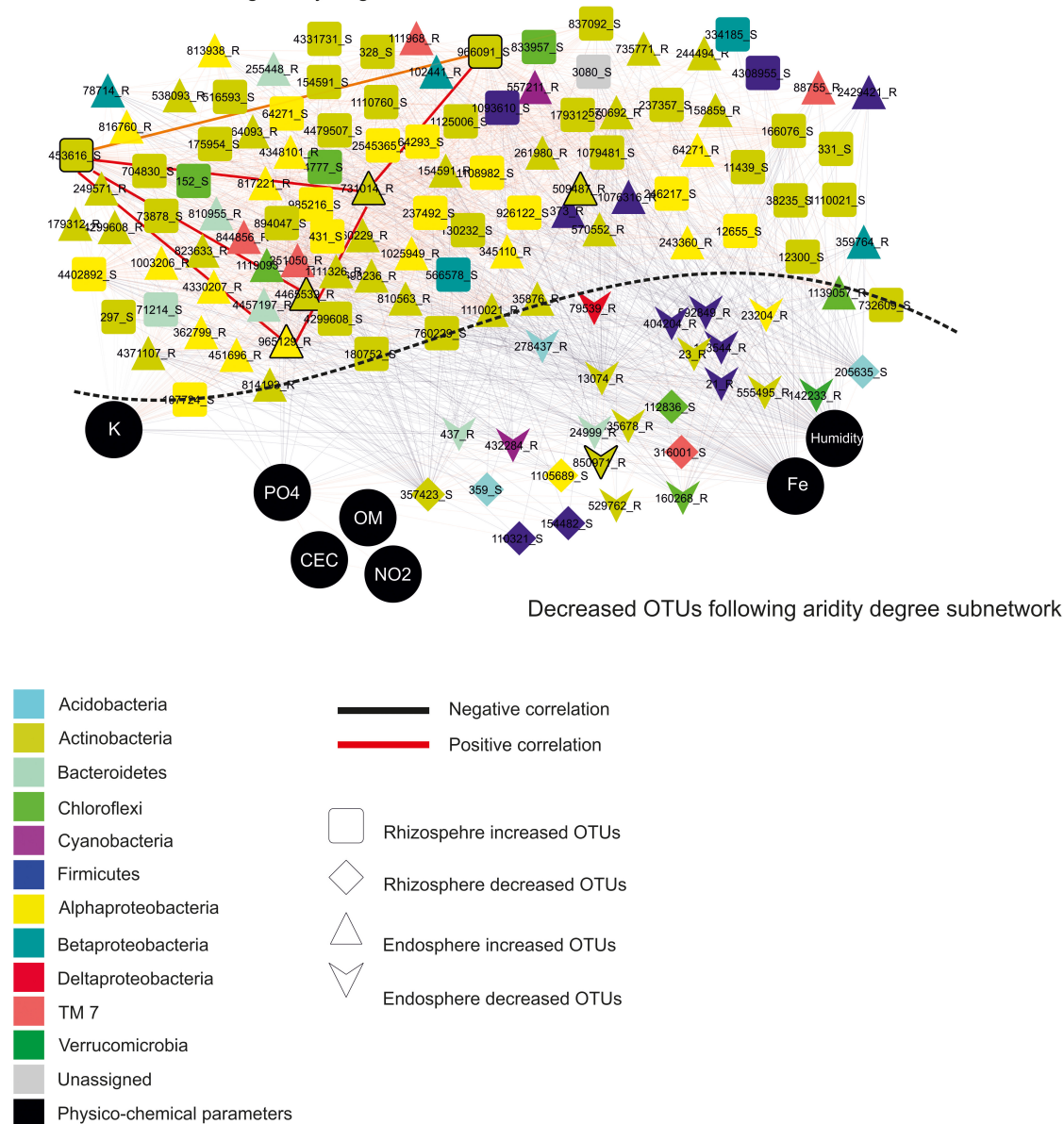


FIGURE 4 | Network topology plots of *Opuntia* bacterial communities associated with the rhizosphere and endosphere: Increased OTUs following the aridity degree subnetwork and decreased OTUs following the aridity degree subnetwork. Nodes: physicochemical parameters are represented by black circles, square: rhizosphere-increased OTUs, diamond: rhizosphere-decreased OTUs, triangle: endosphere-increased OTUs, and heart: endosphere-decreased OTUs. Lines connecting two nodes represent significant correlations: red represents a positive correlation (co-presence), and black represents a negative correlation (mutual exclusion).

with the rhizosphere and endosphere of spineless *Opuntia ficus-indica*. Proteobacteria, Actinobacteria, Firmicutes, Chloroflexi, Acidobacteria, Bacteroidetes, Cyanobacteria, and Verrucomicrobia bacterial phyla dominated both the rhizosphere and endosphere of spineless cacti (Figure 1A). Actinobacteria, Bacteroidetes, Firmicutes, and Proteobacteria have been reported to be the dominating phyla in diverse plant-associated communities including *Arabidopsis* (Lundberg et al., 2012), *Populus* (Shakya et al., 2013), maize (Peiffer et al., 2013),

grape (Zarraonaindia et al., 2015), rice (Edwards et al., 2015), and cacti and agave species (Desgarenes et al., 2014; Coleman-Derr et al., 2016; Fonseca-García et al., 2016, 2018). Furthermore, Acidobacteria has been identified as among the most represented phyla associated with the rhizosphere and the root endosphere of *M. geometrizans* and *O. robusta* (Fonseca-García et al., 2016). Moreover, Chloroflexi and Cyanobacteria were known by their multiple adaptations to environmental harshness such as soil biological crusts in arid environments (da Rocha et al., 2015;

TABLE 3 | Top twenty-two bacterial hubs for the increased and decreased OTUs following the aridity gradient subnetworks associated with *Opuntia*.

	OTU	Degree	Increased	Decreased	Taxonomy (phylum/class; family)	Species (accession number)	Similarity (%)
Rhizosphere	328	89	x		Actinobacteria; Parviterribacteraceae	<i>Parviterribacter kavangonensis</i> D16/0/H6 (NR_148601)	95.28
	966091*	86	x		Actinobacteria; Geodermatophilaceae	<i>Blastococcus colisei</i> BMG 822 (NR_149176)	99.33
	1093610	84	x		Firmicutes; Alicyclobacillaceae	<i>Tumebacillus luteolus</i> UC13 (NR_145584)	98.28
	166076	81	x		Actinobacteria; Rubrobacteraceae	<i>Rubrobacter spartanus</i> HPK2-2 (NR_158052)	93.10
	4402892	71	x		Alphaproteobacteria; Rhodospirillaceae	<i>Niveispirillum fermenti</i> CC-LY736 (NR_126263)	92.31
	453616*	71	x		Actinobacteria; Geodermatophilaceae	<i>Geodermatophilus pulveris</i> BMG 825 (NR_152017)	97.98
	237492	70	x		Alphaproteobacteria; ylobacteriaceae	<i>Chelativorans composti</i> Nis3 (NR_113183)	96.14
	704830	67	x		Actinobacteria; Geodermatophilaceae	<i>Geodermatophilus africanus</i> CF 11/1 (NR_108882)	98.43
	357423	57		x	Actinobacteria; Micromonosporaceae	<i>Krasilnikovia cinnamomea</i> 3-54(41) (NR_041337)	98.88
Endosphere	570692	123	x		Actinobacteria; Micromonosporaceae	<i>Phytohabitans flavus</i> K09-0627 (NR_113542)	98.88
	1110021	101	x		Actinobacteria; Geodermatophilaceae	<i>Modestobacter muralis</i> MDVD1 (NR_145554)	99.10
	251050	86	x		Epsilonproteobacteria; Helicobacteraceae	<i>Helicobacter brantae</i> MIT 04-9366 (NR_043799)	81.35
	1119093	81	x		Chloroflexi; Sphaerobacteraceae	<i>Sphaerobacter thermophilus</i> DSM 20745 (NR_074379)	85.65
	13074	76		x	Alphaproteobacteria; Sphingomonadaceae	<i>Sphingomonas agri</i> HKS-06 (NR_159163)	97.05
	844856	76	x		Firmicutes; Clostridiaceae	<i>Alkaliphilus metalliredigens</i> QYMF (NR_074633)	81.15
	1025949	71	x		Alphaproteobacteria; Phyllobacteriaceae	<i>Phyllobacterium myrsinacearum</i> NBRC 100019 (NR_113874)	99.32
	154591	70	x		Actinobacteria; Pseudonocardiaceae	<i>Actinophytocola oryzae</i> GMKU 367 (NR_116313)	98.65
	4465539*	68	x		Actinobacteria; Promicromonosporaceae	<i>Promicromonospora alba</i> 1C-HV12 (NR_148779)	99.33
	965129*	63	x		Alphaproteobacteria; Sphingomonadaceae	<i>Sphingomonas carotinifaciens</i> L9-754 (NR_159247)	99.09
	509487*	56	x		Actinobacteria; Pseudonocardiaceae	<i>Pseudonocardia hierapolitana</i> PM2084 (NR_126236)	99.33
	4371107	56	x		Actinobacteria; Conexibacteraceae	<i>Conexibacter stalactiti</i> YC2-25 (NR_157993)	98.49

*Relative abundance of OTU > 1% of all sequences.

Maestre et al., 2015; Čapková et al., 2016; Zhang et al., 2016). Within the domain Archaea, these populations were not abundant in both the rhizosphere and endosphere of *Opuntia ficus-indica*. Findings similar to ours have been previously reported in plant-associated communities of agaves and cactus studies (Fonseca-García et al., 2016, 2018). However, different situations were also reported where Archaea were abundant such as in rice (Edwards et al., 2015) and olive (Müller et al., 2015). In this study, profiles of prokaryotic distribution associated with spineless *Opuntia ficus-indica* were clearly distinct between inside and outside roots (rhizosphere and endosphere samples), which is the major selective force shaping plant–microbe interactions in arid and semi-arid habitats. This is consistent with findings

described for agaves and cacti (Desgarennés et al., 2014; Coleman-Derr et al., 2016; Fonseca-García et al., 2016, 2018).

The Impact of the Aridity Gradient on Microbial Community Composition in Spineless Cacti

Under the background of climate change, the decrease in precipitation, the increase in temperature, and drought frequency in dryland environments are among the major factors influencing the composition of soil microbial communities, as well as yield and crop quality. In this study, we investigated the effect of the geographic location and the degree of aridity on

the prokaryotic microbiome associated with spineless *Opuntia ficus-indica*. Hence, we analyzed the prokaryotic community structure from the rhizosphere and endosphere sampled from four locations across an aridity gradient including humid, semi-arid, upper-arid, and lower-arid (Figure 2). Our results revealed a significant variation of the prokaryotic community composition as a function of the aridity gradient and geographic location in both rhizosphere and endosphere samples. Alphaproteobacteria and Actinobacteria were significantly enriched with the increased degree of aridity in both the rhizosphere and endosphere. Betaproteobacteria and Verrucomicrobia phyla were only enriched in the endosphere and rhizosphere, respectively. However, Firmicutes and Betaproteobacteria decreased in the rhizosphere; Cyanobacteria and Deltaproteobacteria in endosphere. Thereby, we suggest that the prokaryotic community profiles in the rhizosphere and endosphere were altered considerably and differently according to the degree of aridity and the geographic location. Previous studies with diverse experiments evaluating the impact of drought stress on soil and root-associated microbiome revealed that Actinobacteria phyla, typically dominant in dryland environments, were significantly enriched in response to drought stress (Santos-Medellín et al., 2017; Ochoa-Hueso et al., 2018). Similar to our findings, Maestre et al. (2015) reported that Alphaproteobacteria increased with aridity.

The impact of the aridity gradient on spineless cactus-associated microbiome was also demonstrated at the OTU level where major OTUs detected in the rhizosphere and endosphere are as presented in Figure 3. Among these dominant OTUs, we identified that rhizosphere and endosphere increased the OTUs across the aridity gradient, which may play a significant role in stress and adaptation to aridity. Rhizosphere-increased OTUs were related to genera *Blastococcus*, *Geodermatophilus*, *Actinoplanes*, and *Promicromonospora* (Actinobacteria); endosphere-increased OTUs to genera *Promicromonospora*, *Pseudonocardia* (Actinobacteria), *Sphingomonas*, and *Agrobacterium* (Alphaproteobacteria class). *Sphingomonas*, *Burkholderia*, *Ralstonia*, *Bacillus*, *Geodermatophilus*, *Streptomyces*, *Amycolatopsis*, *Promicromonospora*, *Agrobacterium*, *Massilia*, *Pseudomonas*, and *Erwinia* are well-known as plant growth-promoting rhizobacteria or PGPR (Gopalakrishnan et al., 2015; Lamizadeh et al., 2016; Sathya et al., 2017; Gupta et al., 2018). Two rhizobacteria related to the genus *Bacillus* were isolated from Brazilian cacti, displaying plant growth-promotion abilities under drought conditions (Kavamura et al., 2013). *Sphingomonas*, *Burkholderia*, *Ralstonia*, *Bacillus*, *Rubrobacter*, *Blastococcus*, *Streptomyces*, *Amycolatopsis*, *Promicromonospora*, and *Pseudomonas* have been recognized as endophytic bacteria (Kandel et al., 2017; Singh and Dubey, 2018; Kuźniar et al., 2019). Endophytic bacteria and rhizoplane communities in cacti from the desert areas in Mexico have been identified by previous studies, revealing the effect of the association of bacteria, archaea, and fungi on seed germination and survival of cacti in their harsh natural environment (Puente et al., 2004a,b, 2009a,b; Fonseca-García et al., 2016).

Factors Affecting the Assembly of Microbial Communities in Spineless Cacti Following the Aridity Gradient

We identified increased and decreased OTUs in inside and outside roots (rhizosphere and endosphere samples) across the aridity gradient by referring to their differential abundance (Supplementary Figure S3 and Supplementary Table S4). Decreased OTUs were affiliated to Actinobacteria, Acidobacteria, Firmicutes, Chloroflexi, Alphaproteobacteria (in both rhizosphere and endosphere samples), Verrucomicrobia, Bacteroidetes, Cyanobacteria (only in endosphere), and TM7 (only in the rhizosphere). However, increased OTUs belonged to Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Bacteroidetes, Firmicutes and Chloroflexi (in both rhizosphere and endosphere), TM7, and Cyanobacteria (in endosphere). Dominant increased bacterial OTUs were related to Actinobacteria and Alphaproteobacteria. They were previously reported by other researchers studying the effect of drought stress on rice root-associated microbiome (Santos-Medellín et al., 2017). Our results indicated that microorganisms related to the increased bacterial OTUs could play an important role in the tolerance to aridity in the rhizosphere and endosphere associated with spineless cacti in the arid zone. These results suggest that microorganisms affected by aridity are sample type specific.

Co-occurrence correlation was performed firstly between increased and decreased OTUs and secondly between physicochemical soil parameters and increased and decreased OTUs according to the degree of aridity in both the rhizosphere and endosphere (Figure 4). A positive correlation between increased bacterial OTUs in the endosphere and rhizosphere was detected in co-occurrence patterns. However, potassium presents a negative correlation with them. Decreased bacterial OTUs were correlated positively with environmental variables such as iron, humidity, and phosphate, suggesting that they were affected by these physicochemical parameters. In water-limited soil, the decrease in ion contents including sodium, calcium carbonate, and potassium was reported (Bachar et al., 2010). In diverse experiments analyzing the influence of several factors on soil bacteria, chemical properties including pH and ion content have stressed an important role in describing community structure (Chodak et al., 2015; Gunnigle et al., 2017; Hartmann et al., 2017; Naylor and Coleman-Derr, 2018). Bacteria and nutrients are able to be transported more facily via water which is limited in dry environments (Abu-Ashour et al., 1994). Dominant bacterial OTUs among all increased bacterial OTUs identified in the subnetwork of increased aridity degree in rhizosphere and endosphere samples are related to Actinobacteria and Alphaproteobacteria. These results suggest that these groups are able to tolerate drought conditions. Some bacterial species related to the Actinobacteria phylum are able to form spores to resist to desiccation and survive under drought conditions (Singh et al., 2007). Several studies have reported that the enrichment of Alphaproteobacteria and Actinobacteria in the rhizosphere where carbon is available was increased due to root exudates (Smalla et al., 2001; Fierer et al., 2007). PhyloChip

hybridization analysis revealed that Alphaproteobacteria and Actinobacteria were more abundant in semi-arid deserts (Ding et al., 2013). Bacterial species isolated from the rhizosphere of three cactus plant species (*Mammillaria carnea*, *Opuntia pilifera*, and *Stenocereus stellatus*) were mainly affiliated with Alphaproteobacteria, Actinobacteria, and Firmicutes (Aguirre-Garrido et al., 2012). Torres-Cortés et al. (2012) demonstrated that Actinobacteria was the most dominant phyla in the rhizosphere of the cactus species *Mammillaria carnea* during the dry season using the pyrosequencing method. Our findings displayed that bacterial species related to *Blastococcus*, *Geodermatophilus*, *Pseudonocardia*, *Promicromonospora*, and *Sphingomonas* genera formed five dominant hubs. *Blastococcus* and *Geodermatophilus* were increased especially in the rhizosphere whereas *Pseudonocardia*, *Promicromonospora*, and *Sphingomonas* were more abundant in the endosphere across the aridity gradient. These hubs could play a principal role in the arid zones associated with spineless cacti. Indeed, several studies reported that these strains were often associated with deserts and biocrust habitats and their adaptation to extreme environmental conditions, such as high salt concentration, low relative humidity, and high UV radiation, has been described (Hussain et al., 2019; Pombubpa et al., 2019). Moreover, bacterial species related to these genera have been defined as endophytes associated with arid plants and some of them possess the ability to protect the host plants and promote their growth (Trujillo et al., 2015; Singh and Dubey, 2018). These endophytic bacteria may play crucial roles in maintenance and sustenance of specific arid habitats.

CONCLUSION

In the present work, we used a next-generation sequencing approach to assess bacterial and archaeal diversity and composition in rhizosphere and endosphere samples associated with *Opuntia ficus-indica* spineless cacti along an increased aridity gradient in Tunisia. Our findings revealed that microbial composition and abundance were linked to the rhizosphere and endosphere. Interestingly, Actinobacteria, Firmicutes, and Chloroflexi phyla were more abundant in the rhizosphere and inversely Cyanobacteria, Proteobacteria (especially Gammaproteobacteria and Betaproteobacteria classes), and TM7 phyla were more dominant in the endosphere. Along the climatic aridity gradient, Alphaproteobacteria and Actinobacteria were significantly increased in both rhizosphere and endosphere. However, Betaproteobacteria and Verrucomicrobia were enriched in the endosphere and rhizosphere, respectively. Similarly, Firmicutes and Betaproteobacteria decreased in the rhizosphere; Cyanobacteria and Deltaproteobacteria in the endosphere. Indeed, specific OTUs were detected in each bioclimatic zone. These results suggest that the aridity gradient potentially shaped the diversity and composition of bacteria and archaea in the rhizosphere and endosphere associated with spineless cactus. Interestingly, in rhizosphere and endosphere samples increased and decreased OTUs along the aridity gradient were identified and their co-occurrence in correlation with soil parameters was investigated. Five hubs related to *Blastococcus* and *Geodermatophilus* (in rhizosphere) and to *Pseudonocardia*,

Promicromonospora, and *Sphingomonas* (in endosphere) may be considered as indicators of aridity. Thus, we predict that the climatic aridity will shift significantly the cactus microbial diversity in the rhizosphere and endosphere and occurring taxa being specialized to this zone. The effect of other abiotic (salinity, elevation, spatial...) and biotic (diverse species) parameters on the bacterial and fungal microbiome associated with cacti will be monitored.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Bacterial sequence data of the DGGE bands has been submitted to GenBank and the assigned accession numbers were from MK208464 to MK208475. 16S Raw reads have been deposited in the Short Read Archive of NCBI under project no. PRJNA511384.

AUTHOR CONTRIBUTIONS

FK, MG, AM, and SS conceived and designed the study. AC coordinated the sampling and performed the soil physicochemical analyses. NM performed the molecular analyses. FK and MG performed the bioinformatic and statistical analyses. FK wrote the original draft of the manuscript. All authors read and contributed to the review and editing of 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/fmicb.2020.01622/full#supplementary-material>

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Phenolic Acids Released in Maize Rhizosphere During Maize-Soybean Intercropping Inhibit *Phytophthora* Blight of Soybean

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Interspecies interactions play a key role in soil-borne disease suppression in intercropping systems. However, there are limited data on the underlying mechanisms of soil-borne *Phytophthora* disease suppression. Here, a field experiment confirmed the effects of maize and soybean intercropping on *Phytophthora* blight of soybean caused by *Phytophthora sojae*. Experimentally, the roots and root exudates of maize were found to attract *P. sojae* zoospores and inhibit their motility and the germination of cystospores. Furthermore, five phenolic acids (*p*-coumaric acid, cinnamic acid, *p*-hydroxybenzoic acid, vanillic acid, and ferulic acid) that were consistently identified in the root exudates and rhizosphere soil of maize were found to interfere with the infection behavior of *P. sojae*. Among them, cinnamic acid was associated with significant chemotaxis in zoospores, and *p*-coumaric acid and cinnamic acid showed strong antimicrobial activity against *P. sojae*. However, in the rhizosphere soil of soybean, only *p*-hydroxybenzoic acid, low concentrations of vanillic acid, and ferulic acid were identified. Importantly, the coexistence of five phenolic acids in the maize rhizosphere compared with three phenolic acids in the soybean rhizosphere showed strong synergistic antimicrobial activity against the infection behavior of *P. sojae*. In summary, the types and concentrations of phenolic acids in maize and soybean rhizosphere soils were found to be crucial factors for *Phytophthora* disease suppression in this intercropping system.

Keywords: intercropping, *Phytophthora sojae*, phenolic acids, interference, infection behavior

INTRODUCTION

Intercropping, or the practice of growing two or more crops in the same field, is widely used in Asia, Latin America, and Africa, providing as much as 15–20% of the global food supply (Machado, 2009; Lithourgidis et al., 2011). Intercropping can increase yield stability by increasing species diversity of farmland ecosystems but also effectively alleviate the prevalence of and damage by

pests and diseases (Ratnadass et al., 2012; Lv et al., 2018). Many previous studies have shown that intercropping could control the occurrence of airborne crop diseases by forming a physical barrier, diluting pathogens, and improving field microclimates while effectively inhibiting soil-borne diseases through root interactions (Zhu et al., 2000, 2005; Yang et al., 2014). For example, maize/pepper, tomato/chives, watermelon/rice, and wheat/broad bean intercropping can reduce the damage from pepper *Phytophthora* blight, tomato bacterial wilt, watermelon Fusarium wilt, and wheat take-all, respectively (Yu, 1999; Hao et al., 2010; Wang et al., 2016).

Interspecies interactions that occur during intercropping can lead to the suppression of soilborne diseases. Plant roots interact with many soil-inhabiting microbes that can colonize them and provide plants with key functions for plant longevity and fitness (Pascale et al., 2020). Root-derived exudates act as vital food sources or signals for microbes, and they not only support microbial proliferation in the rhizosphere but are also responsible for the formation of distinct microbial assemblages between soil and the rhizosphere (Berendsen et al., 2012; Lombardi et al., 2018; Olanrewaju et al., 2019). For example, benzoxazinoids and triterpenes from plant root exudates could optimize the microbial community in the plant rhizosphere, which helped plants to resist pathogens (Hu et al., 2018; Huang et al., 2019). Coumarins can attract *Pseudomonas* into the plant rhizosphere and then also reshape the composition of the microbiome community around the roots (Stringlis et al., 2019; Pascale et al., 2020). Intercropped maize has been found to cause a twofold increase in flavonoid exudations and increased soybean nodulation by *Rhizobium* (Li B. et al., 2016).

Apart from supporting beneficial associations with soil-inhabiting microbes, non-host plant roots could also interfere with the infections caused by pathogenic microorganisms in intercropping systems (Hao et al., 2010; Lv et al., 2018; Rolfe et al., 2019). Previous studies have found that maize roots could attract *Phytophthora capsici* zoospores, simultaneously inhibiting zoospore swimming and cystospore generation, which helps peppers to resist *Phytophthora* blight (Yang et al., 2014). This phenomenon has also been found in the interaction between other non-host plant species and *Phytophthora* (Fang et al., 2016; Jiang et al., 2017), and it may be an important factor that is involved in soil-borne *Phytophthora* pathogen suppression in intercropping systems. Thus, we infer that the key compounds of non-host root exudates may mediate the interactions of pathogen infection processes, and their underlying mechanisms remain to be further studied.

Fungal and oomycete pathogens have been known to orient hyphal growth and zoospore swimming toward chemical stimuli from the host plant (Turra et al., 2015). Additionally, root exudates also act as chemoattractants to recruit beneficial microorganisms (Lombardi et al., 2018). For example, isoflavones released by soybean roots attract the nodulating symbiont *Bradyrhizobium japonicum* as well as the pathogen *Phytophthora sojae*, which causes *Phytophthora* blight in soybean (Morris et al., 1998). The chemotaxis of pathogenic and beneficial microbes to plant roots could be used in intercropping systems to help the non-host plant to inhibit pathogens. Previous studies have

found that *Phytophthora* zoospores display chemotaxis toward the roots of many non-host plants, including chives, rape, and maize, indicating that this attraction phenomenon occurs widely and may be caused by some common compounds in root exudates such as sugars, amino acids, phenolic acids, etc. (Yang et al., 2014; Fang et al., 2016; Jiang et al., 2017). Phenolic acids, which are defined chemically as carboxylic acids derived from either benzoic or cinnamic acid skeletons and can be divided into hydroxybenzoic acid and hydroxycinnamic acid (Stalikas, 2007), are aromatic secondary plant metabolites that are widely distributed in plant root exudates (Herrmann, 1989; Shahidi and Ambigaipalan, 2015). A number of previous studies have found that phenolic acids can promote the growth of plant pathogens and play an important role in the continuous soil sickness of many plants (Ye et al., 2004; Zhou et al., 2012). However, some studies also reported the antifungal activity of phenolic acids *in vitro* (Zhang et al., 2006; Wu et al., 2009). These different effects of phenolic acids on pathogens may result from the chemical structure of phenolic acids associated with various plants. Hence, we hypothesize that the special type or concentration of phenolic acids may be an important factor during the suppression of the *Phytophthora* infection process.

In the present work, we used the “corn/soybean-*Phytophthora sojae*” model as the research object to conduct the following study. First, we performed a field study to understand the inhibition of *Phytophthora* blight in soybean by maize/soybean intercropping. We then observed the interaction between roots/root exudates and pathogens and identified the phenolic acids in the rhizosphere soils of maize and soybean. Finally, we tested the antimicrobial activity of phenolic acid compounds on the infection behavior according to their concentrations in the rhizosphere soils of maize and soybean revealing the underlying mechanism of infection behavior suppression in *P. sojae* by non-host plants.

MATERIALS AND METHODS

Plant and Pathogen Materials

The maize (B73) and soybean (Williams) used in the present study were provided by the State Key Laboratory for Conservation and Utilization of Bio-Resources at Yunnan Agricultural University. *P. sojae* (P6497) was provided by the Seed Pathology and Pharmacology Laboratory of China Agricultural University. A zoospore suspension was obtained using a previously described trapping method with a few modifications (Lan et al., 2007). *P. sojae* was grown on V8 medium in a 25°C incubator under a 12-h light-dark cycle for 7 days (Hua et al., 2008). Eight samples were taken from the colony edges, seeded into a 250 mL culture flask containing 10% liquid V8 medium, and shaken in the dark at 25°C and 140 rpm for 48 h. All the cultures were transferred to new culture dishes and washed with sterile water four times. After the washing, the hyphae were added to 15 ml of soil extract and cultured at 25°C in the dark for 12–15 h, and then the zoospores were released. A zoospore suspension (10^6 mL⁻¹) was prepared after

the zoospores were filtered through gauze and counted using a hemocytometer.

Field Experiment

A field trial was conducted in 2019 at the Harbin Experimental Station of Heilongjiang Academy of Agricultural Sciences (45°50'N, 126°51'E), Heilongjiang Province, China. To determine the effect of the soybean and maize intercropping system on soybean *Phytophthora* blight suppression, we conducted field experiments using a single factor randomized block design with three replicates. The treatments are shown in **Figure 1**, and they include soybean monoculture and maize/soybean intercropping. The area of the individual plots was $3.6 \times 5.6 \text{ m}^2$. A maize/soybean intercrop was planted in a west-east row orientation in alternating 120-cm-wide strips, which included a 60 cm maize strip (two rows of maize with 40 cm inter-row spacing and 20 cm intra-row distance) and a soybean strip (two rows of soybean with 30 cm of inter-row spacing and 15 cm of intra-row distance). The wide gap between the maize and soybean strips measured 25 cm. We then used the same inter- and intra-row spacing (30 and 15 cm) for soybean in the monoculture. Each treatment was replicated three times. All the plots were located in the same field and arranged using a randomized block design. When the soybean plants had grown to the three-leaf stage, the cotyledon hypocotyl method described by Zuo (2004) was used to inoculate the soybean in the field according to **Figure 1**. In brief, the soybean plants were

cut with a blade at 0.5 cm below the cotyledon, and then the inoculum was injected into the wound. The incidence rates of soybean *Phytophthora* disease in the center and border rows were surveyed to show the ability of the zoospores to spread in and across rows. The incidence was calculated by using the following formula:

Disease incidence rate of soybean in the center row = Number of infected plants in center row / Total number of investigated plants in center row $\times 100\%$.

Disease incidence rate of soybean border row = Number of infected plants in border row / Total number of investigated plants in border row $\times 100\%$.

Simultaneously, all the aboveground samples were sun-dried and weighed to calculate the aboveground soybean biomass.

Interaction Assay Between Maize Roots and *P. sojae*

A special apparatus was used to monitor the interaction between the spores of *P. sojae* and the maize roots (Yang et al., 2014). In brief, a U-shaped chamber was formed by placing a bent capillary tube on a glass slide and covering it with a coverslip. The maize roots, which were approximately 2 cm long, were excised with a sterile razor blade. The root cap side of the maize roots was inserted into a zoospore suspension (10^6 mL^{-1}) in the chamber. The behavior of the zoospores in the rhizosphere

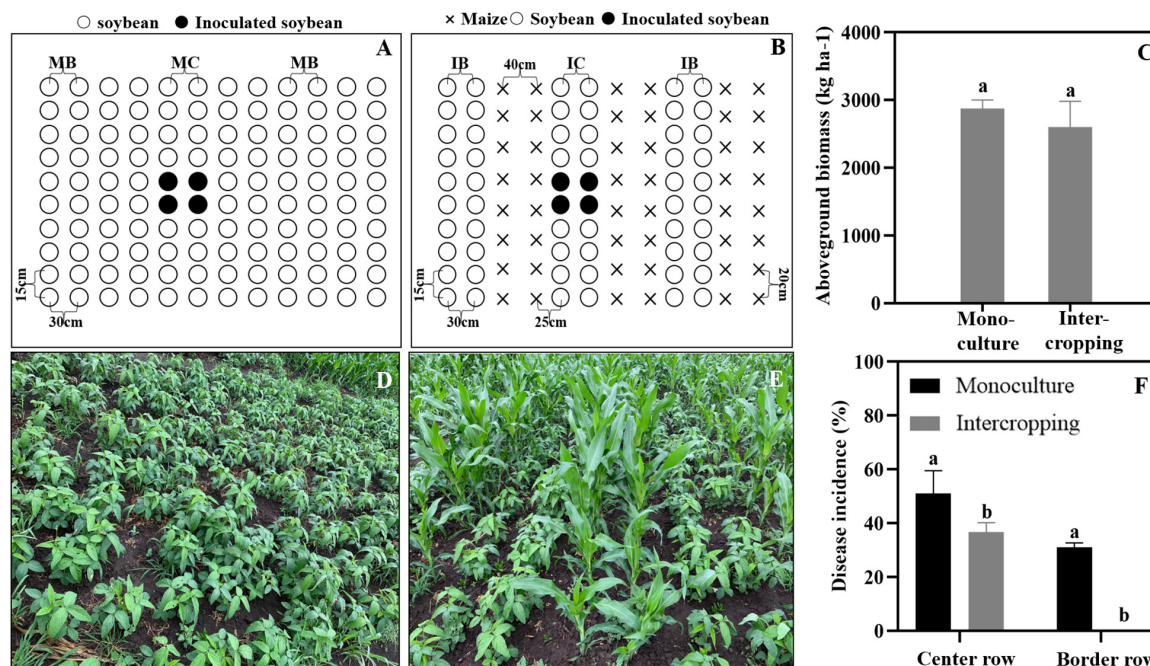


FIGURE 1 | Maize and soybean intercropping patterns in the field and their effects on soybean *Phytophthora* blight. **(A,D)** Soybean monoculture; **(B,E)** Maize and soybean intercropping. **(C)** Aboveground biomass of soybean in monoculture and intercropping schemes. **(F)** Disease incidence of soybean *Phytophthora* blight in monoculture and intercropping systems. IB and MB indicate the border lines (indicator line) in the intercropping and monoculture fields. IC and MC indicate the center lines (inoculation line) in intercropping and monoculture fields. Significant differences are based on ANOVA tests ($P < 0.05$). The error bars indicate the standard errors of the means ($n = 3$).

was recorded every minute for a period of 5 min by taking five photographs of the rhizosphere along the root cap and elongation zone under a light microscope (Leica DM2000, Germany) and adjusting the magnification (40 times or 100 times) according to the test requirement for photo collection. A capillary tube was inserted into a chamber containing the same zoospore suspension concentration as the control. The numbers of total cystospores and germinating cystospores in the rhizosphere were counted. Then, the inhibition ratio of the swimming zoospores and the cystospores germination were calculated using the follow formulas: Inhibition ratio of zoospores swimming = (Number of zoospores in the control – Number of zoospores in the treatment)/Number of zoospores in the control \times 100%; and Inhibition ratio of cystospores germination = Number of germinated cystospores in the control – Number of germinated cystospores in the treatment/Number of germinated cystospores in the control \times 100%, respectively. The chemotactic ratio (CR) was calculated as “the numbers of the zoospores and cystospores on the test root” divided by “the numbers of the zoospores and cystospores in the control” (Halsall, 1976). A CR > 1 indicates positive chemotactic activity. Each interaction assay was replicated three times.

Antimicrobial Activity of Maize Root Exudates and Phenolic Acids Against *P. sojae*

Collection of Maize Root Exudates

Maize plants were cultured using a previously described method (Yang et al., 2014), and root exudates were collected by water culture method. The maize seeds were surface sterilized with 3% sodium hypochlorite for 10 min and sown in black plastic pots with 40% humus soil and 60% field soil (poor soil outside the planting area). The 60% poor field soil were used to ensure the similarity with field soil where pathogen suppression was observed. And the 40% humus was used to supplement nutrient to avoid the effect of nutritional competition on root exudates. One maize seed was sown in each pot and irrigated with water. When the maize reached the three-leaf stage, the maize plant from each pot was removed and washed, and the maize roots were immersed in 200 mL of distilled water for 4 h to collect the root exudates. The collected liquids were filtered and extracted twice with ethyl acetate and concentrated under reduced pressure (Rotavapor R-200, Buchi). Finally, the concentrate was weighed and re-dissolved in 2 mL of methanol and filtered through a 0.22- μ m filter. The concentration of the maize root exudate stock was 0.1 mg/mL and that of the collected fluids before concentration was 0.001 mg/mL. The root exudates were separated into two parts and prepared for antimicrobial assays and phenolic acid compound identification.

Antimicrobial Activity of Maize Root Exudates Against *P. sojae*

The collection of maize root exudates for zoospores was performed according to Yang et al. (2014). The maize root exudate stock was diluted 2, 5, 10, 20, 30, and 50 times, and distilled water containing the same concentration of methanol

was used as a control treatment. The chemotaxis of *P. sojae* zoospores toward maize root exudates was observed according to the method reported by Fan et al. (2002) with a few modifications. The specific method is shown in **Figure 2**. A square groove was made with a capillary measuring 1 mm in diameter, and then the square groove was placed on a glass slide (length 25 mm \times width 25 mm \times height 1 mm). A zoospore suspension at a concentration of 1×10^6 cells/mL was added to the groove. One end was treated with a capillary tube to which a diluted root exudate was added. A capillary containing the same concentration of methanol at one end was used as a control, and the zoospore behavior was observed under a microscope.

The antimicrobial activity of maize root exudates against zoospore motility and cystospore germination was tested as described by Yang et al. (2014). In brief, 10 μ L of root exudate and 40 μ L of a zoospore suspension (10^6 /mL $^{-1}$) or cystospore suspension (10^6 /mL $^{-1}$) was immediately mixed on the glass slides. Then, the final concentration of the root exudates was diluted 5, 10, 25, 100, 150, and 250 times for concentrations of 100, 50, 20, 10, 5, 3.5, and 2 μ g/mL on the slides. The slides were placed in Petri dishes containing moist filter paper and incubated at 25°C in the dark. Photographs of immobilized zoospores and germinated cystospores were taken under a microscope. The percentage of zoospores that encysted into cystospores was then recorded every 1 min for a period of 5 min, and the percentage of germinated cystospores was calculated after 1.5 h of incubation. Each treatment had three replicates.

Identification of Phenolic Acid Compounds in Maize Root Exudates and Rhizosphere Soil

Gas Chromatography-Mass Spectrometry (GC-MS) Analyses of Root Exudates

The root exudates were derived by methoxypyridine and N-methyl-N-(trimethylsilyl)trifluoroacetamide. Then, the gas chromatography-mass spectrometry (GC-MS) fingerprints of the root exudates were obtained on a SHIMADZU GCMS-QP2010 instrument (SHIMADZU, Japan). The root exudates were separated on an SH-Rxi-5Sil MS capillary column (221-75954-30, 30 m \times 0.25 mm \times 0.25 μ m, SHIMADZU). The pressure was maintained at 49.5 kPa, giving a column flow of 1 mL/min. The injection volume was 1 μ L in splitless mode, and the injector temperature was 250°C. The initial column temperature was 40°C (hold 2 min), and it was increased at a rate of 3°C/min to 80°C and then increased to 260°C at a rate of 5°C/min, at which it was then held for 30 min. The ion source temperature was 230°C with an interface temperature of 250°C. Helium (99.999% purity) was used as the carrier gas at a flow rate of 1 mL/min. Mass spectra were obtained in electron impact (EI) ionization mode at 70 eV by monitoring the full-scan range (m/z 50–500). The compounds were identified by matching the mass spectra obtained with those of the reference compounds stored in the NIST14 library except for the compounds that appeared in the control. The characteristic fragments of the root exudate phenolic acids with more than 80% similarity were compared with those of the phenolic acid standards.

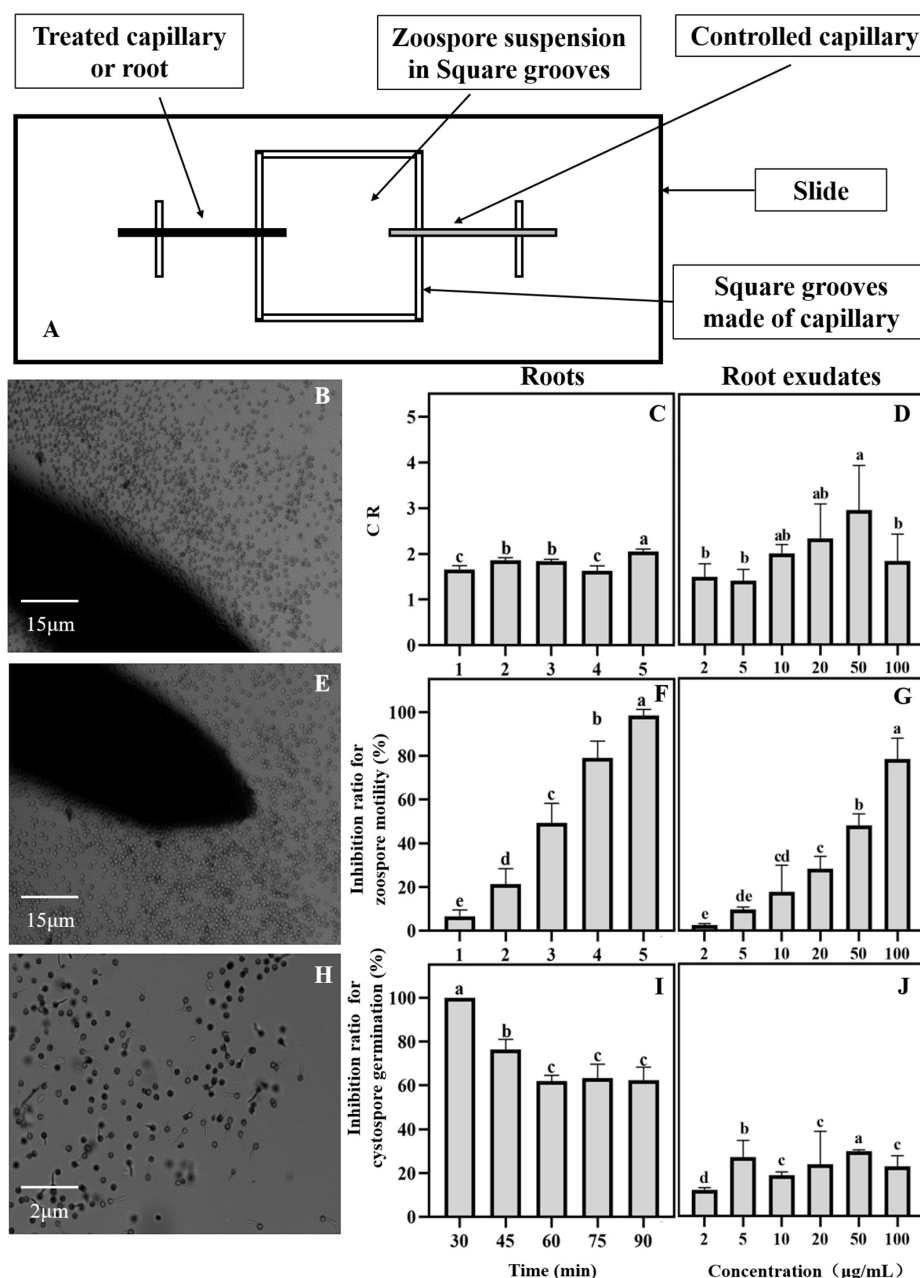


FIGURE 2 | Effects of maize roots and exudates on zoospore behavior of *Phytophthora sojae*. (A) Chemotactic test device for maize root and exudates against *P. sojae* zoospores; (B~J) Effect of maize roots and root exudates on zoospore chemotaxis roots: (B,C); root exudates: (D), swimming root: (E,F); root exudates: (G), and cystospore germination root: (H,I); root exudates: (J). Significant differences are based on ANOVA test ($P < 0.05$). The error bars indicate the standard errors of the means ($n = 3$).

High-Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS) Analysis of Rhizosphere Soil

The collection of rhizosphere soil was based on the methods of Bai et al. (2015) with slight modifications. In brief, maize or soybean plants were manually harvested from the pots, and large soil aggregates were removed by shaking the roots. The roots of all 15 plants derived from a single pot were pooled

into a 50 ml EP tube containing 20 mL of sterile Silwet L-77 amended PBS buffer (PBS-S; 130 mM NaCl, 7 mM Na_2HPO_4 , 3 mM NaH_2PO_4 , pH 7.0, and 0.02% Silwet L-77) and washed on a shaking platform for 20 min at 180 rpm. The washing buffer was subjected to centrifugation ($1,500 \times g$, 20 min), and the resulting pellet was defined as rhizosphere soil and then air dried. For the quantitative analysis of phenolic acids, 20 g of soil was extracted with 50 mL of 1 N NaOH and shaken for

12 h at room temperature (Kong et al., 2008). The filtrate was adjusted to pH 2.5 using HCl followed by centrifugation at 1200 g for 20 min. The phenolics were extracted from the acidified solution with ethyl acetate and re-dissolved in MeOH before being analyzed by HPLC-MS (Souto et al., 2000). In addition, phenolic acid analysis was performed in unplanted soil and was used as a control.

Based on the GC-MS results, five phenolic acids were identified from the root exudates and further selected to determine their presence and concentrations in maize and soybean rhizosphere soils by HPLC-MS. Cinnamic acid (Shanghai Yuanye Biotechnology Co., Ltd.), *p*-coumaric acid (Shanghai Yuanye Biotechnology Co., Ltd.), vanillic acid (Sigma-Aldrich Shanghai Trading Co., Ltd.), *p*-hydroxybenzoic acid (Shanghai Aladdin Biochemical Technology Co., Ltd.) and ferulic acid (Beijing Suo Laibao Technology Co., Ltd.) were purchased. The rhizosphere soil was analyzed using a Waters UPLC-MS system fitted with an Acquity UPLC System and a triple quadrupole mass spectrometer. Separation was performed on an Acquity UPLC BEH C18 column (1.7 μ m, 2.1 mm \times 50 mm). The solvents were as follows: solvent A, 0.1% glacial acetic acid (Aladdin Biochemical Technology, LC/MS grade) in water (Fisher Scientific, Shanghai, LC/MS grade) and solvent B, acetonitrile (Merck, HPLC grade). A multistep gradient was used for all the separations with an initial injection volume of 5 μ L and a flow rate of 0.4 mL/min. The multistep gradient was as follows: 0–4 min 10–38% (v/v) solution B, 4.1–4.5 min 38–90% (v/v) solution B, 4.6–5.5 min 90% (v/v) solution B, and 5.6–8 min 90–10% (v/v) solution B. The column temperature was maintained at 40°C. The total run time was 8 min. The mass spectral ionization, fragmentation, and acquisition parameters were optimized on a tandem quadrupole mass spectrometer using electrospray ionization (ESI) in negative mode (Table 1). Quantification was performed in multiple reaction monitoring (MRM) mode with dwell and interscan delay times of 0.2 and 0.1 s, respectively. Data were acquired and processed using Masslynx software (version 4.0, Waters, Milford, MA, United States). The relative concentrations of phenolic compounds in the maize rhizosphere soil were calculated from the standard curves, which were generated from the areas of the different standard concentrations by HPLC-MS.

Antimicrobial Activity of Phenolic Acids Against *P. sojae*

The antimicrobial effect of phenolic acids from the maize exudates on the infection behavior of *P. sojae* (chemotaxis, zoospore motility, cystospore germination, and hyphal growth) was determined at different concentrations (cinnamic acid: 0.5, 1, 5, 10, and 20 mg/L, *p*-Coumaric acid: 1, 5, 10, 15, and 20 mg/L, and other phenolic acids: 1, 10, 20, 50, and 100 mg/L) with the same method as that used on the root exudates. In addition, the effect of the mixture (maize rhizosphere: five compounds, soybean rhizosphere: three compounds) and each single compound on *P. sojae* were further tested at concentrations of 0.1, 0.5, 1, 5, and 10 times their actual concentrations in the rhizosphere based on the HPLC-MS findings.

The effect of phenolic acids on *P. sojae* hyphal growth was evaluated as described by Miller et al. (1983) with a few modifications. The specific method employed 60 mL of V8 liquid medium with a 100 mL flask and each flask contained 6 dishes of *P. sojae*; the samples were cultured for 36 h on a shaking table at 28°C and 140 rpm. Then, 600 μ L phenolic acid solutions of different concentrations were added to each flask containing dishes. A 600 μ L methanol solution without phenolic acid was added as the control; each treatment was repeated 3 times. After continuous culturing for 12 h, the liquid medium was removed by filtration; the hyphae were then wrapped in filter paper, dried and weighed to calculate the inhibition rate of the hyphae. The calculation method was as follows: mycelium inhibition rate (%) = (mycelia weight of control 1 - treated mycelia weight)/(mycelia weight of control 1 - mycelia weight of control 2) \times 100%. Control 1 was the mycelial weight after treatment with 600 μ L of methanol solution without phenolic acid, and control 2 was the mycelial weight after culturing for 36 h on a shaking table at 28°C and 140 rpm.

Data Analysis

The experiments were set up using a completely randomized design. The yields, disease incidence in the intercrop and monoculture were analyzed by Independent Sample *t*-test; CR, and inhibition ratio of root exudates and compounds at different concentration were analyzed by one-way analysis of variance (ANOVA). Analyses were performed with an SAS software package (SAS Institute 2011), and the mean values ($n = 3$) were

TABLE 1 | Phenolic acids quantified by UPLC-MS in rhizosphere soil around maize and soybean.

Analytes	RT (min)	Transition	CV (v)	CE (v)	Molecular weight	Actual concentration (μ g/g)		
						Maize	Soybean	Control
<i>p</i> -Hydroxybenzoic acid	0.85	136.968>93.476	28	32	137	17.11 \pm 0.78a	3.29 \pm 1.43b	0.82 \pm 0.38c
<i>p</i> -Coumaric acid	1.34	162.968>119.468	28	32	164	26.13 \pm 0.12	–	–
Cinnamic acid	2.80	147.032>76.967	26	30	148	0.15 \pm 0.02	–	–
Vanillic acid	0.97	166.968>151.995	14	30	168	4.93 \pm 0.13a	1.55 \pm 0.10b	0.11 \pm 0.02c
Ferulic acid	1.56	193.032>133.979	16	30	194	0.51 \pm 0.00b	2.16 \pm 0.06a	0.04 \pm 0.02c

RT, retention time; CV, cone voltage; CE, collision energy. Control, soil without plant culture. The error indicates the standard errors of the means ($n = 3$). Significant differences are based on ANOVA tests. Lower case letters show significant differences in the same phenolic acids at the soil with different plant culture at the 0.05 level.

compared using Duncan's new multiple range test at the 5% level. Figures were drawn using GraphPad Prism 8.

RESULTS

Maize Intercropping With Soybean Can Restrict the Spread of *Phytophthora* Blight

As shown in **Figure 1**, the aboveground biomass of the soybean showed no significant difference between the intercrop and the monoculture (**Figure 1C**). In addition, the disease incidence of soybean *Phytophthora* blight was significantly decreased in the maize/soybean intercropping systems compared with that of the monoculture. The *Phytophthora* blight barely spread across the maize rows, and the disease incidence in center rows was also decreased by 15% (**Figure 1F**).

Maize Roots and Root Exudates Interfere With the Infection Behavior of Zoospores

The interaction between the maize roots and *P. sojae* zoospores was observed under a microscope. The zoospores can be attracted to the maize roots (**Figure 2B**). Based on the CR values, the zoospores showed positive chemotactic activity toward the maize roots (**Figure 2C**). After approaching the maize roots, the zoospores rapidly lost their swimming ability and transformed into cystospores (**Figure 2E**). After 5 min, all the zoospores stopped swimming (**Figure 2F**). The maize roots then effectively inhibited the germination of the cystospores by 62% compared with the capillary tube control at 90 min (**Figures 2H,I**). The maize root exudates also showed a strong ability to attract zoospores (**Figure 2D**), inhibit the motility of the zoospores (**Figure 2G**), and suppress the germination of the cystospores (**Figure 2J**) in a dose-dependent manner. When the concentration of root exudates reached 20 $\mu\text{g/mL}$, which was close to the concentration of the root exudates before concentration, the inhibition rates for zoospore motility and cystospore germination reached 28% and 24%, respectively (**Figures 2G,J**).

Phenolic Acids in Root Exudates and Rhizosphere Soils of Maize and Soybean

Sixteen organic acids, five alkanes, four alcohols, two amines and two esters were identified in the maize root exudates by GC-MS (**Figure 3**). Among them five phenolic acid compounds were consistently identified in the rhizosphere soil of maize with HPLC-MS (**Table 1**). The five phenolic acid compounds were *p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid and cinnamic acid in order of their concentration in the maize rhizosphere soil from high to low (**Table 1**). However, only three phenolic acid compounds, *p*-hydroxybenzoic acid, vanillic acid, and ferulic acid, were identified in the soybean rhizosphere soil by HPLC-MS, and *p*-hydroxybenzoic acid and vanillic acid showed significantly lower concentrations than those in the maize rhizosphere soil (**Table 1**). In addition, the *p*-hydroxybenzoic acid, vanillic acid and ferulic acid were also determined in the

background soil, and the concentration was much lower than in the maize or soybean rhizosphere (**Table 1**), which illustrated that phenolic acids mostly measured in the soil was plant-derived.

Phenolic Acid Compounds Show Various Activities Against *P. sojae* Infection

Among the five phenolic acids, only cinnamic acid was associated with positive chemotaxis with *P. sojae* zoospores (**Figure 4**). However, the five phenolic acids showed dose-dependent inhibitory effects on the zoospore motility, cystospore germination, and hyphal growth of *P. sojae* (**Figure 5**). Cinnamic acid demonstrated the strongest activity against zoospore motility and cystospore germination, which were inhibited by 100% and 49%, respectively, at a concentration of 5 $\mu\text{g/mL}$ (**Figures 5D,I**). The *p*-coumaric acid showed similar antimicrobial activity at 10–15 $\mu\text{g/mL}$ (**Figures 5E,J**). Furthermore, vanillic acid and cinnamic acid showed the highest inhibitory activities against hyphal growth, with inhibition rates of 82% and 80%, respectively (**Figures 5L,N**). The remaining three phenolic acids also showed significant antimicrobial effects on zoospore motility, cystospore germination, and hyphal growth at soil concentrations of 10–50 $\mu\text{g/mL}$. In addition, when used at concentrations at which they are found in maize rhizosphere, phenolic acids such as *p*-coumaric acid and vanillic acid, could significantly interfere with the infection process of *P. sojae*, but they had little effect when used at those concentrations in the soybean rhizosphere (**Tables 2, 3 and Supplementary Table S2**). Then cinnamic acid could attract *P. sojae* zoospores at the rhizosphere concentration of 5 times and 10 times compared to the control (**Supplementary Table S1**). Interestingly, when these phenolic acid compounds were pooled together according to their concentrations in the maize rhizosphere soil, the mixture showed strong activity against all infection stages of *P. sojae*, except chemotaxis, in a dose-dependent manner (**Table 2**). The antimicrobial effect of the mixture on zoospore motility was significantly higher than that of phenolic acid alone. For example, the mixture ($\times 1$) showed higher inhibitory activity against zoospore motility, which was 3.5 times that of the corresponding individual compounds at the same concentration (**Table 2**). However, the mixture of three phenolic acids at the soybean rhizosphere soil concentrations showed a slight effect on the infection processes, with the exception of cystospore germination. The inhibitory effect of the phenolic acid mixtures at the soybean rhizosphere soil concentrations and its proportions on cystospore germination were also significantly lower than they were at the maize rhizosphere soil concentrations (*t*-test, $p < 0.05$) (**Table 3**).

DISCUSSION

Non-host Plant Roots and Root Exudates Interfere With the Infection Behavior of *Phytophthora* Pathogens in Intercropping Systems

A large number of studies have found that maize intercropping with soybean, peppers, potatoes and other crops can not only

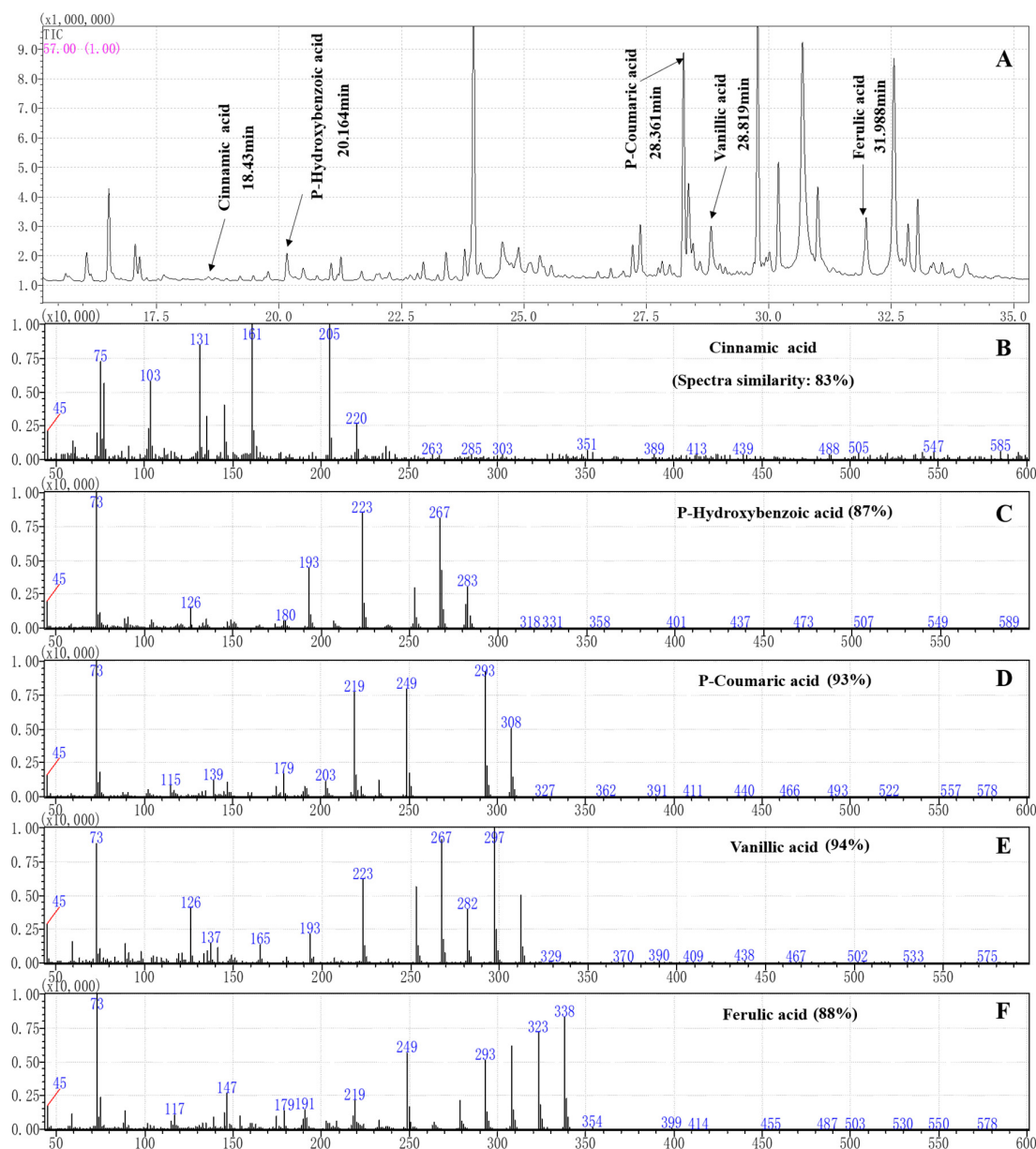


FIGURE 3 | Separation and characterization of phenolic acids from maize root exudates by gas chromatography (GC) mass spectrometry (MS) analysis. **(A)** GC-MS profiles of root exudates showing five peaks at the following retention times (tr): 18.43 min (cinnamic acid), 20.164 min (*p*-hydroxybenzoic acid), 28.361 min (*p*-coumaric acid), 28.819 min (vanillic acid) and 31.988 min (ferulic acid) in the root exudates of maize were identified. **(B–F)** The characteristic ion fragment diagram and spectra similarities of the phenolic acids after derivation.

reduce the occurrence of crop leaf diseases but also effectively inhibit the spread and expansion of soil-borne diseases (Sun et al., 2006; Li et al., 2009; Yang et al., 2014; Fu et al., 2016). In the present study, field experiments also indicated that maize intercropping with soybean could significantly inhibit the spread of *Phytophthora* blight in soybean in and across rows (**Figure 1**). Roots and root exudates play an important role in plant-microbe interactions in the rhizosphere (Bais et al., 2006; De-la-Peña et al., 2008; Doornbos et al., 2012). Our results indicated that maize roots and root exudates could attract *P. sojae* zoospores

and suppress zoospore motility and cystospore germination, causing the pathogens to lose their infection ability (**Figure 2**). Therefore, the interference of maize roots and root exudates with the *P. sojae* infection process may be an important mechanism for inhibiting *Phytophthora* blight of soybean via maize/soybean intercropping. In addition, Yang et al. (2014) and Fang et al. (2016) found that maize and rapeseed roots could inhibit the growth of *Phytophthora* *in vitro* and decrease the incidence rate of *Phytophthora* blight in the field. Hence, the interference of non-host roots and root exudates on *Phytophthora* pathogens

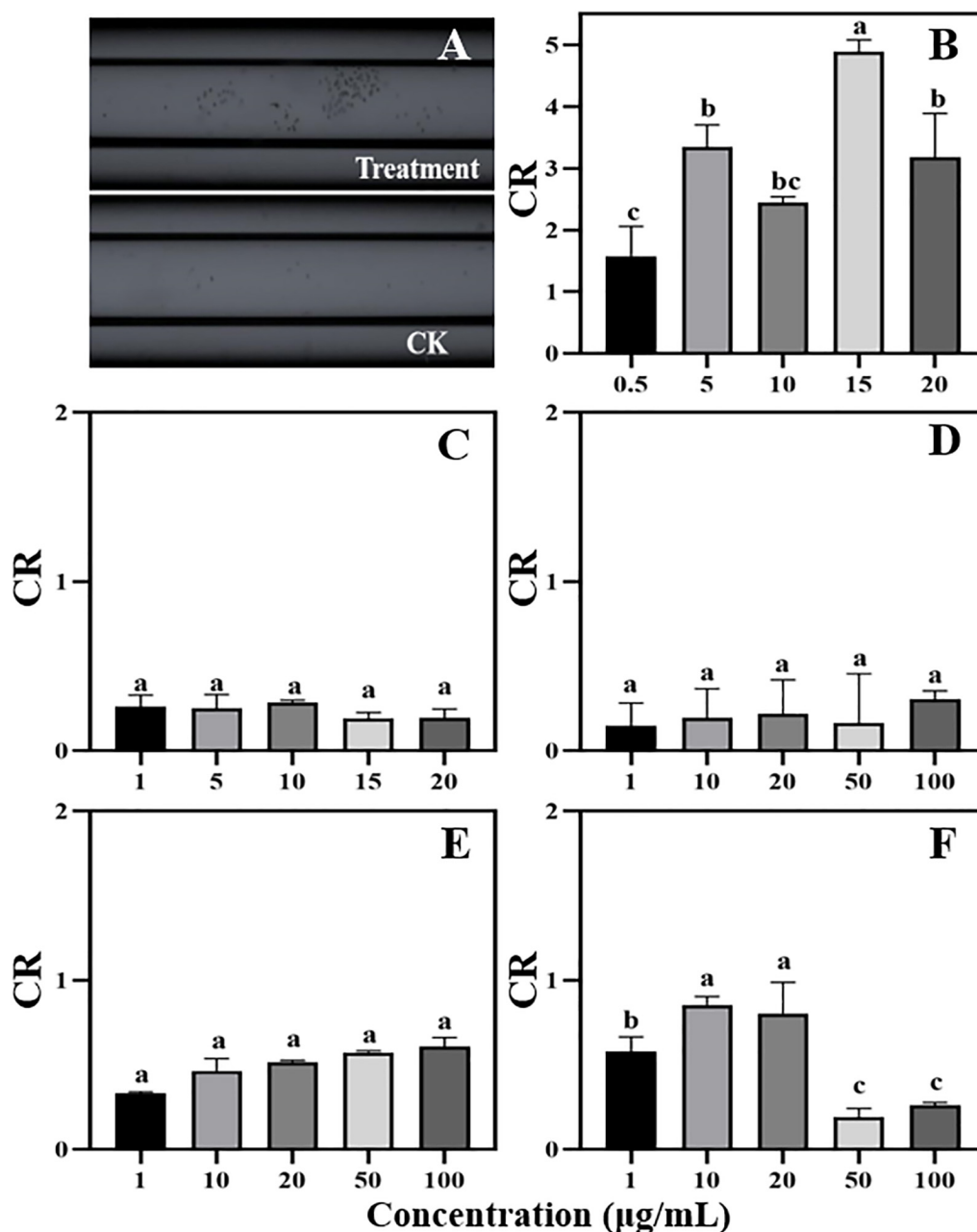


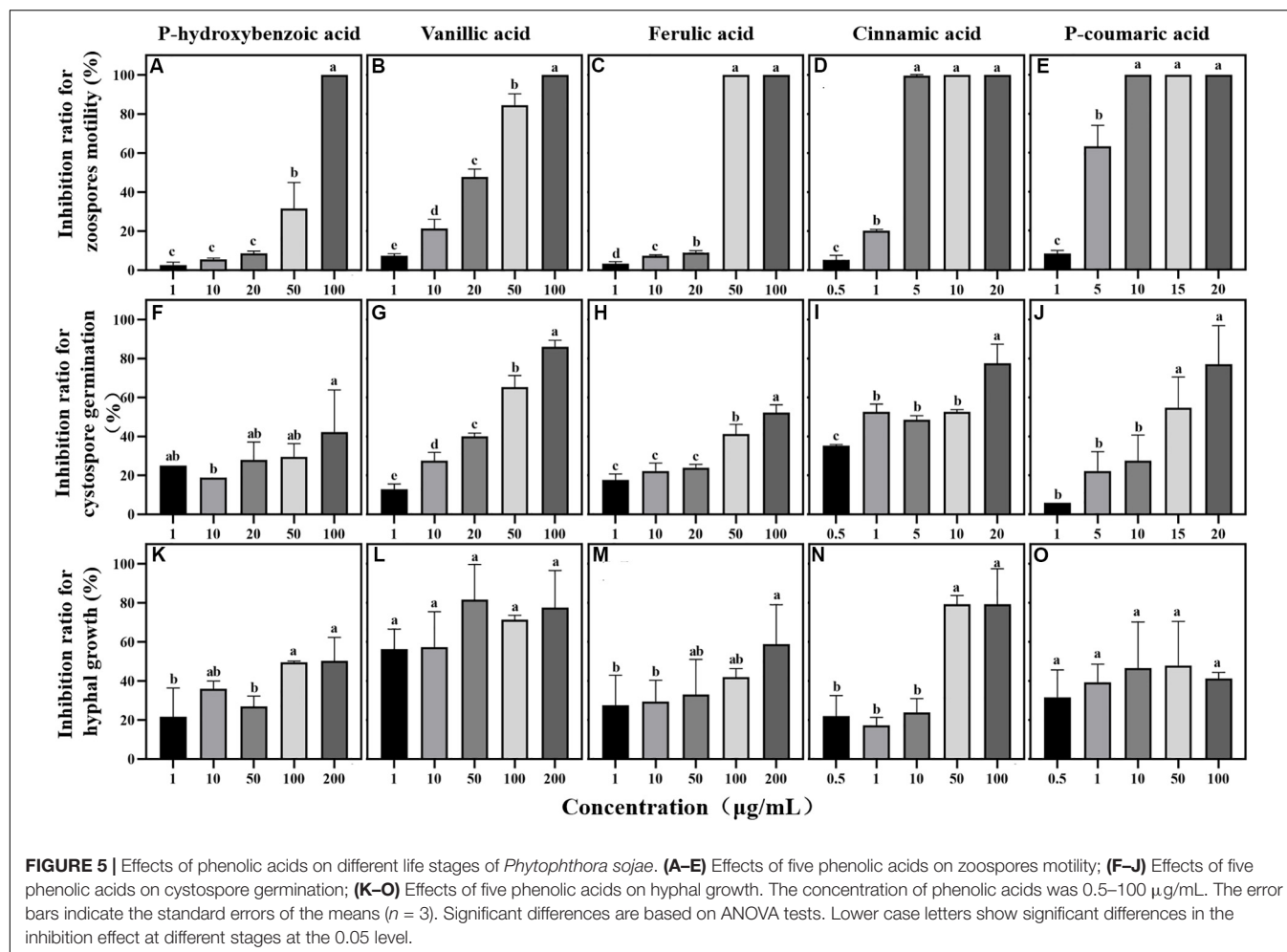
FIGURE 4 | Chemotaxis of zoospores to different concentrations of phenolic acids. (A) Chemotaxis picture (treatment: 5 $\mu\text{g/mL}$ cinnamic acid; CK: 1%MeOH-H₂O); (B) Cinnamic acid (Concentration: 0.5–20 $\mu\text{g/mL}$); (C) *p*-Coumaric acid (Concentration: 1–20 $\mu\text{g/mL}$); (D–F) Vanillic acid, ferulic acid, and *p*-Hydroxybenzoic acid (Concentration: 1–100 $\mu\text{g/mL}$). Significant differences are based on ANOVA test ($P < 0.05$). The error bars indicate the standard errors of the means ($n = 3$).

may play an important role in soilborne disease control via intercropped maize or rotated rapeseed.

Phenolic Acids Show Various Abilities to Interfere With *P. sojae* Infection

Phenolic acids are complex and important secondary metabolites that are widely distributed throughout plant root exudates (Herrmann, 1989; Ryan et al., 1999). In the present study, five

phenolic acid compounds (*p*-coumaric acid, *p*-hydroxybenzoic acid, vanillic acid, ferulic acid, and cinnamic acid) were consistently found in the root exudates and rhizosphere soils (Figure 3). Phenolic acids are important chemical substances in the rhizosphere of plants, and they can act as a signal to attract harmful and beneficial microorganisms within the soil (Liang et al., 2009; Kakkar and Bais, 2014). The zoospores of *P. sojae* displayed positive chemotaxis towards cinnamic acid but showed no response to the other four phenolic acids (Figure 4).



Cinnamic acid could also serve as a signal substance for attracting *R. solanacearum* and accelerating disease progression in tobacco (Li B. et al., 2016). Previous research reported that cinnamic acid was found in the root exudates of a large number of plant species, including tobacco, cucumber, watermelon and rice (Ye et al., 2004; Hao et al., 2010; Ling et al., 2010; Li B. et al., 2016). Hence, cinnamic acid may be attracting *Phytophthora* zoospores to the roots of non-host plants. In addition, the previous research found the chemotaxis of beneficial microorganisms to cinnamic acid (Kape et al., 1991), indicating that non-host roots can also attract beneficial microorganisms to resist pathogens via cinnamic acid, which still requires further study.

Phenolic acids not only attract microorganisms as signal substances in soil but also directly affect the growth and reproduction of microorganisms (Yuan et al., 2018). In the present study, five phenolic acids inhibited the *Phytophthora* infection process in a dose-dependent manner (Figure 5). Among these compounds, cinnamic acid and *p*-coumaric acid, which are hydroxycinnamic acids, more strongly inhibited *P. sojae* zoospore motility and germination than the other phenolic acids (Figure 5). A large number of previous studies also reported the high antimicrobial activity of cinnamic acid and

p-coumaric acid against plant pathogens (Zhang et al., 2007; Hao et al., 2010; Kim et al., 2012). Additionally, *p*-hydroxybenzoic acid has been reported to improve the growth of pathogens at low concentrations and creates a continuous soybean cropping barrier (Zhang et al., 2007). Previous studies also reported that some fungi could metabolize phenolic compounds and use them as carbon sources to obtain nutrition or to detoxify (Shalaby et al., 2012; Jin et al., 2020). Thus, we infer that the difference in the chemical structure may be an important reason for the different effects of phenolic acids on pathogens. Moreover, vanillic acid showed the highest inhibition ratio against *P. sojae* hyphal growth (Figure 5), indicating the different antimicrobial functions of phenolic acids in maize root exudates. This phenomenon has also been found in the interaction between plant pathogens and other antimicrobial compounds such as flavones, benzoxazinoids and antioxidants (Bagga and Straney, 2000; Lee and Bostock, 2007; Wang et al., 2020).

Apart from phenolic acids, organic acids, alkanes, alcohols, amines and esters were identified in the maize root exudates by GC-MS at the present study, which was also reported to have antimicrobial activity against pathogens, such as 3-Methylbutanoic acid in organic acid, tyrosol and D-pinitol in

TABLE 2 | The antimicrobial activity of phenolic acids against infection process of *Phytophthora sojae* according their concentrations in rhizosphere soil of maize.

Concentration	Compound	Chemotaxis	Inhibition ratio (%)		
			Zoospore motility	Cystospore germination	Hyphal growth
× 0.1 (0.1 times)	<i>p</i> -Coumaric acid	0.40 ± 0.12a	2.33 ± 1.20c	12.67 ± 0.33bc	37.33 ± 0.88a
	<i>p</i> -Hydroxybenzoic acid	0.53 ± 0.07a	4.67 ± 0.88b	8.33 ± 2.73c	9.00 ± 4.36b
	Vanillic acid	0.27 ± 0.07a	0.00 ± 0.00d	7.33 ± 2.73c	49.33 ± 6.36a
	Ferulic acid	0.20 ± 0.18a	0.00 ± 0.00d	7.33 ± 0.88c	37.00 ± 10.54a
	Cinnamic acid	0.13 ± 0.07a	4.00 ± 0.33bc	23.33 ± 4.05a	32.33 ± 13.20ab
mixture		0.20 ± 0.12a	10.33 ± 0.88a	21.00 ± 4.00ab	32.33 ± 3.92ab
× 0.5 (0.5 times)	<i>p</i> -Coumaric acid	0.53 ± 0.07a	8.33 ± 1.76b	10.33 ± 3.18b	49.67 ± 1.45a
	<i>p</i> -Hydroxybenzoic acid	0.33 ± 0.07ab	8.33 ± 2.96b	10.00 ± 4.00b	23.33 ± 7.36b
	Vanillic acid	0.13 ± 0.07b	4.33 ± 1.45b	10.67 ± 2.19b	50.00 ± 3.61a
	Ferulic acid	0.47 ± 0.07a	4.67 ± 2.73b	8.67 ± 1.45b	25.33 ± 4.67b
	Cinnamic acid	0.33 ± 0.07ab	6.67 ± 2.33b	37.33 ± 7.84a	36.33 ± 9.87ab
mixture		0.07 ± 0.07b	44.67 ± 0.88a	27.33 ± 11.67ab	43.67 ± 7.31ab
× 1 (1 times)	<i>p</i> -Coumaric acid	0.20 ± 0.07b	17.00 ± 4.73b	13.67 ± 2.73d	69.33 ± 15.41a
	<i>p</i> -Hydroxybenzoic acid	0.20 ± 0.12b	7.00 ± 2.08c	13.33 ± 2.03d	24.33 ± 8.09c
	Vanillic acid	0.07 ± 0.07b	6.67 ± 2.85c	23.67 ± 3.76c	33.67 ± 9.39bc
	Ferulic acid	0.07 ± 0.07b	6.67 ± 0.33c	27.67 ± 4.91bc	37.33 ± 4.91bc
	Cinnamic acid	0.97 ± 0.16a	15.67 ± 0.88b	37.33 ± 1.45b	25.67 ± 4.41bc
mixture		0.90 ± 0.10a	60.00 ± 2.52a	50.00 ± 4.51a	52.67 ± 1.67ab
× 5 (5 times)	<i>p</i> -Coumaric acid	0.20 ± 0.07c	22.33 ± 1.76b	14.00 ± 1.53d	80.00 ± 2.52a
	<i>p</i> -Hydroxybenzoic acid	0.73 ± 0.24b	10.00 ± 2.31c	20.00 ± 2.89cd	29.67 ± 2.40c
	Vanillic acid	0.07 ± 0.07c	11.33 ± 1.20c	21.00 ± 3.46cd	40.33 ± 3.48c
	Ferulic acid	0.60 ± 0.07b	10.67 ± 0.88c	29.33 ± 3.28c	45.00 ± 16.52bc
	Cinnamic acid	1.27 ± 0.02a	18.67 ± 1.45b	43.67 ± 6.36b	37.00 ± 3.51c
mixture		1.45 ± 0.02a	87.67 ± 0.67a	60.67 ± 2.40a	66.33 ± 7.88ab
× 10 (10 times)	<i>p</i> -Coumaric acid	0.40 ± 0.07c	29.33 ± 4.37c	57.33 ± 1.45b	90.67 ± 2.03a
	<i>p</i> -Hydroxybenzoic acid	0.53 ± 0.13c	21.33 ± 1.86cd	10.00 ± 1.00d	41.33 ± 7.31b
	Vanillic acid	0.13 ± 0.13c	19.67 ± 2.40d	18.67 ± 4.41c	66.00 ± 7.23a
	Ferulic acid	0.20 ± 0.13c	22.33 ± 3.18cd	56.33 ± 1.67b	31.00 ± 10.82b
	Cinnamic acid	1.30 ± 0.12b	38.00 ± 1.73b	58.00 ± 1.15b	41.00 ± 11.01b
mixture		1.68 ± 0.13a	100.00 ± 0.00a	68.00 ± 1.73a	87.00 ± 2.52a

Mixture is consisted of five phenolic acid compounds according to their concentrations and proportion in the rhizosphere soil of maize. The concentration with (× 0.1), (× 0.5), (× 1), (× 5) and (× 10) indicate that the concentrations of mixture were 0.1, 0.5, 1.0, 5.0 and 10.0 times of the rhizosphere soil concentration (Mixture = 0.15 mg/L cinnamic acid + 26.13 mg/L *p*-coumaric acid + 4.93 mg/L vanillic acid + 0.50 mg/L ferulic acid + 17.11 mg/L *p*-hydroxybenzoic acid). Different letters in the same group indicate significant differences by ANOVA ($P < 0.05$). The error indicates standard error of means ($n = 3$).

TABLE 3 | The antimicrobial activity of phenolic compounds against infection process of *Phytophthora sojae* according their concentrations in rhizosphere soil of soybean.

Compound	Inhibition ratio (%)			
	Chemotaxis	Zoospore motility	Cystospore germination	Hyphal growth
<i>p</i> -Hydroxybenzoic acid	0.25 ± 0.03a	13.00 ± 0.00a	2.33 ± 1.20c	−1.33 ± 2.85b
Vanillic acid	0.15 ± 0.02bc	5.00 ± 1.15b	18.00 ± 0.58b	17.67 ± 4.41a
Ferulic acid	0.11 ± 0.01c	10.00 ± 2.00a	11.33 ± 2.85b	25.00 ± 1.73a
mixture	0.18 ± 0.01b	3.67 ± 0.67b	27.00 ± 4.00a	21.33 ± 4.18a

Mixture is the complex of three phenolic acids according to their concentrations and average proportion in the rhizosphere soil of soybean. The concentration indicates that the concentrations of mixture were 1.0 time of the rhizosphere soil concentration (Mixture = vanillic acid 1.55 mg/L + ferulic acid 2.16 mg/L + *p*-hydroxybenzoic acid 3.29 mg/L). Different letters in the same group indicate significant differences by ANOVA test ($P < 0.05$). The error indicates standard error of means ($n = 3$).

alcohols, and ethanolamine in amines (Lv et al., 2007; Wang et al., 2012; Abdel-Rhman and Rizk, 2016; Liu et al., 2019). Therefore these compounds may also participate in the suppression of *P. sojae* infection process by maize roots, but their involvement

in this phenomenon needs further study. The antimicrobial substances in root exudates not only affect plant pathogens, but they also alter the soil microbial community around the roots (Hu et al., 2018; Stringlis et al., 2018; Huang et al.,

2019). Previous studies have shown that soil microorganisms can affect pathogen infection process through microbiota-modulated immunity [MMI] and direct microbial competition [DMC] (competition for nutrients and space as well as the secretion of antimicrobials) (Hacquard et al., 2017; Vannier et al., 2019). Hence, root-secreted natural products, such as the various weapons in the chemical arsenal of plants, can help to resist pathogens.

The Type and Concentration of Phenolic Acids in Host and Non-host Plants Mediate the Suppression of *P. sojae* by Intercropping

Whether phenolic acids inhibit or promote the growth of plant pathogens remains unclear (Ye et al., 2004; Zhang et al., 2006). On the one hand, phenolic acids such as cinnamic acid or *p*-coumaric acid (hydroxycinnamic acid) have been reported to have strong antimicrobial activity against crop pathogens (Kim et al., 2004; Hao et al., 2010); on the other hand, *p*-hydroxybenzoic acids, including vanillic acid (hydroxybenzoic acid), reportedly promote pathogen growth and cause soil sickness (Zhou and Wu, 2012a,b; Zhou et al., 2012). Therefore, the difference in the type of phenolic acids may be a factor in the inconsistency of their antimicrobial effects. In the present study, cinnamic acid and *p*-coumaric acid, which are types of hydroxycinnamic acid that have high inhibitory activity against *P. sojae*, were identified in maize rhizosphere soil but not in soybean rhizosphere soil (Table 1). Many previous studies also reported the absence of these hydroxycinnamic acids in the soybean rhizosphere (Zhang et al., 2007; Gao et al., 2014). Hao et al. (2010) found that the *p*-coumaric acid in rice root exudates could significantly inhibit the growth of *Fusarium oxysporum*, but it was not present in watermelon root exudates. These results indicated that non-host plant roots could secrete special phenolic acids (that are absent in the hosts) to suppress the soil-borne pathogen of the host plant, which may be an important mechanism underlying disease control by intercropping.

In addition, *p*-hydroxybenzoic acid and vanillic acid were also found in the soybean rhizosphere but much lower concentrations than in the maize rhizosphere (Table 1). The infection process was significantly disrupted by these phenolic acids at the maize rhizosphere concentrations but showed little effect at the soybean rhizosphere concentrations (Figure 5). *p*-Hydroxybenzoic acid and vanillic acid, which are hydroxybenzoic acids, were found in the root exudates of soybean and exhibited plant pathogenic fungi promotion at low concentrations and inhibition at high concentrations, for fungi such as *Aspergillus*, *Fusarium*, etc. (Zhang et al., 2007; Vio-Michaelis et al., 2012; Heleno et al., 2013). Therefore, the simultaneous presence of phenolic acids in non-host and host root exudates may increase their concentrations in the rhizosphere soil and result in a strong inhibition of *P. sojae* growth.

Synergism via the combination of two or more drugs has been utilized, and this combination/synergistic approach has demonstrated greater antimicrobial ability than single drugs alone (Sun and Johnson, 1960; Hossain et al., 2016). In the

present study, the mixture of phenolic acids in maize rhizosphere soil showed higher inhibition during certain infection stages, especially zoospore motility, than the same concentrations of the individual compounds (Table 2). These results suggested the occurrence of synergistic antimicrobial effects, which have been widely used in pesticides for plant disease control (Chung et al., 2011; Berditsch et al., 2015). For example, a combined formulation of oregano and thyme showed a synergistic effect, resulting in enhanced efficiency against *Aspergillus. flavus*, *Aspergillus. parasiticus* and *Penicillium. chrysogenum* (Hossain et al., 2016). Hence, the synergistic interaction could improve the antimicrobial effect of phenolic acids on the infection behavior of *P. sojae*. In addition, we barely observed a synergistic antimicrobial effect with the mixture of phenolic acids at their soybean rhizosphere soil concentrations and proportions, which further supports this theory.

CONCLUSION

We used maize and soybean intercropping systems as a model to determine whether non-host plant roots and root exudates can attract the zoospores of *Phytophthora* pathogens and then inhibit their growth and ability to cause infections, ultimately reducing the incidence of *Phytophthora* blight in the field. The difference in the type and concentration of phenolic acids between host and non-host plants was an important factor in the interference of non-host plant roots with *P. sojae* infection. Moreover, phenolic acids in maize root exudates exhibit synergistic antimicrobial activity, interfering with the infection behavior of *P. sojae* in soybean but not in soybean rhizosphere soil. This “non-host specificity” strategy can be used in agricultural systems to achieve sustainable and ecological disease management.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

SZ and YXL conceived the ideas and designed the methodology. HZ, YY, YL, and JW performed the field experiment. HZ and YY performed GC-MS and HPLC-MS experiments. HZ, YY, YWL, and HW performed the biological activity test of standards. XM, HH, MY, and XH collected the data. HZ and YY analyzed the data. HZ, YY, SZ, and YXL wrote 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/fpls.2020.00886/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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Harnessing the Microbiomes of Suppressive Composts for Plant Protection: From Metagenomes to Beneficial Microorganisms and Reliable Diagnostics

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Soil-borne diseases cause significant yield losses worldwide, are difficult to treat and often only limited options for disease management are available. It has long been known that compost amendments, which are routinely applied in organic and integrated farming as a part of good agricultural practice to close nutrient cycles, can convey a protective effect. Yet, the targeted use of composts against soil-borne diseases is hampered by the unpredictability of the efficacy. Several studies have identified and/or isolated beneficial microorganisms (i.e., bacteria, oomycetes, and fungi) from disease suppressive composts capable of suppressing pathogens (e.g., *Pythium* and *Fusarium*) in various crops (e.g., tomato, lettuce, and cucumber), and some of them have been developed into commercial products. Yet, there is growing evidence that synthetic or complex microbial consortia can be more effective in controlling diseases than single strains, but the underlying molecular mechanisms are poorly understood. Currently, a major bottleneck concerns the lack of functional assays to identify the most potent beneficial microorganisms and/or key microbial consortia from complex soil and compost microbiomes, which can harbor tens of thousands of species. This focused review describes microorganisms, which have been isolated from, amended to or found to be abundant in disease-suppressive composts and for which a beneficial effect has been documented. We point out opportunities to increasingly harness compost microbiomes for plant protection through an integrated systems approach that combines the power of functional assays to isolate biocontrol and plant growth promoting strains and further prioritize them, with functional genomics approaches that have been successfully applied in other fields of microbiome research. These include detailed metagenomics studies (i.e., amplicon and shotgun sequencing) to achieve a better understanding of the complex system compost and to identify members of taxa enriched in suppressive composts. Whole-genome sequencing and complete assembly of key isolates and their subsequent functional profiling can elucidate the mechanisms

of action of biocontrol strains. Integrating the benefits of these approaches will bring the long-term goals of employing microorganisms for a sustainable control of plant pathogens and developing reliable diagnostic assays to assess the suppressiveness of composts within reach.

Keywords: compost, organic farming, microbiome, biocontrol, metagenomics, genome assembly, strain collection, mechanism of action

INTRODUCTION

Soil-borne plant pathogens including fungi, oomycetes, bacteria, and viruses, as well as parasitic nematodes, can cause severe yield losses. Soil-borne diseases such as pre- and post-emergence damping-off, root, stem collar and crown rots, and vascular wilting can be found in many crops and are primarily caused by members of the oomycetes (e.g., *Pythium* sp. and *Phytophthora* spp.) and fungi (e.g., *Rhizoctonia solani*, *Sclerotium* spp., *Fusarium* sp., and *Verticillium* sp.), but also by bacteria (e.g., *Ralstonia solanacearum*, *Pectobacterium*, and *carotovorum*), viruses (e.g., beet necrotic yellow vein virus, soil-borne wheat mosaic virus, and peanut clump virus), and nematodes (e.g., *Meloidogyne* sp.) (Koike et al., 2003; Agrios, 2005; Noble and Coventry, 2005; Fry and Grünwald, 2010; Andika et al., 2016). Pathogens may survive in soil for many years as dormant resting stages (i.e., spores). Chemical fungicide treatments are often not effective enough against soil-borne diseases (Reddy, 2016; You et al., 2020) and are met by increasing levels of criticism and public concerns about negative effects, highlighting the urgency to search for viable alternatives. A combined approach to avoid severe crop yield losses due to soil-borne pathogens includes breeding and selection of appropriate crop varieties, crop rotation, soil drainage, avoidance of soil compaction, appropriate sowing dates, and application of organic amendments such as composts (Abawi and Widmer, 2000; Bailey and Lazarovits, 2003; Ghorbani et al., 2009; Reddy, 2016).

In this review we focus on compost, a valuable recycling product that is widely used in agriculture, viticulture, horticulture as well as private gardening, as an integrated part of a good agricultural practice. Composts contain significant amounts of nutrients including phosphorus, magnesium, potassium, and calcium. Furthermore, compost amendments improve organic matter content, soil structure, water holding capacity, microbial biomass, and activity (Eden et al., 2017). In addition, they can suppress soil-borne plant diseases, which, if not defeated, often result in serious yield losses. Composts can also contribute to improved resistance of plants to foliar diseases (Zhang et al., 1998; Vallad et al., 2003).

The disease-suppressive potential of composts against a wide spectrum of pathogens has been known for many decades and has been summarized by several authors (Bailey and Lazarovits, 2003; Noble and Coventry, 2005; Bonanomi et al., 2010; Noble, 2011), with some reviews focusing on the role of microorganisms in disease suppression (Fuchs, 2009; Hadar and Papadopolou, 2012; Mehta et al., 2014). For instance, in a meta-analysis on publications for the

time period from the 1970s until 2006, Bonanomi et al. (2007) found a disease-suppressive effect of composts in more than 50% out of 1,016 case studies, while disease-promoting effects were rather rare (below 12% of the case studies). Similarly, reviewing reports of 79 container and 54 field experiments, Noble (2011) reports success rates of compost of 74% in container and 83% in field experiments, whereas a disease promoting effect was only found in 8 or 2% of the experiments, respectively.

Despite the overwhelming potential of composts to reduce soil-borne diseases in general, predictability of the success of the application of a certain compost against a particular pathogen is limited. Comparing the suppressiveness of 18 composts in seven different plant–pathogen systems, Termorshuizen et al. (2006) found overall success rates of 54%, but variability between pathogens was high, with success rates ranging from 6% for *Phytophthora cinnamomi* to 71% for *Phytophthora nicotianae*. Similarly, the review by Bonanomi et al. (2007) on 1,016 case studies revealed that the success rates of compost applications varied substantially between plant–pathogen systems, with success rates between 32% (*Rhizoctonia* spp.) and 74% (*Fusarium* sp.). To optimally exploit the disease-suppressive potential of composts in practice, predictability is highly desirable, but is so far hampered by the complexity of compost microbiota, which can contain thousands of different species or strains.

Compost is an organic material resulting from the mostly aerobic decomposition of organic matter by microorganisms. The composition of the starting material can be highly diverse, including different kinds of manure, wood, green waste, food waste, digestate (i.e., the remains from anaerobic decomposition in biogas production), or waste from specialized industries (e.g., olive mills and paper mills) (Fuchs et al., 2006). Professional producers of composts often add small amounts of former compost lots at the beginning of the composting process to inoculate microorganisms that promote the composting process. The composition of the microbial community highly depends on the initial compost material and undergoes a number of substantial alterations during the different phases (Mehta et al., 2014). A first mesophilic phase with temperatures of 25–40°C is generally followed by a thermophilic phase (40–70°C) during which thermophilic bacteria (e.g., *Bacillus* sp. and *Thermus* sp.) predominate. This phase is very important to destroy potentially harmful organisms, including human and plant pathogens as well as weed seeds (Bollen et al., 1989; Johansen et al., 2013). In the following cooling phase (second mesophilic phase), mesophilic organisms recolonize the substrate, either from protected niches such as edges of compost

piles, from spores, or by external re-inoculation. In this phase, degraders of cellulose, including bacteria (e.g., *Cellulomonas* sp., *Clostridium* sp., and *Nocardia* sp.) as well as fungi (e.g., *Aspergillus* sp., *Fusarium* sp., and *Paecilomyces* sp.), become important (Insam and de Bertoldi, 2007). In the maturation or curing phase, the microbial community is again completely altered. Often fungi become predominant over bacteria due to higher competitiveness in conditions of poor nutrient availability. During storage, the microbial composition undergoes further alterations, while physico-chemical parameters remain relatively stable (Danon et al., 2008). Thus, the maturation stage and the storage of a mature compost are the main determinants for the microbial composition of the final compost product (Insam and de Bertoldi, 2007). However, the microbial composition of composts can also be influenced by the site of composting (e.g., composting facilities and field edges), the humidity management, initial C/N ratios, as well as turning techniques and intensities, which are all affecting the temperatures reached during the composting process (Eiland et al., 2001; Tiquia, 2005; Wang et al., 2015). Since many factors can affect compost quality, checking physical, chemical and biological properties of composts before application is essential, and quality guidelines have been set up in many countries by the government and the composting industry (e.g., Abächerli et al., 2010).

In some cases, abiotic factors have been shown to cause disease suppression by composts (e.g., high levels of ammonia in non-mature composts, high pH, fungitoxic compounds like hydroxyl-oleic acids, siderophores, and salts) (de Bertoldi, 2009). Yet, many studies have demonstrated a loss of disease suppressiveness in sterilized composts (Gorodecki and Hadar, 1990; Hoitink et al., 1997; Cotxarrera et al., 2002; Reuveni et al., 2002; Tilston et al., 2005), which indicates a crucial role of the living microorganisms in disease suppressiveness of composts. The ability of composts to suppress pathogens has been attributed either to the total microbial quantity and diversity (general disease suppression) or to individual organisms (specific disease suppression) (Berendsen et al., 2012).

It has been demonstrated that a change in the microbial community structure of soils occurs after the amendment with compost (Noble and Coventry, 2005) either by importing new microorganisms and/or by influencing the native microbial community. Several microorganisms isolated from or identified in compost have the potential to suppress soil-borne diseases and are summarized in the present review. Yet, attempts to generally predict the suppressiveness of composts against specific pathogens based on the presence/absence of known biocontrol strains and/or on other biotic (e.g., enzyme activity and microbial respiration) or abiotic markers (e.g., carbon, ammonia, and nitrate) have not been successful so far (de Bertoldi, 2009; Hadar and Papadopolou, 2012). Furthermore, farmers often report superior disease-suppressive effects of composts compared to application of single strains (Jacques Fuchs, personal communication), even though the efficacy of biocontrol strains isolated from composts has been demonstrated under field conditions in several cases (Cao et al., 2011; Xue et al., 2015). There is growing evidence

that an optimal disease suppression by composts might rather result from a consortium of microorganisms than from individual, specific strains. This is in accordance with many recent studies showing superior disease suppression of microbial consortia compared to individual strains (Berendsen et al., 2018).

Recent advances in next-generation sequencing (NGS) technology, such as amplicon and shotgun sequencing, have allowed characterizing the composition of compost microbiomes in great detail (Blaya et al., 2016). NGS technologies thus hold great potential to enable researchers to identify microorganisms and communities with a potential disease-suppressive effect. In addition, the *de novo* assembly of complete genomes of isolates that show a suppressive effect, offer unprecedented opportunities to move beyond studying the composition of compost microbiomes toward obtaining a mechanistic understanding of the beneficial effects that individual strains and consortia of strains can exert, and to elucidate direct causal effects (i.e., specific genes or pathways involved in suppression). The great potential of this approach has been shown for both strains isolated from natural soils that exert suppressive effects against pathogens (Mendes et al., 2011; De Vrieze et al., 2015; De Vrieze et al., 2020), as well as for strains isolated from the phyllosphere (Gore-Lloyd et al., 2019).

Here, we summarize the current knowledge on disease-suppressive isolates from compost for the biocontrol of plant pathogens and highlight the potential of an integrated systems approach combining experimental and NGS techniques to identify key microorganisms and microbial communities involved in suppressiveness of compost against plant diseases.

SUPPRESSIVE MICROORGANISMS ASSOCIATED WITH COMPOST

Several microorganisms isolated from or identified in compost have the potential to suppress soil-borne diseases. So far, these comprise taxa that are easily culturable under laboratory conditions (Schloss and Handelsman, 2004), which allows a later use as biocontrol agents or biofertilizers. Several organisms also occurring in composts (e.g., *Bacillus amyloliquefaciens*, *Streptomyces* sp., *Bacillus subtilis*, and *Trichoderma harzianum*) have been developed into commercial products (Bundesamt für Landwirtschaft [BLW], 2020; Speiser et al., 2020).

Several modes of action conveying a disease-suppressive effect have been described (Freimoser et al., 2019). Direct biological mechanisms include the overall increase in biomass and activity and therefore competition for nutrients and space among the different communities ("competition") (Hadar and Mandelbaum, 1986; Mandelbaum, 1990; Diáñez et al., 2005; Bonanomi et al., 2010; Bonilla et al., 2012), the direct attack of the pathogen through the production of secondary antimicrobial metabolites ("antagonism") (Mehta et al., 2014), as well as the secretion of chitinases, glucanases, and proteases ("hyperparasitism") (Nelson, 1983; Kwok et al., 1987; de Bertoldi, 2009). More indirect mechanisms are the activation of disease resistance genes in plants (i.e., induced systemic resistance, ISR) (Zhang, 1996;

Zhang et al., 1998; Krause et al., 2003; Aldahmani et al., 2005; Horst et al., 2005; Kavroulakis et al., 2005; Ntougias et al., 2008), as well as an overall improvement of plant nutrition and vigor leading to enhanced disease resistance (Hameeda et al., 2006; Zhao et al., 2017).

So far, few studies have employed cultivation-independent approaches to characterize the microbiota of disease-suppressive composts using the 16S, 18S, and ITS rRNA markers (Yu et al., 2015; Blaya et al., 2016; Vida et al., 2016; Ros et al., 2017, 2020; Corato and De Corato, 2019; Corato et al., 2019; Scotti et al., 2020). The combined outcome of these studies shows that the taxa identified as highly abundant (see **Tables 1, 2**, method “abundant”) greatly vary depending on the studied plant–pathogen systems and the starting material of the compost. For instance, Blaya et al. (2016) found a higher abundance of Bacteroidetes, alpha- and gamma-Proteobacteria and Chloroflexi, as well as non-pathogenic *Fusarium* and *Zopfiella* in composts that were most suppressive against *Phytophthora nicotianae* in pepper. In contrast, Acidobacteria Gp14, Actinobacteria, and Cystobasidiomycetes were more abundant in composts with strong suppression of *Pythium* wilt disease in cucumber (Yu et al., 2015).

Due to PCR amplification biases in amplicon-based studies (Sambo et al., 2018), a considerable fraction of organisms contributing to diseases suppression of compost is likely hitherto unexplored. Yet, at the moment, these marker-based studies are the only feasible high-throughput method for a large number of samples. Very few studies have employed a shotgun approach. For instance, Antunes et al. (2016) have described the microbial community structure during a composting process using shotgun as well as amplicon-based metagenomics. They found that the two data sets were mostly in accordance on higher taxonomic levels, whereas the largest discrepancies could be found on the species level. More compost microbiome studies are needed to get a better understanding of these complex systems, to elucidate which bacterial or fungal species correlate with disease suppression, and to increase the number of functionally relevant isolates as starting point for the design of robust consortia for biocontrol applications.

Bacteria

Bacterial communities in compost are usually characterized by a high abundance of the phyla Proteobacteria, Firmicutes, Bacteroidetes, and Actinobacteria (Neher et al., 2013). These phyla are commonly present in composts (and soils) regardless of their maturity and chemical and physical properties (Yu et al., 2015).

Within the Gammaproteobacteria, several *Pseudomonas* spp. and *Enterobacter* spp. strains are known to suppress *Pythium* damping-off (Chen et al., 2012) (**Table 1**). *Pseudomonas* is one of the most diverse (phylogenetically and functionally) Gram-negative bacterial genera with the largest number of known species (Gomila et al., 2015). It is ubiquitous in a wide range of habitats and exhibits a high degree of physiological and genetic adaptability (Mulet et al., 2012). For instance, *Pseudomonas* strains were abundant in a compost with a disease-suppressive effect toward *Pythium* in cucumber (Chen

et al., 2012). *Enterobacter cloacae* has been isolated from compost and showed promising anti-*Ganoderma* activity (Chin et al., 2017). Besides *Pseudomonas* and *Enterobacter*, other genera of the Gammaproteobacteria can also contribute to disease-suppressive traits of composts. For instance, *Aeromonas media* strains were preferentially isolated from the rhizosphere of plants grown in substrates amended with suppressive compost (Oberhaensli et al., 2017). *A. media* improved suppressiveness of compost against *P. ultimum* in cress (*Lepidium sativum* L.) potting experiments. The largest effect was achieved when the bacterial strains were added to compost with a low suppressiveness, which increased the suppressiveness to the level of highly suppressive compost (Oberhaensli et al., 2017).

Within the phylum Firmicutes, *Bacillus* (e.g., *B. subtilis*) and related genera have demonstrated a beneficial effect on the disease suppression activity of compost (Nakasaki et al., 1998; Cao et al., 2011; Antoniou et al., 2017) (**Table 1**). *Bacillus amyloliquefaciens* amended to compost increased the suppression of *Fusarium* wilt disease in watermelon (Zhao et al., 2017), banana (Xue et al., 2015), as well as in cucumber (Du et al., 2017). Other *Bacillus* strains isolated from disease-suppressive composts include *B. licheniformis* (Du et al., 2017), *B. subtilis* (Tsolakidou et al., 2019), *B. tequilensis* (Chin et al., 2017), and *B. velezensis* (Syed-Ab-Rahman et al., 2018) (**Table 1**).

Within the phylum Actinobacteria, several *Streptomyces* strains including *S. aureoverticillatus*, *S. coeruleorubidus*, *S. griseoruber*, *S. lusitanus*, and *S. variegatus* have been isolated from compost and showed antagonistic activity toward several phytopathogens (e.g., *Pythium*, *Fusarium*, and *Phytophthora*) (Cuesta et al., 2012).

Within the phylum Bacteroidetes, Sphingobacteria as well as Flavobacteria (e.g., *Flavobacterium balustinum*) have been associated with disease suppression (Kwok et al., 1987). Similarly, the higher abundance of the subgroup Gp14 of the phylum Acidobacteria in compost was associated with suppressiveness (Yu et al., 2015) (**Table 1**).

Oomycetes and Fungi

Several beneficial oomycetes and fungi have been associated with suppressiveness of compost (**Table 2**). A higher abundance of Cystobasidiomycetes has been found in composts with a stronger disease-suppression ability compared to composts without or with a reduced ability (Yu et al., 2015). The fungal antagonist *Verticillium biguttatum* was isolated from compost and its addition to compost increased the suppressiveness in bioassays with *Rhizoctonia solani* and sugar beet or potato (Postma et al., 2003). Non-pathogenic *Pythium* taxa in combination with compost significantly reduced damping-off in rooibos caused by pathogenic *Pythium* (Bahramisharif et al., 2013). Addition of the biocontrol agent *Trichoderma asperellum* strain T-34 to compost reduced *Rhizoctonia* damping-off in cucumber plants (Trillas et al., 2006), as well as *Fusarium* wilt in tomato and carnation (Cotxarrera et al., 2002; Sant et al., 2010). Ros et al. (2017) have evaluated the effect of compost fortified with the biological control agent *Trichoderma* on pepper infected with *Phytophthora nicotianae*. They found that the amended *Trichoderma* likely

TABLE 1 | List of identified beneficial bacteria either isolated from, amended to or abundant in suppressive compost, their respective plant–pathogen system(s) and (if known) the proposed mechanism of action conveying the suppressive effect.

Beneficial organism	Method	Pathogen	Plant	Mechanism	References
<i>Aeromonas media</i>	Isolated and amended	<i>Pythium ultimum</i>	Cress	Unknown	Oberhaensli et al. (2017)
Acidobacteria Gp14	Abundant	<i>Pythium</i> sp.	Cucumber	Unknown	Yu et al. (2015)
<i>Acinetobacter</i> sp.	Isolated	<i>Phytophthora capsici</i> , <i>P. citricola</i> , <i>P. palmivora</i> , <i>P. cinnamomi</i>	–	Unknown	Syed-Ab-Rahman et al. (2018)
<i>Bacillus amyloliquefaciens</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>cucumerinum</i> (FOC)	Cucumber	Possibly protease, cellulase and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity	Du et al. (2017)
	Isolated	<i>Phytophthora capsici</i> , <i>P. citricola</i> , <i>P. palmivora</i> , <i>P. cinnamomi</i>	–	Unknown	Syed-Ab-Rahman et al. (2018)
JDF35	Amended	<i>Fusarium oxysporum</i>	Watermelon	Promotion of plant growth (possibly via tryptophan-dependent synthesis of auxins and extracellular phytase activity and increased availability of N, P and K in the soil)	Zhao et al. (2017)
NJN-6	Amended	<i>Fusarium oxysporum</i>	Banana	Unknown	Xue et al. (2015)
<i>Bacillus licheniformis</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>cucumerinum</i> (FOC)	Cucumber	Possibly protease, cellulase and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity	Du et al. (2017)
	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp., <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Bacillus subtilis</i> SQR 9	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp., <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Bacillus tequilensis</i> CE4	Isolated	<i>Ganoderma boninense</i>	–	Unknown	Chin et al. (2017)
<i>Bacillus velezensis</i>	Isolated	<i>Phytophthora capsici</i> , <i>P. citricola</i> , <i>P. palmivora</i> , <i>P. cinnamomi</i>	–	Unknown	Syed-Ab-Rahman et al. (2018)

(Continued)

TABLE 1 | Continued

Beneficial organism	Method	Pathogen	Plant	Mechanism	References
<i>Burkholderia</i> spp.	Abundant	<i>Rosellinia necatrix</i>	Avocado	Unknown	Vida et al. (2016)
<i>Chryseobacterium</i> sp.	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp., <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Enterobacter</i> spp.	Abundant	<i>Pythium</i> sp.	Cucumber	Unknown	Chen et al. (2012)
	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp., <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Enterobacter cloacae</i> subsp. <i>dissolvens</i> B3	Isolated	<i>Ganoderma boninense</i>	–	Unknown	Chin et al. (2017)
<i>Flavobacterium balustinum</i>	Amended	<i>Rhizoctonia</i>	Bark	Unknown	Kwok et al. (1987)
<i>Lechevalieria</i> sp.	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Nocardiosis</i> spp.	Abundant	<i>Rhizoctonia solani</i> , <i>Sclerotinia minor</i>	Cress	Unknown	Scotti et al. (2020)
<i>Ochrobactrum</i> sp.	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp. <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Paenibacillus polymyxa</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>cucumerinum</i> (FOC)	Cucumber	Possibly protease, cellulase and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity	Du et al. (2017)
<i>Pseudomonas</i> spp.	Abundant	<i>Pythium</i> sp.	Cucumber	Unknown	Chen et al. (2012)
	Abundant	<i>Rhizoctonia solani</i> , <i>Sclerotinia minor</i>	Cress	Unknown	Scotti et al. (2020)
	Abundant	<i>Rosellinia necatrix</i>	Avocado	Unknown	Vida et al. (2016)
<i>Serratia marcescens</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> (FORL)	Tomato	Unknown	Kavroulakis et al. (2010)

(Continued)

TABLE 1 | Continued

Beneficial organism	Method	Pathogen	Plant	Mechanism	References
<i>Stenotrophomonas maltophilia</i>	Isolated	<i>Verticillium dahliae</i> , <i>Fusarium oxysporum</i> f. sp. <i>Lycopersici</i> (FORL)	Tomato	Production of diffusible secondary metabolites (more than volatile organic compounds), indole-3-acetic acid (IAA) production, ACC deaminase activity	Tsolakidou et al. (2019)
<i>Streptomyces albogriseolus</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Streptomyces aureoverticillatus</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Streptomyces coeruleorubidus</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Streptomyces griseoruber</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Streptomyces lusitanus</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)
<i>Streptomyces variegatus</i>	Isolated	<i>Fusarium oxysporum</i> f. sp. <i>melonis</i> , <i>Phytophthora cinnamomi</i> , <i>Pythium debaryanum</i> , <i>Sclerotinia sclerotiorum</i> , <i>Thanatephorus cucumeris</i> , <i>Agrobacterium tumefaciens</i>	–	Possibly synthesis of antibiotic compounds	Cuesta et al. (2012)

There are large variations between taxa identified in isolation (method “isolated”) and NGS (method “abundant”) studies, which can be attributed in parts to the fact that NGS approaches also sample unculturable microorganisms.

TABLE 2 | List of identified beneficial fungi and oomycetes either isolated from, amended to or abundant in suppressive compost, their respective plant–pathogen system(s) and (if known) the proposed mechanism of action conveying the suppressive effect.

Beneficial organism	Method	Pathogen/Parasite	Plant	Mechanism	References
<i>Coniothyrium minitans</i>	Amended	<i>Sclerotinia minor</i>	Lettuce	Possibly nutrient/space competition	Rabeendran et al. (2006)
<i>Cystobasidiomycetes</i>	Abundant	<i>Pythium</i> sp.	Cucumber	Unknown	Yu et al. (2015)
<i>Dothideomycetes</i>	Abundant	<i>Rosellinia necatrix</i>	Avocado	Unknown	Vida et al. (2016)
Non-pathogenic <i>Fusarium oxysporum</i>	Amended	<i>Rhizoctonia solani</i>	Carnation	Unknown	Postma et al. (2003)
	Abundant	<i>Phytohthora nicotianae</i>	Pepper	Unknown	Blaya et al. (2016)
Non-pathogenic <i>Pythium</i> sp. <i>RB II</i>	Isolated and amended	<i>Pythium mamillatum</i> , <i>Pythium pyrlobum</i> , <i>Pythium irregular</i> , <i>Phytophthora cinnamomi</i>	Rooibos	Possibly nutrient competition	Bahramisharif et al. (2013)
Non-pathogenic <i>Pythium acanthicum</i>	Isolated and amended	<i>Pythium mamillatum</i> , <i>Pythium pyrlobum</i> , <i>Pythium irregular</i> , <i>Phytophthora cinnamomi</i>	Rooibos	Possibly nutrient competition	Bahramisharif et al. (2013)
Non-pathogenic <i>Pythium cederbergense</i>	Isolated and amended	<i>Pythium mamillatum</i> , <i>Pythium pyrlobum</i> , <i>Pythium irregular</i> , <i>Phytophthora cinnamomi</i>	Rooibos	Possibly nutrient competition	Bahramisharif et al. (2013)
<i>Trichoderma asperellum</i>	Amended	<i>Phytophthora nicotianae</i>	Pepper	Mycoparasitism, antibiosis, nutrient/space competition	Ros et al. (2017)
	Isolated and amended	<i>Fusarium oxysporum</i>	Tomato	Unknown	Cotxarrera et al. (2002)
T34	Amended	<i>Fusarium oxysporum</i>	Carnation	Possibly induction of plant disease resistance, increased root system and available surface area, facilitated water uptake	Sant et al. (2010)
T34	Amended	<i>Rhizoctonia solani</i>	Cucumber	Chitinase activity	Trillas et al. (2006)
<i>Trichoderma harzianum</i>	Amended	<i>Phytophthora nicotianae</i>	Pepper	Mycoparasitism, antibiosis, nutrient/space competition	Ros et al. (2017)
<i>Verticillium biguttatum</i>	Amended	<i>Rhizoctonia solani</i>	Radish	Unknown	Nelson (1983)
	Amended	<i>Rhizoctonia solani</i>	Sugar beet, Potato	Unknown	Postma et al. (2003)
<i>Zopfiella</i>	Abundant	<i>Phytohthora nicotianae</i>	Pepper	Unknown	Blaya et al. (2016)

There are large variations between taxa identified in isolation (method “isolated”) and NGS (method “abundant”) studies, which can be attributed in parts to the fact that NGS approaches also sample unculturable microorganisms.

conveys higher disease suppressiveness than the native compost microbiota.

CURRENT CHALLENGES AND LIMITATIONS

The application of compost and compost derived microorganisms for disease control entails various challenges and limitations (e.g., pathogen-specificity, unknown mechanisms, temporal variability of the same compost, large microbial diversity, and registration), which will be discussed in the following in detail.

In a large number of studies, a compost suppressive to one pathogen was ineffective or rarely even conducive to

other pathogens, which suggests that suppressiveness is often pathogen-specific (Bonanomi et al., 2010) and that consortia of beneficial microorganisms may have to be specifically tailored to target different pathogens. Moreover, some pathogens appear to be very enigmatic. For instance, the pathogenicity of *R. solani* is still poorly understood despite the enormous number of studies that have aimed to find an effective control agent (total $n = 670$; $n = 272$ for composts) (Bonanomi et al., 2010). In general, most experiments have been conducted on fungal and oomycete pathogens so far (Noble, 2011).

The ability of certain composts to suppress diseases does not only vary across pathogens (Scheuerell et al., 2005; Termorshuizen et al., 2006), but even when using composts of very similar composition and at the same inclusion rate (volume compost/volume substrate) (Noble and Coventry,

2005). This variability impairs repeatability and reliability of experiments even if they are performed with highly similar batches of compost. This most likely can be attributed to the enormous variability of compost characteristics and the impossibility of obtaining composts with a standardized (i.e., identical) composition, as well as to changes in the microbial composition over time and during storage (van Rijn et al., 2007). The enormous diversity of the microbial compost communities poses big challenges for the identification of potential beneficial strains and consortia. Furthermore, it is challenging to identify direct, and in particular, indirect interactions among microbes jointly contributing to disease suppression (Chen et al., 2012). A large proportion of the compost microbes may not be involved in obvious binary interactions (e.g., pathogen – antagonist), but rather influence interactions between other organisms (i.e., tritagonists) (Freimoser et al., 2016). In order to develop individual strains or combinations of strains into biological control products, they first need to be isolated and for a large part of them this is not possible due to very specific or unknown requirements for successful cultivation (see section “An Integrated Strategy to Harness Compost Microbiomes for Plant Protection”). Furthermore, their registration as biopesticides (in the class of microbial pesticides) is required. The prerequisites for their registration can vary across countries and can be laborious, which further hampers their commercialization. Since these strains originate from compost, which has experienced elevated temperatures during the composting process, they often show a high heat resistance and can be very persistent (Jurado et al., 2014). As for any other biocontrol strain, questions related to biosafety and in particular the potential to colonize mammalian hosts have to be carefully studied. Despite the necessity to ensure biosafety, data requirements to register microbial biocontrol agents in the European Union are extensive, time-consuming and expensive (Köhl et al., 2019), thus detaining companies from submitting registration dossiers and resulting in very low numbers of newly approved microbial biocontrol organisms. In the case of synthetic microbial consortia, each single strain has to be approved by authorities, which even multiplies hurdles for registration. In other countries like the United States, more tailored registration procedures result in many more newly approved microbial biocontrol organisms (Köhl et al., 2019). Striving for similar procedures in the European Union by the biocontrol industry has not been successful so far (Anonymous, 2018; Köhl et al., 2019).

Considering the complex nature of composts, a multitude of factors likely contributes to suppressiveness. So far, no defined set of taxa has explained disease suppression and their composition will likely be context (host, pathogen, soil, or substrate) dependent. We consider an integrated systems approach very promising to overcome these challenges and outline such an approach in the following section. Possible important outcomes of such a systems approach may include identification and isolation of new biocontrol strains and their mode of action, development of microbial consortia with suppressiveness superior to single strains, development of diagnostic tools to allow a targeted application of composts

against soil-borne diseases, and the development of strategies to selectively promote key microbial organisms in composts.

AN INTEGRATED STRATEGY TO HARNESS COMPOST MICROBIOMES FOR PLANT PROTECTION

In order to establish a basis to increasingly harness compost microbiomes for plant protection, we propose an integrated systems approach (**Figure 1**). This approach combines the power of experiments and functional assays (**Figure 1**, yellow boxes), and the value of a growing collection of isolated strains (**Figure 1**, blue container) with detailed metagenomics studies (i.e., amplicon and shotgun sequencing) to achieve a better understanding of the complex system compost and to identify members of taxa enriched in suppressive composts. Further, this approach includes whole-genome sequencing of potentially suppressive isolates and subsequent functional genomics studies (i.e., comparative genomics, (meta-)transcriptomics, and (meta-)proteomics), which can elucidate the mechanisms of action of biocontrol strains and represent a basis to amend composts with optimal strain combinations (biocontrol and plant growth promoting strains) and design specific diagnostic applications (**Figure 1**, green box).

The development of robust functional assays, which allow to accurately evaluate the suppressiveness of compost, is one of the most important tasks. Due to the above mentioned pathogen-specificity of suppressive composts, such assays should be developed for a broad range of plant–pathogen systems (Bahramisharif et al., 2013; Oberhaensli et al., 2017), and ideally should be miniaturized to allow sufficient throughput at manageable costs.

Once such robust assays are available, potentially beneficial microorganisms conveying the disease-suppressive effect can be isolated from suppressive composts (**Figure 1**). These strains would largely expand on the rather limited repertoire of suppressive microorganisms that have been described so far (see **Tables 1, 2**). In order to reduce the extremely high species diversity in composts and substrates/soil that can amount to tens of thousands of operational taxonomic units (OTUs) or exact sequence variants (ESVs), it is likely beneficial to target specific niches, for instance the rhizoplane (i.e., the region where the root surface is in contact with soil). Plants are able to shape their rhizoplane microbiome (Hu et al., 2018) and possibly attract microorganisms involved in disease suppression (Berendsen et al., 2012). Therefore, isolation experiments targeting the rhizoplane allows researchers to focus on the likely most relevant species (**Figure 1**, orange boxes). For instance, *A. media* was the dominant species in the rhizoplane of cress grown in a suppressive compost as compared to cress grown in non-suppressive composts. The addition of *A. media* to non-suppressive compost increased the suppressiveness against *P. ultimum* (Oberhaensli et al., 2017). Importantly, the creation of a collection of rhizoplane strains isolated from protected plants represents a critical first objective of the integrated systems approach and a highly valuable resource not only for

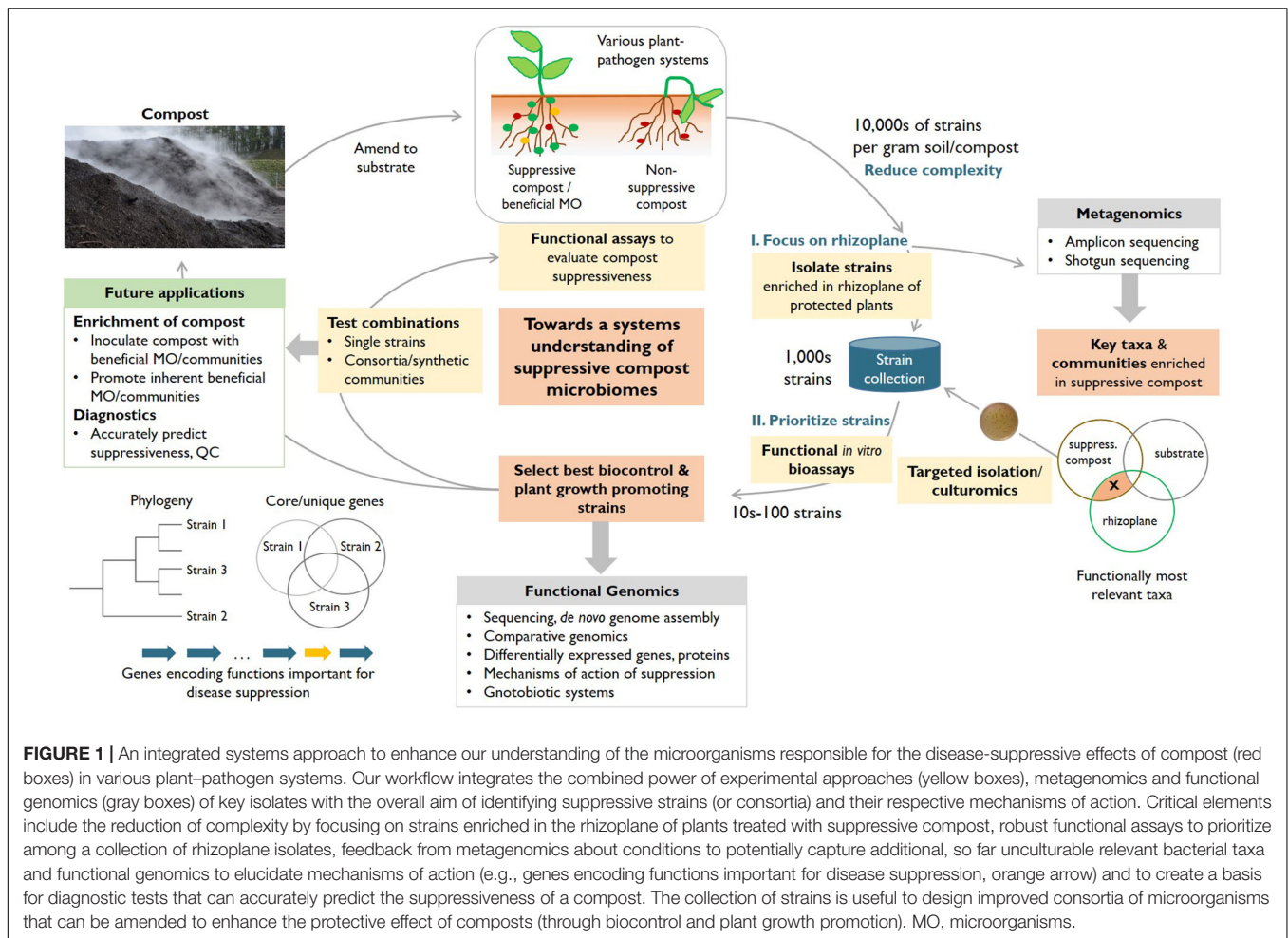


FIGURE 1 | An integrated systems approach to enhance our understanding of the microorganisms responsible for the disease-suppressive effects of compost (red boxes) in various plant–pathogen systems. Our workflow integrates the combined power of experimental approaches (yellow boxes), metagenomics and functional genomics (gray boxes) of key isolates with the overall aim of identifying suppressive strains (or consortia) and their respective mechanisms of action. Critical elements include the reduction of complexity by focusing on strains enriched in the rhizoplane of plants treated with suppressive compost, robust functional assays to prioritize among a collection of rhizoplane isolates, feedback from metagenomics about conditions to potentially capture additional, so far unculturable relevant bacterial taxa and functional genomics to elucidate mechanisms of action (e.g., genes encoding functions important for disease suppression, orange arrow) and to create a basis for diagnostic tests that can accurately predict the suppressiveness of a compost. The collection of strains is useful to design improved consortia of microorganisms that can be amended to enhance the protective effect of composts (through biocontrol and plant growth promotion). MO, microorganisms.

subsequent functional genomics studies but also for an effort to develop optimized consortia of biocontrol and plant growth promoting strains.

Patterns of differential enrichment in the rhizoplane of protected plants (as observed for *A. media*) further contribute to the reduction of complexity and prioritization of the potentially most relevant microorganisms. Such an enrichment can also be identified using amplicon-based metagenomic diversity studies (16S, 18S, and ITS), which allow an assessment of the microbial community composition (including unculturable microorganisms) and dynamics for a large number of samples. For instance, Blaya et al. (2016) found a higher relative abundance of Ascomycota including the genera *Fusarium* and *Zopfiella* in highly suppressive composts against *Phytophthora* root rot. These microbiome studies can thus also help to further guide efforts to isolate key taxa and choosing the appropriate culturing techniques, as well as the design of synthetic communities (see below).

Controlled lab experiments with isolated and cultured strains (e.g., from the rhizoplane) are a crucial step toward a better understanding of their functional potential. However, the majority of microorganisms appear unculturable using conventional approaches, known as the “great plate count

anomaly” (Staley and Konopka, 1985). Recent advances in culturomics, i.e., efforts to culture a larger number of isolates from microbiomes (Lagier et al., 2018; Sarhan et al., 2019), minimize the gap between the number of culturable species and the actual microbial richness and can even identify species that are not detected by sequencing-based approaches (Lagier et al., 2012). Culture conditions need to be adapted to the potentially beneficial taxa identified in the sequencing-based microbial community analysis in order to maximize the number of culturable and potentially new bacteria, fungi, and oomycetes. While such culturomics based approaches are challenging, they have greatly expanded the number of strains obtained from the human gut microbiome for example (Lagier et al., 2018). A similar approach also enabled the identification of the novel antibiotic teixobactin against Gram-positive bacteria (Ling et al., 2015), which was isolated from the previously uncultured soil bacterium *Eleftheria terrae* that was successfully grown in its natural habitat with a diffusion chamber (i.e., isolation chip) (Nichols et al., 2010). Individual strains are then characterized and prioritized according to their suppressiveness in functional assays (Hilber-Bodmer et al., 2017). Further, public strain collections may represent a valuable resource to explore the disease-suppressive potential of additional strains from the same

species or genus. Eventually, the beneficial strains need to be tested *in vivo* (i.e., *in planta*), since several studies have shown that *in vitro* assays often fail to capture the complexity of a natural system including plant-associated phenotypes (Bulgarelli et al., 2013). Since disease suppression likely does not derive from a single strain, these experiments should explore the composition of various consortia to point toward a particularly potent microbial community for plant protection (De Vrieze et al., 2018; Tsolakidou et al., 2019). Consortia often result in better plant growth promotion or protection in comparison to single strains (Berendsen et al., 2018).

Whole-genome sequencing (using long reads NGS technologies such as Pacific Biosciences and Oxford Nanopore Technologies), complete *de novo* genome assembly, and genome annotation of the most promising candidates will be the basis for an in-depth elucidation of modes of action and provide important information for predicting metabolic features (Schmid et al., 2018). This approach represents an optimal basis for subsequent downstream genome mining and 'omics analyses (Dunlap et al., 2013; Van Der Voort et al., 2015; Mullins et al., 2019). Based on these annotated genomes, genes potentially involved in the process of disease suppression, and thus, the respective mechanisms of action underlying a suppressive effect, can be identified. This can entail a genome mining effort to identify genes from gene families already known to play a role in biocontrol (these include volatiles, secretion systems and their effectors, proteases, chitinases, and glucanases). Alternatively, the gene inventories of beneficial strains and closely related non-beneficial strains can be compared using comparative genomics approaches, or even more informative, the genomes of suppressive strains versus the genomes of mutant strains that have lost the ability to suppress a pathogen. For instance, whole-genome sequencing of both wild-type and a pigmentless mutant strain of the antagonist *Metschnikowia pulcherrima* allowed to identify Snf2 as a regulator of antifungal activity (Gore-Lloyd et al., 2019). Thereby, links from the genotype to a phenotype can be suggested, as has been shown in systems for potential biocontrol strains and different host plant varieties (De Vrieze et al., 2020). An important approach to reduce the number of candidate genes provided from genome comparisons are transcriptomics studies. If carried out on relevant conditions (e.g., suppression versus no effect), they can help to identify the most relevant differentially expressed genes and allow to generate testable hypotheses about the mechanism of action involved. Ideally such experiments are carried out using gnotobiotic systems, which represent a controlled environment using sterilized substrate, and thus, confounding factors (e.g., other soil microbiome strains) are eliminated (Timmusk and Wagner, 1999). Such systems allow investigating the interactions between a certain pathogen, the infected plant and the suppressiveness of a beneficial strain. For instance, a well-studied system is the activated ISR in *Arabidopsis thaliana* caused by rhizosphere inhabiting strains of *Pseudomonas fluorescens* (Bakker et al., 2007). Furthermore, the design and testing of various synthetic communities in these gnotobiotic systems can help explain the observed protection of the plant against the respective pathogen. Further, traits of a consortium in comparison to single strains can

be explored. A drawback of this method is that some suppressive features may only be active under specific environmental conditions. For instance, several microbial antagonists decrease their chitinase production in the presence of simple sugars (de la Cruz et al., 1993; Lorito, 1993; Gupta et al., 1995). Thus, in order to efficiently employ suppressive strains, the appropriate abiotic and biotic conditions need to be known.

Targeted functional (Dougherty et al., 2012; Yeh et al., 2013) or shotgun metagenomic approaches can further inform about potential community functions. For instance, new glycoside hydrolases, which play important roles in degradation of biomass, were discovered in a microbial compost community using shotgun metagenomics (Dougherty et al., 2012). Similarly, compost metagenomes can be mined for genes potentially involved in disease suppression based on homologous genes in databases. Genes with suppressive traits can then be used as markers to screen composts for their disease suppression potential.

In order to elucidate actual functional activity, meta-transcriptomics, in this case of the entire community instead of isolated strains, is a promising technology. It can significantly reduce the complexity by targeting the expressed genes rather than the entire genetic information that is contained in a metagenome. This has been successfully demonstrated in soil studies (Shrestha et al., 2009; Hultman et al., 2015) and in a time-series of a composting process (Antunes et al., 2016). A comparison of gene expression profiles of suppressive and non-suppressive composts could provide a better understanding of the underlying mechanisms of disease suppression. Proteomics is another promising technology for future compost studies since it provides more accurate data about the expression, modification and interaction status of the actual players that carry out most functions in cells, i.e., the proteins (Ahrens et al., 2010; Aebersold and Mann, 2016). In this context, meta-proteomic studies could further extend the functional data sets collected from compost microbiomes. Although such an approach still presents considerable challenges for highly complex microbiomes such as soils and composts, they have the potential to link microbial community compositions and functions (Wilmes and Bond, 2006). For instance, Liu et al. (2015) elucidated the main biodegradation pathways in a composting system and could link them to specific organisms. Similarly, proteins involved in disease suppression could be attributed to beneficial organisms in suppressive compost. Notably, a meta-proteomic analysis will largely benefit from the growing inventory of whole genome sequences from well-characterized entries of strain collections, as this will allow creating better protein search databases, for which the genomic sequence information is required. With complete genomes in hand, proteogenomics (Omasits et al., 2017) will be instrumental to identify as of yet missed biocontrol genes such as short lipopeptides (Van Der Voort et al., 2015) allowing to further extend the inventory of the genetic determinants of antagonism. Finally, the analysis of the metabolome represents an important aspect to monitor the ultimate response of an organism to its environment. Metabolites are conserved across taxa and can therefore be investigated even if no reference genomes are available. Thus, metabolomics represents another powerful tool

for the identification of disease-suppressive mechanisms. For instance, Vinci et al. (2018) showed an enhanced phosphorus and nutrient uptake in maize leaves, and thus improved photosynthetic activity, when treated with *B. amyloliquefaciens* in combination with composts.

Ultimately, the combined information gained from the described approaches will help to develop specific diagnostic tests that can predict the suppressive potential of a given compost for individual plant–pathogen systems, which would further advance the large-scale application of composts to suppress soil-borne diseases. A combined approach including bioassays, metagenomics, strain collections and subsequent sequencing and functional genomics of key isolates is crucial. While metagenomic studies can find patterns in microbial community structures, and thus, can point toward potentially beneficial taxa, isolates with suppressive traits are indispensable for conducting controlled experiments and building up a collection of strains from various plant–pathogen systems. These compost strain collections form the basis to identify consortia of beneficial microorganisms that act synergistically to achieve an overall enhanced biocontrol effect.

In conclusion, the application of compost for disease suppression has a huge potential. However, so far the overall number of isolated strains with documented suppressive effects is rather low and the interactions between the beneficial microbiota, pathogens, and host plants, as well as the mechanisms of action

are mostly unknown. A deeper mechanistic understanding will allow to develop tailored solutions for specific phytosanitary problems, for example, by testing composts for suitable microbial compositions or by targeted enrichment with microorganisms adapted for the respective application.

DATA AVAILABILITY STATEMENT

All relevant data is contained within the article.

AUTHOR CONTRIBUTIONS

SL wrote the manuscript with substantial input from BT (compost background), FF (mechanism of action), and CA (functional genomics). TO carried out the initial literature search and created the final figure with CA. JM, JF, FW, and TO provided critical feedback to improve the manuscript. All authors contributed to the figure, tables, and literature search. All authors contributed to the article and approved the submitted version.

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Field Exploitation of Multiple Functions of Beneficial Microorganisms for Plant Nutrition and Protection: Real Possibility or Just a Hope?

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Bioproducts, i.e., microbial based pesticides or fertilizers (biopesticides and biofertilizers), should be expected to play an ever-increasing role and application in agricultural practices world-wide in the effort to implement policies concerned with sustainable agriculture. However, several microbial strains have proven the capacity to augment plant productivity by enhancing crop nutrition and functioning as biopesticides, or vice-versa. This multifunctionality is an issue that is still not included as a concept and possibility in any legal provision regarding the placing on the market of bioproducts, and indicates difficulties in clearly classifying the purpose of their suitability. In this review, we overview the current understanding of the mechanisms in plant-microbe interactions underlining the dual function of microbial strains toward plant nutrition and protection. The prospects of market development for multifunctional bioproducts are then considered in view of the current regulatory approach in the European Union, in an effort that wants to stimulate a wider adoption of the new knowledge on the role played by microorganisms in crop production.

Keywords: biofertilizers, biopesticides, microbial consortia, multifunctional bioproducts, plant growth-promoting microorganisms, endophytes

INTRODUCTION

Chemical, synthetic plant protection products (PPPs) and mineral fertilizers are criticized mainly for their potentially negative effects on human health (Bennekou, 2019) and/or the environment (Norse, 2005; Huang et al., 2017) including non-renewable resources depletion (Chojnacka et al., 2020), and overall negative impact on biodiversity (Mozumder and Berrens, 2007; Sánchez-Bayo and Wyckhuys, 2019). Because of these concerns, the application of sustainable crop production methods is required by consumers as well as by legal provisions (Marrone, 2019).

In this context, although microbial-based pesticides or fertilizers (hereafter biopesticides and biofertilizers, respectively) would not be expected to fully replace chemical pesticides and mineral fertilizers, they could play an ever-increasing role and application in agricultural practices world-wide (Alabouvette et al., 2012; Kurek and Ozimek, 2013).

The multifunctional use of bioinocula represents an issue that is emerging from several researches (Harman, 2011; Lopes et al., 2018), and could further support the development, marketing and application of microbial-based products. Yet, this prospective is neither included as a concept nor as a possibility in any legal provision dealing with the marketing of bioproducts, while it has started to be appraised in bioproducts for human consumption (Ma et al., 2019).

In this review we are summarizing recent findings on the multiple effects of microorganisms suitable as biofertilizers or biopesticides, in light of the intricate interactions between plants and microorganisms, in an effort to foster the discussion on new products that could find a better acceptance by farmers because of their multifunctional properties.

PLANT-MICROBIAL INOCULA INTERACTIONS AS THE BASIS FOR MULTIFUNCTIONAL BIOPRODUCTS

The interaction between plants and beneficial fungi involves elicitors released by them which include several metabolites, including volatiles (Shoresh et al., 2010; Morath et al., 2012). These compounds function as signal transduction in plants, and as a result both the plant proteome and transcriptome are affected, as it has been observed with *Trichoderma* (Marra et al., 2006; Shoresh and Harman, 2008; Lorito et al., 2010; Lombardi et al., 2020) or arbuscular mycorrhizal fungi (Jung et al., 2012; Cameron et al., 2013; Rivero et al., 2015; Adolfsen et al., 2017). The effect of these modifications is translated into increased plant growth, particularly under stress, improved nutrient use efficiency, acquisition of a systemic resistance to diseases that goes beyond the commonly induced systemic and acquired resistances (Shoresh et al., 2010; Cameron et al., 2013). Qualitatively similar effects are induced in plants by rhizobacteria: the interactions involve different chemical compounds (Abriouel et al., 2011; Fickers, 2012; Lopes et al., 2015; Jasim et al., 2016) as well as priming (Brencic and Winans, 2005; van Wees et al., 2008). Mechanisms include induction of the plant innate immune response system (Jain et al., 2011) or acquired systemic resistance (Iavicoli et al., 2003; Choudhary and Johri, 2009), alteration of plant functional traits (Friesen et al., 2011) and prevention of pathogen settling (Bakker et al., 2012).

On the other hand, growth promotion in bacteria derives mainly from the synthesis of several plant growth hormones (Arkhipova et al., 2005; Xie et al., 2014; Radhakrishnan and Lee, 2016) or their indirect regulation through production of volatile organic compounds (Tahir et al., 2017; Rath et al., 2018) and 1-aminocyclopropane-1-carboxylate deaminase (Glick et al., 2007), as well as the solubilization or mineralization of mineral nutrients (Malusá et al., 2016). A key role in interaction between plants and microorganisms seems to be played by pattern recognition receptors (PRRs), localized in the plants' plasma-membrane, which allow to recognize beneficial microbe/pathogen-associated molecular patterns (Boller and Felix, 2009; Zipfel, 2014; Trdá et al., 2015).

Nevertheless, the relation between plants and beneficial microorganisms inocula occurs within a wider framework of interactions, including those with the plant microbiome (Berg et al., 2017; Fadji and Babalola, 2020) as well as with the soil physical, chemical and biological characteristics (Bardi and Malusá, 2012; Vimal et al., 2017), which all contribute to increase the complexity in developing sustainable management practices and agricultural products such as biofertilizers and biopesticides as well as for better exploiting their characteristics.

BIOPESTICIDES AND PLANT GROWTH PROMOTION

Several biopesticides have been developed to protect plants from pests since the mid-twentieth century (Copping and Menn, 2000; de Faria and Wraight, 2007) and among them several entomopathogenic fungi (e.g., *Beauveria* spp., Zimmermann, 2007) and bacteria (e.g., *Bacillus thuringiensis*, de Almeida Melo et al., 2016) are currently used in crop protection. However, recently published studies have provided evidence for the involvement of entomo- or myco-pathogenic microorganisms in promoting plant growth, thus opening new opportunities of their multifunctional use (Vega et al., 2009; Lacey et al., 2015; **Table 1**). Examples with entomopathogenic fungi include the significant increase in onion yields after *Metarhizium anisopliae* sprays (Maniania et al., 2003) or in growth of soybean seedlings (Khan et al., 2012) or maize plants (Liao et al., 2014) or cotton (Lopez and Sword, 2015) after soil inoculation with different entomopathogenic species. The mechanism of growth promotion is related to the transfer of nitrogen, also from the parasitized pest, which occurred in both leguminous and gramineous species (Behie et al., 2012). However, production of siderophores (Jirakkakul et al., 2015) or increased uptake of iron (Sánchez-Rodríguez et al., 2015) have also been demonstrated to occur in plants colonized with the entomopathogenic *B. bassiana*. The production of the auxin indole-3-acetic acid was likewise found to be associated to several *Metarhizium* and *Beauveria* strains (Liao et al., 2017). Nevertheless, as for the expression of the full efficacy in insect pests' control, the ability of fungal entomopathogens to promote plant growth has resulted to depend on the inoculation method (Jaber and Enkerli, 2016, 2017) or the inoculation rate (García et al., 2011).

Increased plant growth mediated by entomopathogenic fungi could result from the suppression of the plant diseases (Kulda and Bacon, 2008; Jaber, 2015) or from a combination of reduced disease severity and more vigorous development of the plants as observed with *Beauveria* and *Metarhizium* strains and fungal or virus pathogens (Sasan and Bidochka, 2013; Jaber and Salem, 2014). In these cases, the mechanisms could derive from the capacity of entomopathogenic fungi to elicit the expression of photosynthesis- and energy metabolism-related proteins as well as plant defense responses (Gómez-Vidal et al., 2009).

Among pathogen biocontrol fungi, the dual effect of *Trichoderma* application has been observed in several studies. *T. harzianum* T-22 proved to solubilize *in vitro* insoluble rock phosphate likely by both chelation and reduction processes,

since no release of organic acids nor acidification were observed (Altomare et al., 1999). Trichoderma-based products were shown to modulated rhizosphere microbial populations, improving nutrient uptake efficiency, yield, and nutritional quality of leafy vegetables (Fiorentino et al., 2018) or of strawberry plants (Lombardi et al., 2020). Dipping roots of strawberry cuttings in a suspension of *T. asperellum* prior to planting followed by foliage applications during the vegetation season stimulated plant growth (+ 24%) and health (Kowalska et al., 2012). The effect was reverberated on the control of *Botrytis cinerea* also on stored fruits, extending their shelf-life without symptoms of damage up to 7 days. *Trichoderma* spp. isolates significantly reduced the infection of germinating seeds and carrots seedlings by *Pythium* spp. and efficiently influenced the growth of the seedlings as compared to the standard chemical seed dressing (Sobolewski et al., 2013). Similarly, foliar application of *T. asperellum* increased seed yield and weight and improved lipid content of organic oilseed rape (*Brassica napus* L.) (Kowalska, 2014). The mechanism of these plant growth promoting effects could be explained by the growth stimulation, observed with a *T. viridae* strain, particularly of lateral roots and inhibition of the elongation of hypocotyls, resulting in about fourfold increase of dry biomass in comparison to the control (Znajewska et al., 2018).

Among the bacteria exploited for protection against pathogens, the *Bacillus* and *Pseudomonas* genera have common commercial use and frequently are exploited also for plant growth promotion (Santoyo et al., 2012). A rich literature exists on these microorganisms (e.g., Kumar et al., 2011; Chowdhury et al., 2015; Islam et al., 2019) thus the reader is advised to refer to it. However, it is interesting to note that the entomopathogenic *B. thuringiensis*, was able to *in vitro* solubilize low-soluble inorganic phosphate and simultaneously produce IAA when formulated in k-carrageenan (Vassilev et al., 2006). This formulation boosted plant growth and P-uptake when introduced into a soil-plant system, stimulating the establishment and development of the co-inoculated endomycorrhizal fungus *Glomus deserticola* (Vassilev and Vassileva, 2004).

BIOCONTROL POTENTIAL OF BIOFERTILIZERS

Many plant growth promoters used for inoculation in cropping systems might serve as biocontrol microorganisms (Chowdhury et al., 2015; French, 2017; Table 1). The biocontrol potential of several P-solubilizers has been verified in several works of Vassilev and co-workers (Vassilev et al., 2006). Inoculation with

TABLE 1 | Microbial strains showing plant protection and growth promotion effects.

Strains	Effect	References
<i>Trichoderma atroviridae</i>	Plant growth promoter Biocontrol of fungal pathogens	Marra et al., 2006
<i>Trichoderma harzianum</i>	Solubilization of phosphates Biocontrol of Fusarium disease	Altomare et al., 1999; Martínez-Medina et al., 2009
<i>Trichoderma viridae</i>	Plant growth stimulator Modulate rhizosphere microbial and improve N uptake	Fiorentino et al., 2018; Znajewska et al., 2018
<i>Trichoderma</i> spp.	Activator of plant physiological processes Elicitor of plant resistance system and plant growth stimulator	Kowalska et al., 2012; Lombardi et al., 2020
<i>Glomus mosseae</i> and <i>Rhizobium leguminosarum</i>	Biocontrol Fusarium root rot	Dar et al., 1997
<i>Bacillus amyloliquefaciens</i>	Biocontrol of root pathogens Induce systemic resistance, protect plants against attacks of pathogenic microbes, viruses, and nematodes	Chowdhury et al., 2015; Borris, 2020
<i>Bacillus subtilis</i>	Plant growth	Arkipova et al., 2005
<i>Bacillus mojavensis</i>	Plant growth modulators	Rath et al., 2018
<i>Bacillus methylotrophicus</i>	Supports plant growth and enhances nutritional metabolites	Radhakrishnan and Lee, 2016
<i>Metarhizium anisopliae</i>	Plant growth and mitigates salt stress Biocontrol of Trips tabaci	Maniania et al., 2003; García et al., 2011; Khan et al., 2012; Lopez and Sword, 2015
Clavicipitaceous endophytes	Suppression of the plant diseases	Kuldau and Bacon, 2008
<i>Metarhizium robertsii</i>	Promotes root growth Antagonism to Fusarium solani	Sasan and Bidochka, 2013; Liao et al., 2017
<i>Beauveria bassiana</i> and <i>Metarhizium</i>	Reduce fungal and virus disease	Jaber and Salem, 2014
<i>Beauveria bassiana</i> and <i>Metarhizium brunneum</i>	Insect pests' control and promote plant growth	Lopes et al., 2015; Jaber and Enkerli, 2016
<i>B. bassiana</i>	Alleviates Fe chlorosis Biocontrol of downy mildew	Jaber, 2015; Sánchez-Rodríguez et al., 2015
<i>Pseudomonas</i> spp.	Plant growth-promoting Mobilization of insoluble forms of K Biocontrol of Phytophthora infestans	Santoyo et al., 2012; Meena et al., 2014; De Vrieze et al., 2018
<i>Pseudomonas fluorescens</i>	Induces systemic resistance	Iavicoli et al., 2003
Microbial consortia	Biocontrol of Sclerotinia sclerotiorum	Jain et al., 2011
Rhizobacteria	Priming, induction of the plant immune response system prevention of pathogen settling Pathogen suppression	van Wees et al., 2008; Jain et al., 2011; Bakker et al., 2012; Islam et al., 2019
<i>B. bassiana</i> , <i>Lecanicillium dimorphum</i>	Modulates plant defense responses and energy metabolism	Gómez-Vidal et al., 2009
<i>Phanerochaete chrysosporium</i>	P-solubilizing filamentous fungi against Fusarium wilt	Khan and Khan, 2001
<i>Paenibacillus kribbensis</i>	Potassium and phosphate-solubilizing capacity and reduce of several cotton and wheat soil-borne pathogens	Zhang et al., 2013

G. intraradices significantly reduced the impact of the soil-borne pathogen *F. oxysporum* on tomato plants, paralleled by a significant decrease in the number of colony-forming units compared with the control treatment (Vassilev et al., 2008, 2009a). However, the further introduction of a filamentous fungus (*A. niger*) in the formulation, with different plant wastes and rock phosphate as microbial growing substrate, was more effective to control the pathogen. Similar results were achieved with *Phanerochaete chrysosporium* (Vassilev et al., 2009b) or in other field trials with P-solubilizing filamentous fungi against *Fusarium* wilt in tomato (Khan and Khan, 2001). In these cases, the biocontrol function was suggested to be based on production of hydrolytic enzymes or the competition for nutrients and space by the microbial P-solubilizers, as well as by hormones such as indole-3-acetic acid (IAA) and siderophores, being among the metabolites most frequently released by P-solubilizing microorganisms.

The observation that root colonization by AMF is not always associated to improved nutrition and increased vegetative biomass (Smith and Smith, 2011), has prompted to propose that improved stress tolerance is another major benefit of the symbiosis (Gianinazzi et al., 2010) and AM fungi are accepted as functioning in biocontrol (Johansson et al., 2004). Enhanced resistance of mycorrhizal plants to soil-borne pathogen attacks has been associated to the accumulation of phytoalexins, flavonoids, and isoflavonoids in AM-colonized root tissues (Ziedan et al., 2011; Jung et al., 2012). Interestingly, the bioprotective effect of *Glomus mosseae* against the soilborne pathogen *Fusarium oxysporum* f.sp. *lycopersici* was observed in different cultivars and genotypes which differed in their susceptibility to both the AMF and the pathogen (Steinkellner et al., 2012). Nevertheless, the response of phylogenetically diverse plants (i.e., tomato, soybean, and maize) to two mycorrhizal fungi – *Funneliformis mosseae* and *Rhizophagus irregularis* – depended on both the plant and the AMF species involved (Fernández et al., 2014). Although fungal pathogens reduce root colonization by AMF, the latter were shown to provide protection through increased enzymes activity, including those directly involved in the regulation of the symbioses. The biological protection of AMF has been also proved on plant parasitic nematodes, under greenhouse conditions: the population of *Meloidogyne incognita* or *Pratilenchus penetrans* was lowered by 45 and 87%, respectively, in mycorrhized roots in comparison to non-mycorrhized roots (Vos et al., 2012).

The effect of interactions between AMF and other agronomical practices shows how external factors can contribute to the expression of biocontrol potential. The interaction between AMF and different level of P availability was observed in the occurrence of *Alternaria solani* symptoms (Fritz et al., 2006). Mycorrhized tomato plants had significantly less *A. solani* symptoms than non-mycorrhizal plants, but increased P supply, which was paralleled to a reduction in mycorrhiza formation, led to a higher disease severity in mycorrhizal plants. On the other hand, individual co-inoculation of four *Glomus* species with *T. harzianum* affected the colony-forming capacity of the latter, but the combined inoculation – particularly with *G. intraradices* – resulted in a general synergistic effect on disease

control (Martínez-Medina et al., 2009). The inoculation of bean plants with *Glomus mosseae*, besides decreasing propagule number of *Fusarium solani* in the rhizosphere, decreased pathogenic root rot by 34–77% (Dar et al., 1997). However, when co-inoculated with *Rhizobium leguminosarum*, the mycorrhized plants were more tolerant of the fungal root pathogen.

The induction of defense activity by AMF has been also proved in above ground tissues. *Helicoverpa arimigera* larvae feeding on leaves of tomato mycorrhized plants had a reduction of 62.3% in weight relative to non-inoculated plants, likely as a result of a priming effect related to jasmonate pathway (Song et al., 2013). Nevertheless, it could be speculated that the effect on above-ground herbivores derives also from reduced levels of metabolites connected to central catabolic and amino acid metabolism, particularly prominent in sink leaves, which prompted to suggest deteriorations rather than improvements in the nutritional value of colonized plants for higher trophic levels (Fester et al., 2011).

Several genera and species of bacteria (e.g., *Pseudomonas* or *Bacillus*) and fungi (e.g., *Penicillium* or *Aspergillus*) ubiquitous in different soils are known to assist plants growth by mobilization of insoluble forms of K (Meena et al., 2014), with mechanisms similar to those found in P-solubilizers (Sheng and He, 2006). It is thus not unexpected that a strain of *Paenibacillus kribbensis* having potassium and phosphate-solubilizing capacity was also found to reduce the development of several cotton and wheat soil-borne pathogens *in vitro* (Zhang et al., 2013).

The potential function of plant-growth-promoting rhizobacteria in biocontrol has been long known and can be traced to bacterization studies with fluorescent pseudomonads beginning in the 1970s (Weller, 2007). Since then, a huge amount of studies has allowed to characterize the process of root colonization and the biotic and abiotic factors that are affecting it as well as the identification of genes and traits in bacterial fitness underlying the mechanisms of pathogen suppression (e.g., Labuschagne et al., 2010; Sayyed et al., 2013; Islam et al., 2019). However, notwithstanding this knowledge, the major difficulties and weakness in a broad use of PGPR strains in agricultural practices reside in formulation and registration of the bacteria for commercial use (Malusá et al., 2012; Bashan et al., 2014; Borriss, 2020).

REGULATORY FUTURE PERSPECTIVES OF MULTIFUNCTIONAL BIOPRODUCTS

A sustainable agriculture is a central pillar of the United Nations Sustainable Development Goals (United Nations, 2015), which can be pursued by the wide adoption of microbial-based products in agronomical practices. The regulatory and policy pressure posed by this international agreement could potentially transform the market of bioproducts into a key segment of the world economy. Such potential is confirmed by recent market analysis reports, which valued at about 10.2 billion USD by 2025 the global biopesticide market, with an annual growth rate of about 15% (Anonymous, 2019b) and projected 3.15

billion USD by the end of 2026 for the biofertilizers market, at an annual growth rate of about 11% (Anonymous, 2019a). However, it is intriguing that for biopesticides, the market value projected for 2025 was already expected to be reached by 2017 (Marrone, 2007).

Most microbial-based PPPs present on the market have been designed for annual crops (mainly legumes and cereals), but there is an increasing demand for these products in fruit and vegetable crops, particularly for organic production. On the other hand, even though biofertilizers would not fully replace mineral fertilizers (Adesemoye et al., 2009), their application, possibly in association with organic fertilizers or other carbon-based products (Saeid and Chojnacka, 2019), could substitute to a large extent mineral or synthetic inputs, having also a positive impact on plant protection strategies.

However, the legal framework regulating the production and marketing of bioproducts can pose a bottleneck to their wider adoption because it reflects the incomplete knowledge on microbial-based products as well as precautions in their safety assessment. Considering the current situation in the EU, known to have a well-developed regulatory framework on agricultural inputs, it emerges that the biopesticide registration process and data requirements are similar to those needed for chemical pesticides (Regulation Eu 1107/2019). Even though a legal provision (Parliament, 2009) has introduced in the EU a compulsory integrated pest management since 2014 for all crops, the unfamiliarity with biologically based pest management of the risk assessors and regulators has not fostered the modification of the authorization process, taking into consideration the peculiarities of the biopesticides mechanisms of action, as it had already been suggested by prominent researchers (Chandler et al., 2011). However, recently, a specific working group has been organized to this aim, and also the European Food Safety Agency has actively operated to find new assessment methods (Council of the European Union, 2019). Interestingly, similar bottlenecks have been hampering biopesticides' development and use also in the Indian context, paralleled with a legislation aimed to support bioproducts for organic farming which resulted in an unfair competition from sub-standard or misbranded biopesticides (Keswani et al., 2019a).

In case of biofertilizers, the rules have been enacted patchily in the world (Malusá and Vassilev, 2014) and in the European Union only in 2019 a provision has been enacted, though limiting the marketing to just four kinds of biofertilizers: three related to N nutrition (based on symbiotic *Rhizobium* spp. and free-living *Azotobacter* spp. and *Azospirillum* spp.) and one for P nutrition (based on mycorrhizal fungi) (Regulation Eu 1107/2019, 2019). The limitation of species allowed to be marketed as biofertilizers contrasts with the plethora of genera and species recognized having positive effects on plant nutrition and available for commercial applications (Umesha et al., 2018). Furthermore, the EU Regulation allows only the drying or freeze-drying processes in the formulation of the product, which is also restrictive considering the technological possibilities in this respect (Bashan et al., 2014; Vassilev et al., 2020).

Multifunctional bioproducts would also share with biopesticides and biofertilizers the issue of biosafety with

respect to humans and the environment as, although only wild-type strains are being used for bioinocula development, the risk of pathogen spread cannot be completely excluded, thus requiring certain precautions (Keswani et al., 2019b).

In view of this situation, it appears quite difficult to expect that multifunctional bioproducts could soon be made available nor that manufacturers would advertise – not being it allowed by the legal provisions – either biocontrol or growth promotion features in bioproducts not registered for their respective purposes, even if the strains used could express them. The unlikelihood of a regulatory framework would also hamper the development of products based on microbial consortia that exhibit complementary and synergistic effects, through re-assembling strains with differing modes of action into small communities, thereby providing more consistent protection or growth promotion than with the application of single strains, which are now starting to gain attention as a possible strategy to widen the application of bioproducts (Reddy and Saravanan, 2013; Vassilev et al., 2015; De Vrieze et al., 2018). At the same time, the potential use of bioproducts for alleviating other abiotic stresses (Hassen et al., 2016; Rajendra Prasad et al., 2016), particularly relevant in the world-wide experienced climate change conditions, would also face difficulties due to lack of clear rules for their registration and marketing. The current regulatory framework in EU as well as that of other countries where bioproducts are highly promoted (see several articles in Singh et al., 2016) could be perceived as frustrating the researchers efforts in finding the best solutions to exploit microbial inocula, considering that plants (and animals) are no longer viewed as autonomous entities, but rather as "holobionts" (Bordenstein and Theis, 2015). Nevertheless, we believe that the research activity that is currently endeavored to better understand the biochemical and molecular mechanisms involved in plant-microbe-soil interactions, paralleled with their impact on the plant metabolomics and the interactions with endophytes, should also support the progress in manufacturing and the regulatory development, leading to the design and use of safe bioproducts with greater efficacy in enhancing the productivity of sustainable crops. To this aim, exploitation of endophytes (Fadiji and Babalola, 2020), or of pre-, pro-, and post-biotic approaches (Vassileva et al., 2020) as well as of the plants' capacity to "Cry for Help," i.e., recruit and subsequent assembly of protective specific microbiota (Bakker et al., 2018; Rodriguez and Durán, 2020), could represent possible research avenues to be explored.

AUTHOR CONTRIBUTIONS

JK, JT, and EM designed and drafted the work. KM and BT contributed to the revision of the manuscript. All authors contributed to the article and approved the submitted version.

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Soil Application of a Formulated Biocontrol Rhizobacterium, *Pseudomonas chlororaphis* PCL1606, Induces Soil Suppressiveness by Impacting Specific Microbial Communities

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Biocontrol bacteria can be used for plant protection against some plant diseases. *Pseudomonas chlororaphis* PCL1606 (PcPCL1606) is a model bacterium isolated from the avocado rhizosphere with strong antifungal antagonism mediated by the production of 2-hexyl, 5-propyl resorcinol (HPR). Additionally, PcPCL1606 has biological control against different soil-borne fungal pathogens, including the causal agent of the white root rot of many woody crops and avocado in the Mediterranean area, *Rosellinia necatrix*. The objective of this study was to assess whether the semicommercial application of PcPCL1606 to soil can potentially affect avocado soil and rhizosphere microbial communities and their activities in natural conditions and under *R. necatrix* infection. To test the putative effects of PcPCL1606 on soil eukaryotic and prokaryotic communities, a formulated PcPCL1606 was prepared and applied to the soil of avocado plants growing in mesocosm experiments, and the communities were analyzed by using 16S/ITS metagenomics. PcPCL1606 survived until the end of the experiments. The effect of PcPCL1606 application on prokaryotic communities in soil and rhizosphere samples from natural soil was not detectable, and very minor changes were observed in eukaryotic communities. In the infested soils, the presence of *R. necatrix* strongly impacted the soil and rhizosphere microbial communities. However, after PcPCL1606 was applied to soil infested with *R. necatrix*, the prokaryotic community reacted by increasing the relative abundance of few families with protective features against fungal soilborne pathogens and organic matter decomposition (*Chitinophagaceae*, *Cytophagaceae*), but no new prokaryotic families were detected. The treatment of PcPCL1606 impacted the fungal profile, which strongly reduced the presence of *R. necatrix* in avocado soil and rhizosphere, minimizing its effect on the rest of the microbial communities. The bacterial treatment of formulated PcPCL1606 on

avocado soils infested with *R. necatrix* resulted in biological control of the pathogen. This suppressiveness phenotype was analyzed, and PcPCL1606 has a key role in suppressiveness induction; in addition, this phenotype was strongly dependent on the production of HPR.

Keywords: avocado, *Rosellinia necatrix*, antifungal, biocontrol, soil, rhizosphere, microbial community, suppressiveness

INTRODUCTION

Soil is a complex and variable habitats on earth. Soil organisms have developed different mechanisms to survive, function and replicate into a changing environment, with variable moisture, temperature, and chemical contents. Soil conditions can vary in very short distances, but also there is variability over time; therefore, soil organisms must be able to adapt rapidly to different and changing conditions (Thies and Grossman, 2006). Additionally, most of the upper layer of the soils are under the influence of plant roots. Thus, the plant rhizosphere was previously defined as the zone around the root where microorganisms and processes important for plant growth and health are located (Hiltner, 1904). Rhizosphere soil a kind of layer between roots and the surrounding soil, that takes part in the large fluxes of nutrients and non-nutrient compounds (Belnap et al., 2003). Moreover, plant rhizosphere provides a special habitat that promotes higher microbial growth, abundance, and diversity (Praeg et al., 2019).

It is well known that in soil ecosystems, the establishment of plants helps to stabilize microbial community structures and are further modulated after interactions with the plant rhizospheres (Donhauser and Frey, 2018). Plant rhizospheres can be colonized by a high number of microorganisms, reaching cell numbers higher than the number of plant cells, covering between 7% and 15% of the rhizoplane (Gray and Smith, 2005). Plant roots photosynthetically fix carbon, and deposit this carbon directly into their surroundings. These exudates can be used as nutrients by the microbial community, finally influencing their composition and activities (Raaijmakers et al., 2009; Berendsen et al., 2012; Vorholt, 2012).

Soil microorganisms interacts with plant roots and interfere with plant behavior and microbial communities. Many rhizosphere-associated microorganisms can modify seed germination, seedling vigor, plant growth and development, nutrition, diseases, and productivity (Berg et al., 2016). Among them, soilborne plant pathogens are the major limitation in plant production. This group of pathogens is adapted to live in bulk soil; however, the rhizosphere is the place where the pathogen meets the plant and initiates the infection (Raaijmakers et al., 2009). This is also where the complex rhizosphere community of microorganisms can interact with the pathogen and influence the outcome of pathogen infection. Among these microbes, some bacteria positively affect plants and can be considered beneficial bacteria, many of which are designated plant growth-promoting rhizobacteria (PGPR; Lugtenberg and Kamilova, 2009). PGPR can promote beneficial effects on plants. Indirectly, they can inhibit pathogens through competition, colonizing the

rhizoplane, inducing plant resistance, and solubilizing minerals, which can cause a modification in the rhizosphere. Directly, PGPR can release antifungal compounds and lytic enzymes (Lugtenberg and Kamilova, 2009).

The increase in knowledge on plant beneficial bacteria has prompted interest in the biological control of plant diseases, which has increased recently because the use of chemicals in the environment provoke public concerns, aiming to the need of finding alternatives to the chemicals used for disease control. Historically, strains with biocontrol potential have been isolated from suppressive soils, studied and used against different soil pathogens (Köhl et al., 2019). Successful, reproducible biological control requires knowledge on the interactions at the root environments, in order to understand the conditions where biocontrol can be obtained (Deacon, 1994; Whipps, 1997) and, indeed, may be part of the reason why more biocontrol agents are reaching the market-place (Whipps and Davies, 2000; Whipps and Lumsden, 2001). The beneficial microorganisms must be mass produced and applied to the crops in a way that optimizes their activities in the corresponding habitat. Microbes can be delivered under different ways, including as liquids (sprays, drenches, and root dips) or as dry formulations applied in-furrow at the time of planting (O'Callaghan, 2016). Several biological control agents (BCAs), composed by living microbial products have been commercialized. A few examples of PGPR developed as commercial products for biological control are *Bacillus subtilis* GBO3 (Kodiak®; Gustafson Inc., Dallas, TX, United States), *Pseudomonas fluorescens* A506 (BlightBan®; Nufarm Americas, Burr Ridge, IL, United States), *Pseudomonas. aureofaciens* Tx-1 (Spot-Less®; Eco Soil Systems Inc., San Diego, CA, United States), *P. syringae* ESC-10, and ESC-11 (Bio-save®; Jet Harvest Solutions, Longwood, FL, United States), *Streptomyces griseoviridis* K61 (Mycostop®; Verdera Oy, Espoo, Finland), and *S. lydicus* WYEC 108 (Actinovate®; Novozymes BioAg Inc. WI, United States) (Figueiredo et al., 2010; Dey et al., 2014).

Among the bacterial biocontrol agents reported, the group of *Pseudomonas* spp. have been extensively studied due to its colonizing ability, inducing plant systemic resistance and to produce antifungal compounds (Haas and Défago, 2005; Weller, 2007). The antifungal compounds produced by rhizospheric *Pseudomonas* spp. are usually the basis for its biological effectiveness and include phenazine-1-carboxylic acid (PCA), phenazine carboxamide (PCN), 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin (PRN), pyoluteorin (PLT), 2-hexyl 5-propyl resorcinol (HPR), hydrogen cyanide (HCN), siderophores, and some hydrolytic enzymes such as proteases (Ligon et al., 2000; Raaijmakers et al., 2002; Cazorla et al., 2006). Among the antifungal pseudomonads commonly isolated

from soil and rhizosphere, *Pseudomonas chlororaphis* is a root-associated bacterial species that displays a wide weaponry of antifungals and shows efficient colonizing abilities (Arrebola et al., 2019; Biessy et al., 2019). Due to these characteristics, commercial or formulated *P. chlororaphis* and related species have been developed to fight soil diseases, especially those due to pathogenic fungi; for example, the commercial product Cerall[®], composed of the strain *P. chlororaphis* MA342, which shows biological control against phytopathogenic fungi in wheat, rye, and triticale, such as *Fusarium* and *Septoria* (Koppert Biological Systems, Netherlands). Other examples of commercial products based on *P. chlororaphis* and related strains are Cedomon[®] (*P. chlororaphis* MA 342, BioAgri AB, Sweden), Spot-Less[®] (*P. aureofaciens* Tx-1, Turf Science Laboratories, Carlsbad, CA, United States) or AtEze[®] (*P. chlororaphis* 63-28, Turf Science Laboratories, Carlsbad, CA, United States). These formulated *Pseudomonas* spp. have additional activities since they can also be used as bioinsecticides (Kupferschmied et al., 2013) or as rhizobacteria with plant growth-promoting activity (Chen et al., 2015).

Although *Pseudomonas* spp. are widely assayed against different soil diseases, few studies have analyzed the effect of *Pseudomonas* spp. on non-target soil microbial populations. For instance, in barley plants, a transient modification of 3 weeks were observed after *Pseudomonas* spp. DSMZ13134 application (Buddrus-Schiemann et al., 2010). In cucumber, after the application of *P. fluorescens* 2P24 and CPF10, no differences in the bacterial population structure compared to the control were observed after 8 weeks (Yin et al., 2013). On the other hand, in lettuce, the impact of *Pseudomonas jessenii* RU47 on the rhizosphere microbiota was influenced by the soil type 2 or 3 weeks after treatment (Schreiter et al., 2014). Furthermore, all available literature is mainly related to the effect in herbaceous plants, but there is no available information on the effect of *Pseudomonas* application on soil and rhizospheric microbial populations in woody crops.

Rosellinia necatrix causes white root rot, a devastating disease in woody plants worldwide (Pliego et al., 2012). Since the 1990s, different studies have established the importance of this disease in avocado (*Persea Americana* Mill.) crops in the Mediterranean area (López-Herrera et al., 1998, 1999). However, the control of avocado white root rot is considered very complex; therefore, several studies have focused on microbial species with the ability to control *R. necatrix* as additional tools to help manage this disease in the future (Cazorla et al., 2006; Ruano-Rosa et al., 2010). Different strategies have been followed to obtain bacterial isolates with biocontrol potential of *R. necatrix*. *Pseudomonas* spp. and *Bacillus* spp. are commonly isolated from avocado soil and rhizosphere, and some of these strains show antifungal activity and plant protection against soilborne fungal pathogens (Cazorla et al., 2006, 2007; González-Sánchez et al., 2010). Additionally, previous results reported the development of suppressiveness-induced soil after the amendment of commercial soil with composted almond shells in avocado orchards. Induced suppressiveness was directly related to the increase in abundance of specific members of Gammaproteobacteria (including a group of *Pseudomonas* spp. producing antifungal compounds;

Vida et al., 2016). These results increased the interest in *P. chlororaphis* strains as potential BCAs against different avocado phytopathogens (Cazorla et al., 2006; Pliego et al., 2008; Arrebola et al., 2019).

In the present study, the model biocontrol rhizobacterium *Pseudomonas chlororaphis* PCL1606 (PcPCL1606) was used. This bacterium showed strong antagonism against various phytopathogenic fungi (including *R. necatrix*) and displayed biocontrol against *Fusarium oxysporum* f. sp. *radicis-lycopersici* and *R. necatrix* (Cazorla et al., 2006; González-Sánchez et al., 2013). The production of the antifungal compound HPR is directly related to the effectiveness of biocontrol and antagonistic activity (Cazorla et al., 2006; Calderón et al., 2013). Furthermore, PcPCL1606 showed additional characteristics related to its fitness in soil and plant roots, such as efficient plant root colonization (González-Sánchez et al., 2010), increased fungal stress symptoms on hyphae after cell-to-cell contact, accelerated hyphal death (Calderón et al., 2014), survival in soil and potential competitiveness with other bacteria associated with the rhizosphere, as PcPCL1606 can produce two recently described bacteriocins (Dorosky et al., 2017).

The objective of this work was to elucidate the impact of PcPCL1606 applications in native communities of soil and rhizosphere, and to unravel the key role of PcPCL1606 applications in soil inducing suppressiveness against *Rosellinia necatrix*.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Rhizospheric *P. chlororaphis* PCL1606 (PcPCL1606; NCBI complete genome accession number GCA_000963835.1) isolated from healthy avocado roots of trees growing in a *R. necatrix* infested area (Cazorla et al., 2006) was used as model pathogen in this study (Table 1). Additionally, a previously obtained Gfp-tagged derivative PcPCL1606 strain (PcPCL1606-GFP, resistant to gentamycin at 80 µg/ml; Calderón et al., 2014) was used as a control to assess survival features (Table 1). To test the role of PcPCL1606 in suppressiveness, a derivative deletion mutant in the *darB* gene (Δ *darB*), impaired in antagonism and biocontrol, was used (Calderón et al., 2019).

Tryptone-peptone-glycerol (TPG; Calderón et al., 2014) medium was used to routinely grow *Pseudomonas* strains at 25°C. Agar-agar (Difco Laboratories, Detroit, MI) was added to a final concentration of 1.5% to produce solid media. The isolates were maintained as glycerol (30%) stocks at -80°C and revived on TPG, and a single colony was used to inoculate each culture.

In this work, a semi-commercial formulated product based on PcPCL1606 was used for experimentation. To obtain a formulated PcPCL1606 product, a standard fermentation of the biologically active product (PcPCL1606) was performed by Koppert B.V. (Berkel en Rodenrijs, Netherlands). Briefly, TPG culture medium was used, and a 3-liter bioreactor was inoculated with a starter culture of PcPCL1606. The culture was grown for 24 h at pH 7.0 and 25°C. The fermentation parameters (oxygen supply and pH) were controlled throughout

TABLE 1 | Microorganisms and plasmids used in this study.

Strain	Relevant characteristics ^a	References
Bacterial strains		
<i>Pseudomonas chlororaphis</i>		
PcPCL1606	Wild-type, isolated from Spanish avocado rhizosphere, HPR+, antagonism+, biocontrol+	Cazorla et al., 2006
PcPCL1606-GFP	PcPCL1606 containing the pBAH8 plasmid, expressing the green fluorescent protein (GFP), HPR+, antagonism+, biocontrol+, Gm ^r	Calderón et al., 2014
$\Delta darB$	PcPCL1606-derivative deletional mutant in <i>darB</i> gene, HPR–, antagonism–, biocontrol–, Km ^r	Calderón et al., 2019
Fungal strains		
<i>Rosellinia necatrix</i>		
CH53	Wild-type, isolated from avocado white root rot, High virulence	Pérez-Jiménez, 1997
Plasmids		
pBAH8	pBBR1MCS-5-containing PA1/04/03-gfp mut3-To-T1; Gm ^r	Huber et al., 2002

^aHPR: Production of 2-hexyl, 5-propyl resorcinol detected by thin-layer chromatography plates (Cazorla et al., 2006). Antagonism+, fungal antagonism observed in dual plates; biocontrol+, plant protection against *R. necatrix*. Gm^r, resistant to 80 µg/ml of gentamycin; Km^r, resistant to 50 µg/ml of kanamycin.

the fermentation procedure. Antifoam was required during the fermentation process. The fermentation product was harvested 2 to 3 h after the measured oxygen consumption indicated a shift in secondary metabolism (approximately 24 h of growth). Finally, the fermentation product was formulated in a suspension concentrate, comprising cells of the isolate in TPG medium (approximately 5×10^9 colony forming units (cfu)/ml) and was stored at 4°C until its utilization.

For biocontrol and suppressiveness assays, virulent avocado pathogenic *R. necatrix* CH53 was used (López-Herrera and Zea-Bonilla, 2007; Pliego et al., 2009; **Table 1**). The fungus was grown at 25°C on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) or TPG plates, and the fungal strain was stored in TPG at 4°C as previously described (Gutiérrez-Barranquero et al., 2012).

Greenhouse Inoculation Assays: Mesocosm Experiments

Two independent 1-year-long mesocosm experiments were conducted as previously described (Bonilla et al., 2015) to perform disease assessment, bacterial survival and metagenomic analysis. The independent experiments were named “assay 1” (season 2017/18) and “assay 2” (season 2018/19). These assays started 120 days before *R. necatrix* was inoculated (which was considered day 0) and ended 110 days after pathogen inoculation (the experiments last 230 days in total; **Figure 1**).

Briefly, an experimental microplot platform that mimicked field conditions was designed and constructed for the plant assays at the IHSM-UMA-CSIC “La Mayora” (Algarrobo Costa, Spain, 36°45′37.74″ N – 4°02′26.28″ W). The greenhouse was built as an open structure with double roofing to allow air passage for improved ventilation, and the microplots (mesocosms of 35 liter plant pots) were planted in a white gravel bank to reduce oscillation of the soil temperature. Environmental conditions were monitored during each experiment by using a portable data logger, which recorded the air temperature and relative humidity.

In each independent assay, a total of 102 two-year-old commercial avocado seedling plants (cv. Topa-Topa) were independently transplanted to 35 liter pots filled with a blend

(1:1) of solarized natural soil and peat and randomly placed into the experimental area. Each plant into its pot constitute an independent mesocosm. Seventeen independent avocado plants were used for each of the treatments assayed in these studies as listed in **Table 2**. For each independent treatment, eleven plants were inoculated with *R. necatrix* to study multitrophic interactions during biocontrol, and the remaining six non-inoculated plants were used as controls to study multitrophic interactions without the presence of the pathogen. Fungal inoculation was performed as previously described (Sztejnberg and Madar, 1980; Cazorla et al., 2006). Briefly, four holes per pot were made on the soil surface using a punch, and 16 g of wheat colonized with *R. necatrix* strain CH53 was distributed in the holes before filling them with the surrounding soil.

The first assay, “assay 1,” was designed to test the biocontrol of different treatments with formulated PcPCL1606 as a biologically active product against *R. necatrix* and to study the impact of the application of PcPCL1606 on natural microbial populations with *R. necatrix* inoculation. PcPCL1606 treatments were applied by irrigating a final cell concentration of 1.0×10^{10} cfu suspended in 200 ml of sterile water. Treatments were applied using watering to properly distribute the bacterial cells onto the whole pot surface. One of the bacterial treatments was a preventive application (PcPCL1606 preventive), consisting of a single application with a semi-commercial formulate of PcPCL1606 (PcPCL1606 preventive) performed 50 days before inoculation with *R. necatrix*. A second treatment was a curative application (PcPCL1606 curative), using the same semi-commercial formulate of PcPCL1606, which was added after symptoms appeared. The application was performed 70 days after inoculation with *R. necatrix*. A third treatment was included and consisted of a control treatment designed to compare the accuracy of the bacterial counts in different culture media. For this, the PcPCL1606-GFP derivative strain was used in the experiments, following the preventive protocol described above (**Table 1** and **Figure 1**). A bacterial suspension of PcPCL1606-GFP (growing in liquid TPG medium for 24 h at 25°C and 180 rpm) reached a bacterial concentration of 1.4×10^9 cfu/ml. A total of 10^{10} cfu per plant was applied in this treatment (as described above) and allowed

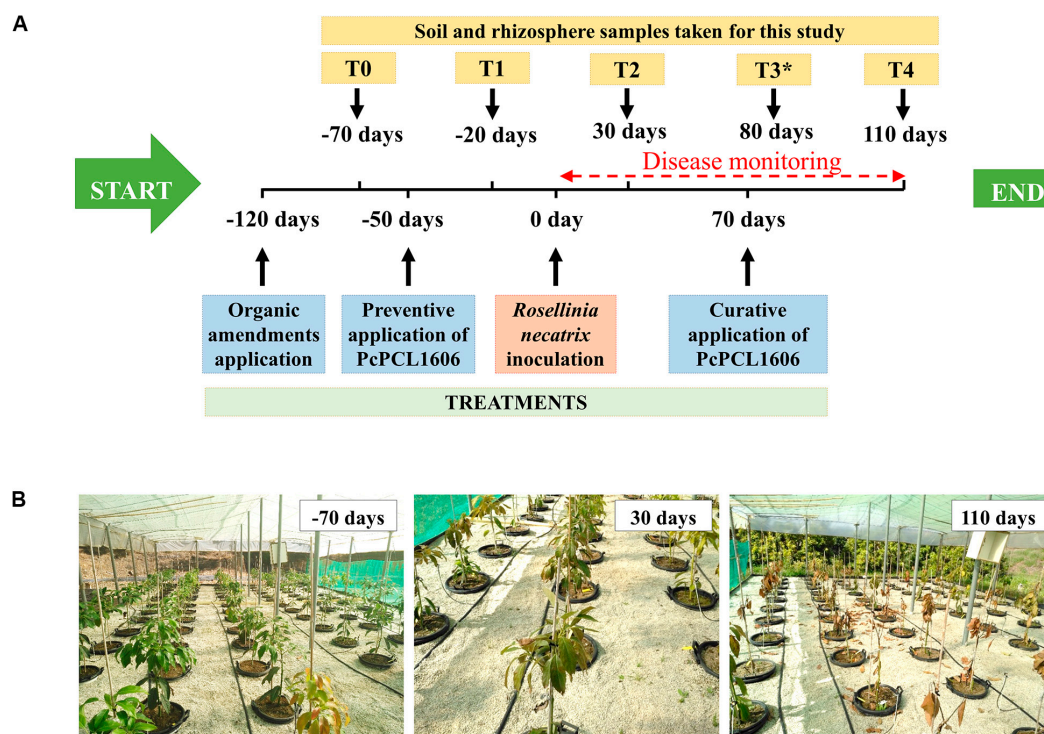


FIGURE 1 | Experimental design of the mesocosm assays. Two independent experiments were performed, treatments in each independent experiment are detailed in **Table 2**. Seventeen independent mesocosms were used for each of the treatments assayed (eleven mesocosms were inoculated with *R. necatrix*, and six mesocosms remained non-inoculated). **(A)** Schematic view of the mesocosms timeline. Experiments started 120 days (–120 days) prior to the *R. necatrix* inoculation (considered as day 0). One hundred and two 2 years-old avocado plants were sown in commercial soil. At this time started the treatments with the positive control, amending composted almond shells to the corresponding pots. At –50 days, the preventive treatment with PcPCL1606 and PcPCL1606-GFP were applied. 70 days after *R. necatrix* inoculation, the curative PcPCL1606 treatment was applied. Disease monitoring was performed just after *R. necatrix* inoculation. Sampling points for further analysis were established at T0 (previous to the preventive treatment), T1 (after preventive treatment), T2 (after *R. necatrix* inoculation), T3* (taken only during biocontrol in “assay 2”) and T4 (end of the experiment). **(B)** Aspect of the mesocosms experiment at –70, 30 and 110 days along the experiment.

TABLE 2 | Main characteristics of the treatments used in the microcosm assay.

Treatment	Assay 1	Assay 2	Code	Composition
Negative control	✓	✓	Control	No organic amendment and no bacteria were added
Positive control (induced suppressiveness)	✓	✓	ASO	Commercial almond shells derived from almond industry were piled and traditionally composted
Formulated PcPCL1606 preventive	✓	✓	PcPCL1606 preventive	PcPCL1606 formulated in liquid, applied 50 days before inoculating <i>R. necatrix</i>
PcPCL1606-GFP preventive	✓	X	PcPCL1606 GFP	PcPCL1606-GFP tagged, applied 50 days before inoculating <i>R. necatrix</i> , like the PcPCL1606 preventive treatment
Formulated PcPCL1606 curative	✓	X	PcPCL1606 curative	PcPCL1606 formulated in liquid, applied after the appearance of disease symptoms, 70 days after inoculation with <i>R. necatrix</i>

✓, Included; X, not assayed.

specific bacterial counts from soil and rhizosphere samples. In a second assay (“assay 2”), only the preventive semi-commercial formulated PcPCL1606 treatment was repeated to confirm its biocontrol efficacy on the pathogen in the previous assay and to study the impact of PcPCL1606 application on the microbial communities during biocontrol (**Table 1** and **Figure 1**).

In both assay 1 and assay 2, two control treatments were included. First, a positive control of biocontrol consisted of developing soil-induced suppressiveness to *R. necatrix* (Vida et al., 2016). For this, 19 liters of composted almond shells (ASO treatment) was placed on the top layer of 16 liters of soil and peat. This positive control treatment was initiated 150 days before *R. necatrix* inoculation to allow the soil to

induce suppressiveness (**Figure 1** and **Table 1**; Vida et al., 2016). The negative control consisted of a group of 17 avocado plants without bacterial treatments.

Disease Assessment

To study the biological control response of the different treatments, plant disease was monitored weekly for the next 110 days after pathogen inoculation (day 0) in both experiments. Aerial symptoms of white root rot in symptomatic plants was measured using a symptom scale (Bonilla et al., 2015): 0, healthy plant; 1, plant with first symptoms of wilt; 2, overall wilted plant; 3, wilted plant with first symptoms of leaf desiccation; and 4, completely dried plant (dead plant) (see **Supplementary Figure S1**). The disease index (DI) was calculated for each treatment as previously described (Cazorla et al., 2006). The experiment was considered finished 110 days after inoculation. The area under the disease progress curve (AUDPC) was calculated for statistical comparison of the treatments (Campbell and Madden, 1990) and analyzed as González-Sánchez et al. (2013).

Soil and Rhizosphere Sampling

Along the experiment, soil, and rhizosphere samples were taken from each of the assayed treatments at the different sampling points to study the effect of the treatments with formulated PcPCL1606 on the microbial community (**Figure 1**). During assay 1, soil and rhizosphere samples were taken at different sampling times. Two sampling points were taken before inoculation with *R. necatrix* (considered day 0): one sample was collected before preventive bacterial treatment (T0, −70 days) and a second sample was collected after preventive treatment (T1, −20 days). Samples were collected at two more times after inoculation with *R. necatrix*: at 30 days after treatment (T2) and at the end of the experiment, 110 days after *R. necatrix* inoculation (T4). Following the indications reported in assay 1 and taking into account the biocontrol results from assay 1, in assay 2, soil and rhizosphere sampling was only conducted 80 days after the inoculation with *R. necatrix* (sampling point T3), when the biocontrol was effective after the preventive bacterial treatment with formulated PcPCL1606.

Sampling was performed as described below. Three plants per treatment were randomly selected. Fifteen-centimeter-deep soil core samples were obtained using a 4-cm-diameter core sampler, avoiding to collect close to the inoculation points. Three equidistant points around each plant were sampled and pooled to provide a single composite sample from each plant. Each composite soil and rhizosphere sample per plant was individually processed and analyzed. In the case of the soil and rhizosphere samples taken from three plants challenged with *R. necatrix* in assay 2 at T3, the 3 samples from the untreated control plants were taken from individual symptomatic plants displaying different disease indexes (with disease index 1, 2, or 3; **Supplementary Figure S1**).

The collected soil and rhizosphere samples were placed in cold storage and transported to the laboratory. From these samples, the roots of avocado, which had adjacent soil, were carefully separated and considered the rhizosphere samples.

The rest of the soil was sieved through a 2 mm pore-size sieve. The bulk soil sample was the sieved soil carefully cleared from the roots was considered the bulk soil sample. Fresh soil and rhizosphere samples were used for culture-dependent and culture-independent approaches to perform microbial population analysis. DNA extraction from the soil and rhizosphere samples was also performed immediately after sample collection.

Microbial Isolation and Plate Counts

Culture-based microbial analysis of *Pseudomonas* present in the soil and rhizosphere samples was performed. For the soil sample analysis, subsamples of 5 g of the bulk soil were suspended in 40 ml of saline solution (0.85% NaCl) with 5 g of sterile gravel (2 to 4 mm in diameter) and mixed at 250 rpm for 30 min on an orbital shaker, which was followed by 20 min of decantation (Bonilla et al., 2015). For the rhizosphere sample analysis, one gram of the fine roots was homogenized for 2 min in a Stomacher bag with 4 ml of saline solution (Bonilla et al., 2015). The supernatants of both the soil and rhizosphere samples were serially diluted 10-fold; 100 µl of each dilution was plated on different selective media, and bacterial counts were recorded after 48 h at 25°C.

To obtain the bacterial counts of fast-growing heterotrophic bacteria, plates of LB medium amended with cycloheximide (100 mg/liter) were used to prevent fungal growth. To count the number of pseudomonads, the previously described *Pseudomonas* selective medium (PSM) was used. PSM is composed of King's B (KB) agar amended with 75 mg of penicillin G, 45 mg of novobiocin, 50 mg of nitrofurantoin and 100 mg of cycloheximide per liter (Larkin and Honeycutt, 2006; Vida et al., 2017).

To study PcPCL1606 survival and to correlate these values with the bacterial count values of PcPCL1606 obtained in PSM with antibiotics, a preventive treatment with PcPCL1606-GFP (gentamicin-resistant strain; **Table 1**) following an identical protocol as described above was performed during assay 1. Soil and rhizosphere samples were taken along assay 1 to compare the bacterial counts in PSM with those in TPG medium amended with gentamycin (80 mg per liter; TPG-Gm). The same soil and rhizosphere samples were processed as described above and plated on PSM and TPG-Gm medium to compare the bacterial counts after 48 h at 25°C. Typical colony morphology of PcPCL1606-GFP and fluorescence validated the counts of Gm-resistant bacteria.

DNA Extraction From Soil and Rhizosphere Samples

At each sampling point, DNA was extracted from soil and rhizosphere samples to specifically detect the presence of PcPCL1606 and *R. necatrix*. Additionally, to analyze the effect of PcPCL1606 application on microbial communities of soil and rhizosphere of avocado plants, samples were taken at T2 (80 days after inoculating the bacteria) in assay 1 from the control and preventive treatment where *R. necatrix* had not been applied and analyzed. To test the effect of the preventive

PcPCL1606 application during biocontrol, samples from T3 of assay 2 (80 days after inoculating the fungal pathogen) in the control and preventive treatment where *R. necatrix* was inoculated were analyzed. DNA was extracted from each of the 3 independent composite samples where formulated PcPCL1606 was applied (PcPCL1606 preventive) and 3 composite samples where it was not applied (Control). Soil and rhizosphere DNA extractions were performed using 2.0 g of soil or rhizosphere sample and a PowerSoil® DNA Isolation Kit (Qiagen Iberia S.L., Madrid, Spain). The DNA extraction quantity and quality ($A_{260}/A_{230} > 1.8$ and $A_{260}/A_{280} > 1.7$) were evaluated using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, United States). Additionally, DNA quality was analyzed by agarose gel electrophoresis and RedSafe™ staining (Labotag, Seville, Spain). DNA was stored at -20°C for further analyses.

Analysis of 16S rDNA and ITS Gene Sequence

Specific detection of PcPCL1606 in each soil and rhizosphere samples at different sampling times (T0, T1, T2, T3 and T4) was performed by PCR-amplification of a partial sequence inside the gene PCL1606_04860 (with certain homology to the sequence of a glutamine-fructose-6-phosphate aminotransferase from *P. aeruginosa*), which contains a specific 378 nt sequence in PcPCL1606. Specific primers 04860F and 04860R (Supplementary Table S1) were used, and the amplification product was revealed after electrophoresis. Specific detection of *R. necatrix* was performed following previously described procedures (Schna et al., 2002).

To analyze the effect of PcPCL1606 on the microbial communities on the uninoculated samples or during the biocontrol process, metagenomic approaches were followed. For metagenomics analysis, the DNA samples taken from each rhizosphere/soil type were sent to be sequenced by ChunLab (Seoul, South Korea) to obtain the microbial DNA sequences of the 16S rRNA gene and ITS hypervariable regions. For this, a partial 16S fragment (428 bp in size) corresponding to the V3-V4 region, was amplified using the PCR primers 341F (CCTACGGGNGGCWGCAG) and 805R (GACTACHVGGGTATCTAATCC), resulting in a 428 bp amplicon (Herlemann et al., 2011). For the ITS amplification, PCR primers ITS1F (CTTGGTCATTAGAGGAAGTAA; Gardes and Bruns, 1993) and ITS2 (GCTGCGTTCTTCATCGATGC; White et al., 1990) targeted the ITS1F-ITS2 region, resulting in a PCR product of 230 bp in size. PCR products were sequenced by using MiSeq technology (Illumina). Sequences were analyzed using EzBioCloud software (ChunLab)¹ as follows. Processing raw reads started with quality check and filtering of low quality ($<Q25$) reads by Trimmomatic version 0.32 (Bolger et al., 2014). After QC pass, paired-end sequence data were merged together using fastq_mergepairs command of VSEARCH version 2.13.4 (Rognes et al., 2016) with default parameters. Primers were then trimmed with the alignment algorithm of Myers

and Miller (1988) at a similarity cut off of 0.8. Non-specific amplicons that do not encode 16S rRNA were detected by nhmmer (Wheeler and Eddy, 2013) in HMMER software package version 3.2.1 with hmm profiles. Unique reads were extracted and redundant reads were clustered with the unique reads by derep_fulllength command of VSEARCH (Rognes et al., 2016). The EzBioCloud 16S and ITS rRNA database (Yoon et al., 2017) was used for taxonomic assignment using usearch_global command of VSEARCH (Rognes et al., 2016) followed by more precise pairwise alignment (Myers and Miller, 1988). Chimeric reads were filtered on reads with $<97\%$ similarity by reference based chimeric detection using UCHIME algorithm (Edgar et al., 2011) and the non-chimeric 16S and ITS rRNA database from EzBioCloud. After chimeric filtering, reads that are not identified to the species level (with $<97\%$ similarity) in the EzBioCloud database were compiled and cluster_fast command (Rognes et al., 2016) was used to perform *de novo* clustering to generate additional OTUs. Finally, OTUs with single reads (singletons) are omitted from further analysis. Finally, the relative abundance of each treatment of eukaryotes/prokaryotes at different taxonomic levels was calculated as the average from three independent samples and was used to perform the comparative distribution analysis.

The secondary analysis, which includes alpha-diversity calculation and biomarker discovery, was conducted by in-house programs of Chunlab, Inc (Seoul, South Korea). All analytics mentioned above were performed in EzBioCloud 16S-ITS based MTP (Microbiome Taxonomic Profile), which is a ChunLab's bioinformatics cloud platform. The Chao1 index (Chao, 1987) and the Shannon index (Magurran, 2013) were performed as previously described. Rarefaction curves were also applied as previously described (Heck et al., 1975). Beta-diversity was calculated from the relative abundance data (at genus level) from all the samples. The Bray-Curtis index was used to calculate samples similarities. For all analyses, the Fitopac 2.1 software (Shepherd, 2010) was used.

White Root Rot Suppressiveness Assays

To test *R. necatrix* inhibition by different treated soils, soil suppressiveness assays were performed using a diffusion chamber experiment (Bonilla et al., 2015; Vida et al., 2016). A fungal disk of *R. necatrix* (0.6-cm in diameter) grown on potato dextrose agar (PDA) was placed on a disk of water-agar medium (1% agar; 5 cm in diameter) and transferred to a nitrocellulose filter (pore size 0.45 μm). These systems were placed on soil samples with different treatments taken from assay 2 at sampling point T3. The diffusion chamber was incubated for 5 days at 25°C . The total area of *R. necatrix* growth was measured using Quantity One 1-D analysis software (Bio-Rad Laboratories, Inc., Madrid, Spain) for each soil. Nine replicate chambers per soil type were analyzed. Composted almond shell (ASO)-amended soil was used as suppressiveness positive control. Unamended and untreated controls (Control) and soil where formulated PcPCL1606 had been applied (PcPCL1606 preventive) were assayed.

To verify the role of PcPCL1606 in the suppressiveness of soil samples under the preventive treatment of PcPCL1606, we prepared heat-treated PcPCL1606 preventive soil (HT).

¹ www.ezbiocloud.net

Briefly, heat-treated soil consisted of heating the soil in 2 autoclave steps as previously described (Vida et al., 2016). To analyze the restoration of suppressiveness, complemented soil was constructed; HT soil was inoculated with 10^2 cfu/g soil of PcPCL1606 from a bacterial culture growing overnight (HT + PcPCL1606).

Due to the important role of the antifungal compound HPR in the biocontrol and antagonism of PcPCL1606 (Calderón et al., 2013), supplementation of the HT soil with the $\Delta darB$ mutant (HT + $\Delta darB$) was also performed. $\Delta darB$ is a mutant deficient in HPR production (not antagonistic and no biocontrol strain) and was used to verify the implication of this compound in suppressiveness (Table 1).

Data Analyses

Data distributions were tested using one-way analysis of variance (ANOVA) followed by Fisher's least significant different test with Bonferroni's correction ($P = 0.05$). All data analyses were performed using IBM SPSS statistics 25 software (SPSS, Inc., Chicago, IL, United States). Based on the standard error, the 95% confidence interval for each response variable was obtained, and the significant differences between the soils were estimated.

RESULTS

Biocontrol of Mesocosm Analyses

In mesocosm studies designed to unravel the biocontrol effect of PcPCL1606 application, the first aerial symptoms of white root rot appeared approximately 30 to 40 days after inoculation with *R. necatrix* in both independent microplot assays. The disease index evolution with time is detailed in Figure 2 for each treatment. From the assayed treatments in both assays, the first to show aerial symptoms of white root rot was the unamended control treatment (Control), which reach a disease index above 80%, with many of the inoculated plants already dead at 110 days after *R. necatrix* inoculation.

During assay 1, the positive control of suppressiveness (ASO treatment) showed a delay in symptom appearance but not in symptom reduction. However, during assay 2, ASO treatment induced a delay in appearance and a decrease in white root rot symptoms (Figure 2). In those experiments, the most evident biocontrol effect was produced by the formulated PcPCL1606 treatments. Preventive and curative PcPCL1606 treatments reduced symptom development and reached disease index values of 65 and 70%, respectively (Figure 2). The treatment with PcPCL1606-GFP showed a delay in symptom development but at the end of the experiment reached a disease index level similar to the untreated control, similar to the results of ASO treatment. The temperature and relative humidity data recorded for each experiment showed some differences among seasons, which usually occurs under real field conditions (Supplementary Figure S2). It is important to remark that during assay 1, several days showed unusually low temperatures approximately 60 days after *R. necatrix* inoculation (March–April 2018), coincident with an increase in disease index in some treatments in assay 1. However, a statistical comparison of AUDPC data revealed

that all of the assayed PcPCL1606 treatments resulted in a significant reduction (ANOVA, $P < 0.05$) in the disease index when compared to the untreated control plants.

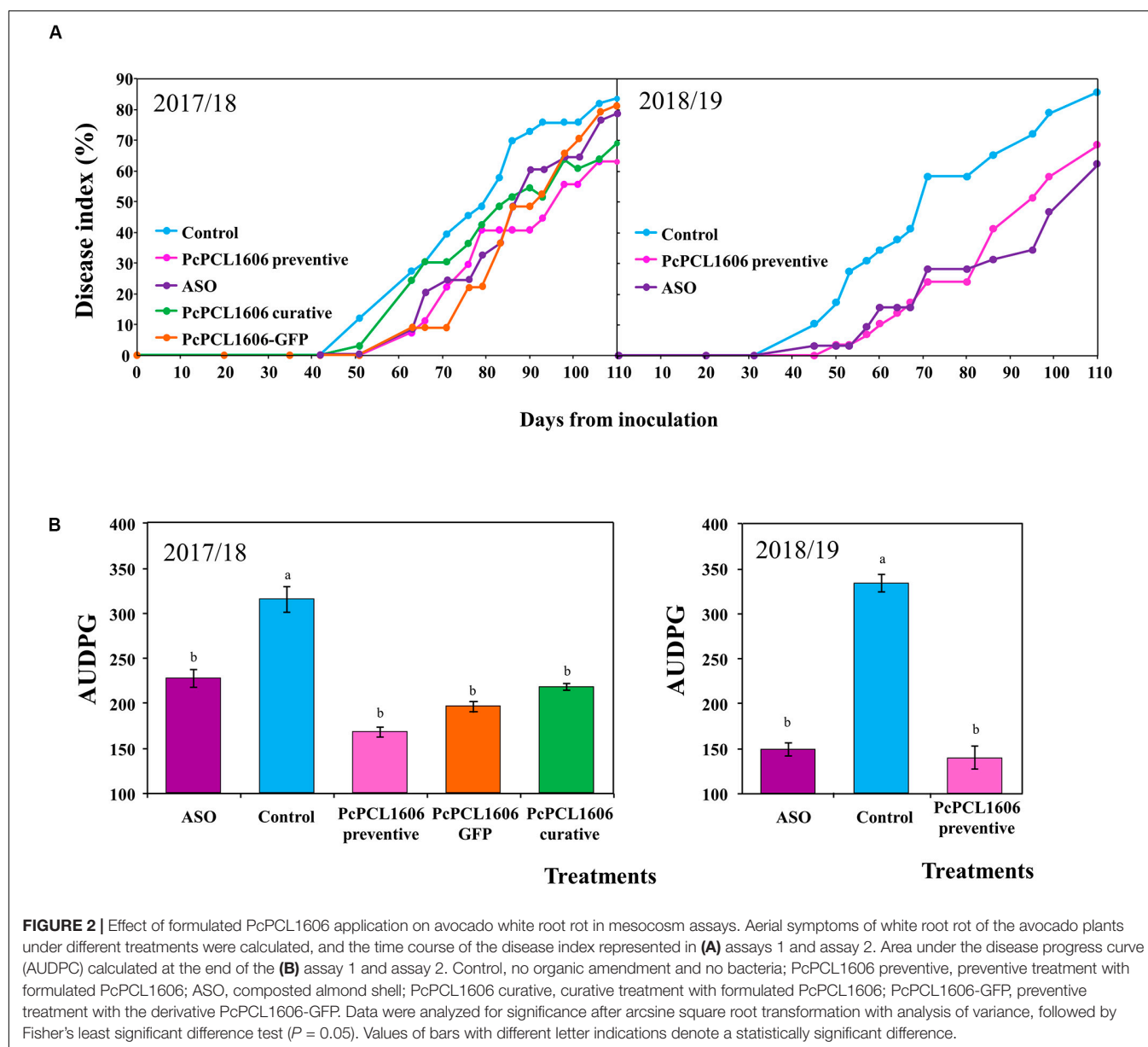
Effect of PcPCL1606 Applications on Soil and Rhizospheric Culturable Microbial Communities

To count the bacterial levels of PcPCL1606 isolated from soil and rhizosphere samples, the PcPCL1606-GFP strain (Table 1) was used for comparison studies. Bacterial counts from samples under preventive treatment with PcPCL1606-GFP were calculated on TPG-Gm medium to specifically select this bacterial strain and PSM. Plate counts revealed the presence of this strain during the experiment, with levels ranging from 10^3 to 10^5 cfu/g and with almost no differences at different sampling times in the rhizospheric and soil samples. A high correlation among the bacterial counts of the two media was obtained, with a regression equation of $y = 0.106 + 1.097x$ ($R = 0.972$) (Supplementary Figure S3).

Bacterial counts in the bulk soil and rhizosphere samples during assay 1 were performed to analyze the effect of PcPCL1606 treatment on the culturable bacterial populations, especially the group pseudomonas. At T0 (50 days after the initiation of the experiments), bacterial counts were very similar among the samples from the soil and rhizosphere, independent of whether they were taken from the untreated control or the ASO treatments. Bacterial counts of total heterotrophic bacteria were approximately 10^6 cfu/g of soil or rhizosphere. Pseudomonas-like counts were also approximately 10^6 cfu/g, with a decrease in count value in rhizosphere samples compared with untreated control plants, with 10^5 cfu/g of rhizosphere (T0, Figure 3).

Thirty days after PcPCL1606 preventive inoculations (T1, 20 days before inoculation with *R. necatrix*), bacterial counts maintained similar values among soil and rhizosphere samples from the different treatments, with values of total heterotrophic bacteria of approximately 10^6 cfu/g and slightly lower values for Pseudomonas-like counts, mainly below 10^5 cfu/g. The samples from untreated control plants had higher total heterotrophic bacterial counts (5×10^6 cfu/g rhizosphere) and, in contrast, showed lower values for Pseudomonas-like counts of approximately 4×10^4 cfu/g of rhizosphere (T1, Figure 3).

In T2 (30 days after inoculation with *R. necatrix*; 80 days after the preventive treatment with formulated PcPCL1606), comparisons among the mesocosm experiments with or without the fungal pathogen were also performed. In general, samples from plants not inoculated with *R. necatrix* showed a slight reduction in total heterotrophic bacterial counts, with higher values of approximately from 7×10^5 cfu/g to 9×10^5 cfu/g in soil and rhizosphere samples from PcPCL1606- and ASO-treated plants (with similar values to those obtained in T1). However, a strong reduction in Pseudomonas-like counts (almost two orders of magnitude less when compared with total heterotrophic bacteria) was also observed, with values ranging from 10^3 to 10^4 cfu/g of sample, with lower values detected in samples from the untreated control plants and higher values in the soil and rhizosphere samples from ASO-treated plants (T2, Figure 3).



In the plants challenged with *R. necatrix*, lower counts of total heterotrophic bacteria were obtained in general when compared with the unchallenged plants at this same sampling time. Higher values ($1-8 \times 10^5$ cfu/g) were also displayed by samples from plants under ASO and PcPCL1606-GFP treatments, and lower values were observed with the untreated control plants, with values of approximately from 1×10^4 cfu/g to 3×10^4 cfu/g. On the other hand, in the samples from soil inoculated with *R. necatrix*, the *Pseudomonas*-like counts were clearly higher when compared with the samples not inoculated with *R. necatrix*. Values ranged from 10^4 to 10^5 cfu/g, reaching almost the same value as total heterotrophic bacteria (samples from untreated control plants). In some cases, the *Pseudomonas*-like levels were not affected by the presence of *R. necatrix*, as shown by the samples taken from the ASO treatment (T2, **Figure 3**).

Finally, the counts of total heterotrophic bacteria at the end of the experiment (T4; 110 days after *R. necatrix* inoculation; 160 days after preventive treatment with PcPCL1606; 30 days after curative treatment with PcPCL1606), when no *R. necatrix* inoculation was performed, were very similar to those reported in T2, but with a slight reduction in almost all the samples. In these samples, the *Pseudomonas*-like counts were approximately two orders of magnitude lower than the total heterotrophic bacteria counts. However, the total heterotrophic bacteria counts were higher in some treatments, such as the curative applications of PcPCL1606 (applied 30 days before this sampling point), as well as in the treatment with composted almond shells (ASO) and in the rhizosphere of the preventive treatment with PcPCL1606 (applied 160 before this sampling point) (T4, **Figure 3**). When the plants were inoculated with *R. necatrix*, bacterial counts

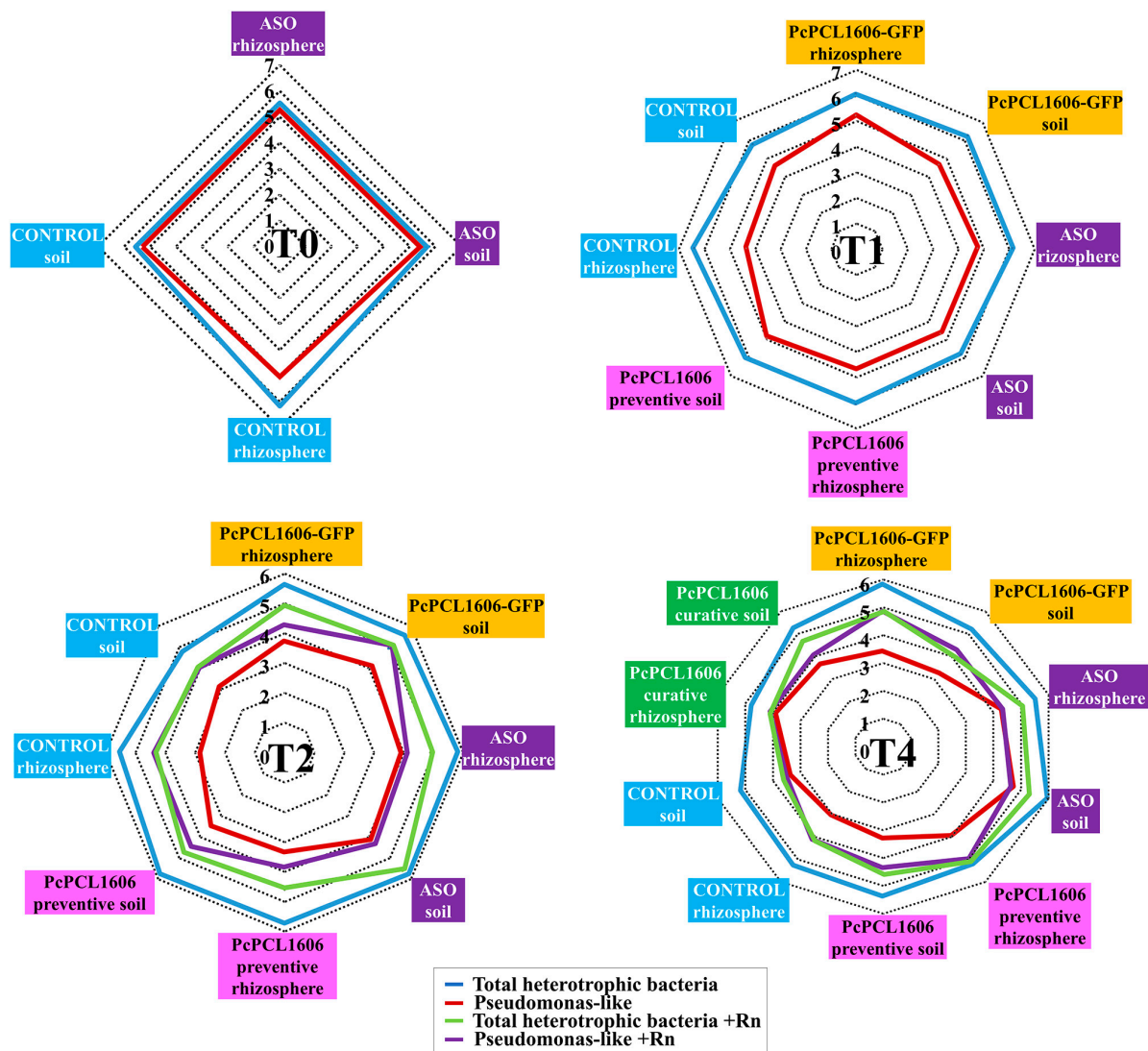


FIGURE 3 | Culturable bacterial populations (total heterotrophic bacteria and pseudomonas-like) during the “assay 1” microcosms experiments. The population densities of fast-growing heterotrophic bacteria and pseudomonas-like were assessed by plate counts at different times (T0, T1, T2, and T4). Bacterial counts obtained from samples taken from inoculated plants with *R. necatrix* are noted as +Rn.

from T4 soil and rhizosphere samples showed a similar behavior to T2, with lower levels of total heterotrophic bacterial counts but higher *Pseudomonas*-like counts. Additionally, in most of the samples, the *Pseudomonas*-like counts reached similar values as the total heterotrophic bacterial counts. Higher total heterotrophic bacterial counts were detected in ASO samples and in rhizosphere samples preventively treated with PcPCL1606. Interestingly, the *Pseudomonas*-like counts were also higher in rhizosphere samples preventively treated with PcPCL1606. Remarkably, typical colonies of PcPCL1606-GFP from soil and rhizosphere samples taken from plants that survived after 160 days after inoculation were recovered on TPG-Gm plates with counts above 10^3 cfu/g.

Because in assay 2, we focused on the confirmation of the biocontrol results previously observed with the PcPCL1606

preventive treatment in assay 1, only one sampling point was taken at T3, when biocontrol was observed. Total heterotrophic bacteria and *Pseudomonas*-like counts (**Supplementary Figure S4**) showed values intermediate to those between T2 and T4 in assay 1, ranging from 10^5 to 10^6 cfu of total heterotrophic bacteria/g and 10^3 to 10^4 cfu of *Pseudomonas*-like/g.

Characterization of the Soil Microbial Community Based on 16S rDNA and ITS Sequencing

Sequencing of 16S rDNA and the ITS variable regions elucidated the relative abundances of microbial clades at different taxonomic levels. Comparative distribution analysis were performed only with the most abundant OTUs ($\geq 1\%$ of relative abundance),

quantified with a sufficient level of precision due to the high level of OTU richness. In all samples, after sequencing of 16S rDNA, a very low relative abundance of *Archaea* was found (<0.1%).

In assay 1 (at sampling time T2, data available at <https://doi.org/10.6084/m9.figshare.12310253.v1>), soil and rhizosphere samples from untreated control plants and from plants under preventive treatment with PcPCL1606 and not challenged with *R. necatrix* were analyzed to study the impact of PcPCL1606 treatments on microbial communities. OTUs with a relative abundance above 1% comprised approximately 65% of the relative abundance of prokaryotic microorganisms in soil and rhizosphere samples from plants under the two different treatments (Figure 4). No relevant changes were observed in the relative abundance of prokaryotic families in any of the analyzed samples, with the seven more abundant OTUs (*Rhodospirillaceae*, *Cytophagaceae*, *Acidobacteriaceae*, *Micropepsaceae*, *Pedosphera_f*, *Opitutaceae* and *Steroidobacter_f*) covering approximately 30% of the relative abundance in all analyzed samples (Figure 4A). The 5 most abundant phyla (above 79% of relative abundance) were *Proteobacteria* (42.89%), *Acidobacteria* (10.86%), *Bacteroidetes* (10.42%), *Verrucomicrobia* (8.95%) and *Actinobacteria* (6.04%). At the class level, *Alpha*- and *Gammaproteobacteria* comprised approximately 30% of the relative abundance in the four analyzed samples. Focusing on the relative abundance of members of the *Pseudomonas* genus, the relative abundance was approximately 0.1% in plants under PcPCL1606 treatment and in soil samples of untreated control plants but was 0.3% in rhizosphere samples from untreated plants. The specific relative abundance of *P. chlororaphis* showed levels below 0.01% of the total relative abundance, except in the rhizosphere of PcPCL1606-treated plants, with 3 times more relative abundance (approximately 0.03%; Figure 4B).

In the same samples, the relative abundance of eukaryotes revealed a similar distribution in the rhizosphere and soil samples treated with PcPCL1606 (Figure 5A). A higher abundance for a family representative of the kingdom of *Chromista* can be observed in samples taken from the untreated control plants. Additionally, in the rhizosphere samples from untreated control plants, a higher relative abundance was observed for an unclassified group and for the class *Sordariomycetes*. Similar results could also be observed at the class level, where the samples from the PcPCL1606 treatment showed a high relative abundance of *Agaricomycetes*, *Sordariomycetes*, *Aphelidiomycetes* and unclassified fungi; however, in the untreated control samples, the relative abundance of *Agaricomycetes* was lower, and the relative abundance of *Chromista* (*Chromista_g_uc* and *Chromista_c*) was higher. In these samples, the relative abundance of *Xylariaceae* was very low (Figure 5B), ranging from 0.02 to 0.04%.

In soil and rhizosphere samples from microplots artificially inoculated with *R. necatrix*, the analysis of the 16S rRNA sequences (data available at <https://doi.org/10.6084/m9.figshare.12309788.v1>), revealed a decrease in the relative abundance of the family *Acidobacteriaceae*, but similar groups of microorganisms were found at the family level (Figure 6A) among the different independent experiments (assay 1 and assay 2). Prokaryotic communities from soil and rhizosphere samples treated with

PcPCL1606 were almost identical among each treatment and differed from the communities detected in the control samples, which were also more similar among each treatment (Figure 6A). The phylum *Proteobacteria* comprised approximately 50% of the relative abundance, where the class *Alphaproteobacteria* was the most abundant (approximately 30%), followed by the class *Gammaproteobacteria* (ranging from 6.8 to 9.3%). The relative abundance of the genus *Pseudomonas* ranged from 0.2 to 0.3%, with higher values in the soil from samples treated with PcPCL1606 (Figure 6B). Interestingly, the relative abundance of the species *P. chlororaphis* was detectable in the samples from the rhizosphere and soil treated with PcPCL1606 (Figure 6B), but with low values of relative abundance (0.01–0.03%, respectively).

The ITS sequences were analyzed to reveal the abundance of eukaryotic microbes on the microplots inoculated with the soilborne phytopathogen *R. necatrix*; this allowed us to identify differences in the composition and relative abundance of fungal microbes. A higher number of eukaryotic families with relative abundances equal to or greater than 1% was found in the rhizosphere samples taken from plants under treatments with PcPCL1606, and a lower number was detected in rhizosphere samples from untreated control plants (Figures 7A,B). Eukaryotic communities from samples of rhizosphere treated with PcPCL1606 were very similar among samples, independent of whether they were inoculated or not with *R. necatrix* (Figures 5A, 7A). In general, the introduction of the fungal pathogen *R. necatrix* disturbed the eukaryotic community, leading to different patterns in the different samples and showing a high relative abundance of *Xylariaceae* and *R. necatrix* (Figures 7A,B). To highlight the differences generated by the pathogen, plants displaying different disease indexes (from 1 to 3) were taken, and the soil and rhizosphere of control plants not treated with PcPCL1606 were sampled. The individual analysis of these soil and rhizosphere samples coming from symptomatic plants in the untreated control plants revealed the profound effect of the pathogen, with an increase in the relative abundance of *Xylariaceae* (the family where *R. necatrix* belongs) with an increase in the disease index (Figures 7B,C). However, almost no detection of *Xylariales* or the species *R. necatrix* was observed from the samples treated with PcPCL1606 (Figure 7C). Interestingly, after treatment with PcPCL1606, soil samples showed a high increase in the family *Entolomataceae* (Figure 7A).

The Shannon diversity index and Chao richness index for prokaryotes and eukaryotes in bulk and rhizosphere soils are shown for assay 1 and assay 2, and overall, no differences in microbial diversities between bulk and rhizosphere soil was observed (Figure 8). The Shannon diversity index for prokaryotes was not influenced by the treatment with PcPCL1606, which was independent of whether *R. necatrix* was present (Figure 8). PcPCL1606 preventive application did not significantly influence prokaryotic diversity (Figure 8A). For eukaryotes, no significant differences were observed in non-artificially infected samples; however, a significant difference was found in the Shannon diversity index among rhizosphere samples treated or untreated with PcPCL1606 (Figure 8A). Prokaryotic and eukaryotic

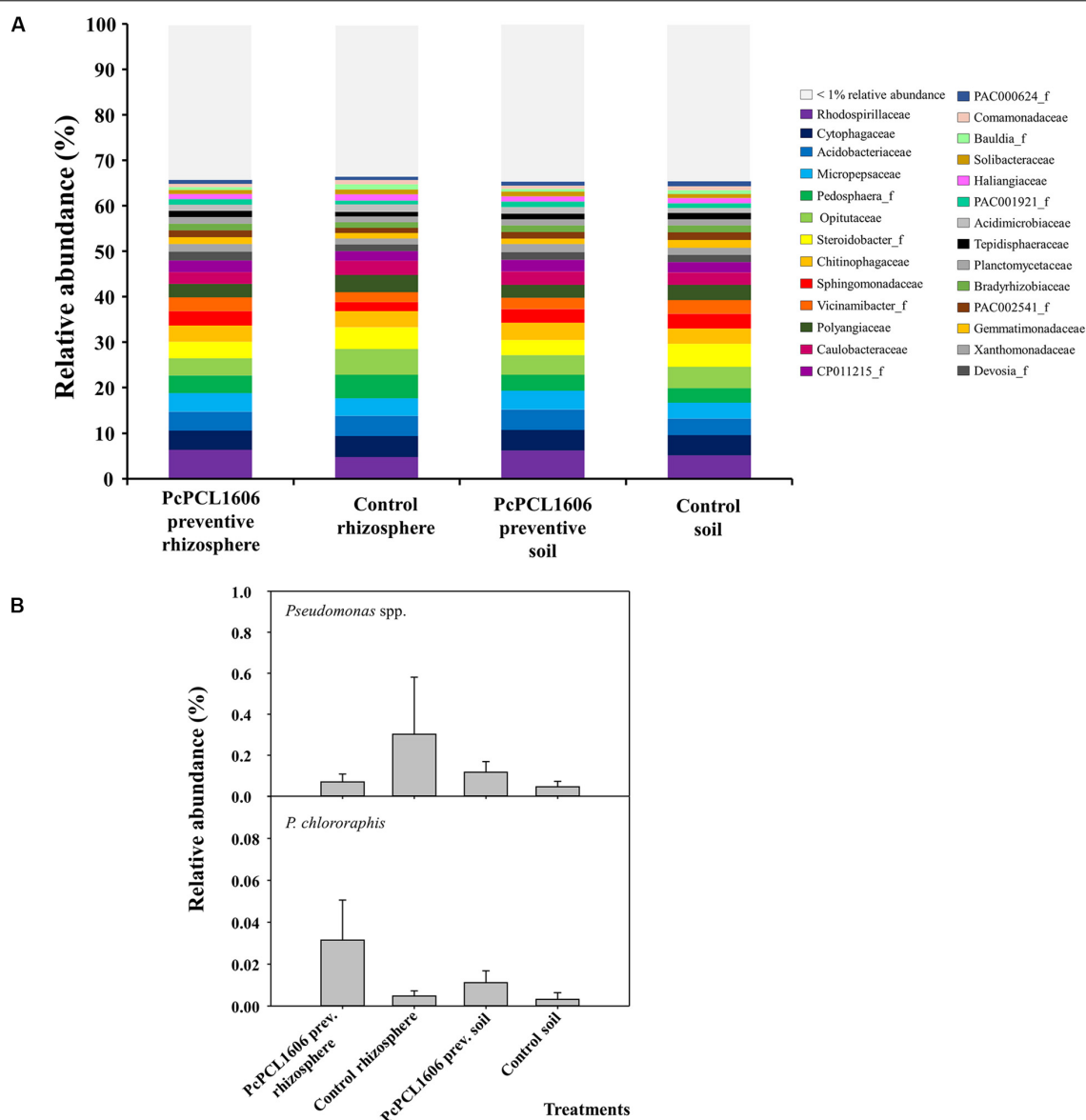


FIGURE 4 | Analysis of prokaryotic communities present in samples of soil and rhizosphere taken from avocado plants non-inoculated with *R. necatrix* during “assay 1” at T2. Negative control of soil (Control soil) and rhizosphere (Control rhizosphere) and samples from formulated PcPCL1606 preventive treatment of soil (PcPCL1606 preventive soil) and rhizosphere (PcPCL1606 preventive rhizosphere) were showed. **(A)** Relative abundance (percentage) of different prokaryotic groups detected by 16S rDNA gene sequencing analysis of soil and rhizosphere DNA at family level. The group with < 1% relative abundance was represented in gray. **(B)** Relative abundance of *Pseudomonas* genus and *Pseudomonas chlororaphis* specie at different treatments.

richness using the Chao index showed no significant differences among the different samples and treatments (**Figure 8B**).

Additionally, the Beta-diversity analysis using Bray-Curtis dissimilarities, showed that a preventive application of PcPCL1606 had no influence on the prokaryotic community structure, clustering together with the non-treated samples (**Supplementary Figure S5A**); however, the presence of *R. necatrix* resulted in a separate clustering of the samples treated or non-treated with PcPCL1606. For the eukaryotic community (**Supplementary Figure S5B**), the results are very similar, separating the samples with the preventive treatment

of PcPCL106 to the samples without bacterial treatment. It is remarkable that eukaryotic communities taken from diseased plants with the lower disease index (samples P1), were allocated in between these two main groups (**Supplementary Figure S5B**).

Role of PcPCL1606 in Soil Suppressiveness

The ability of the different soil samples to inhibit *R. necatrix* was tested using the diffusion chamber assay. The suppressively induced soil after ASO application, as well as combinations with

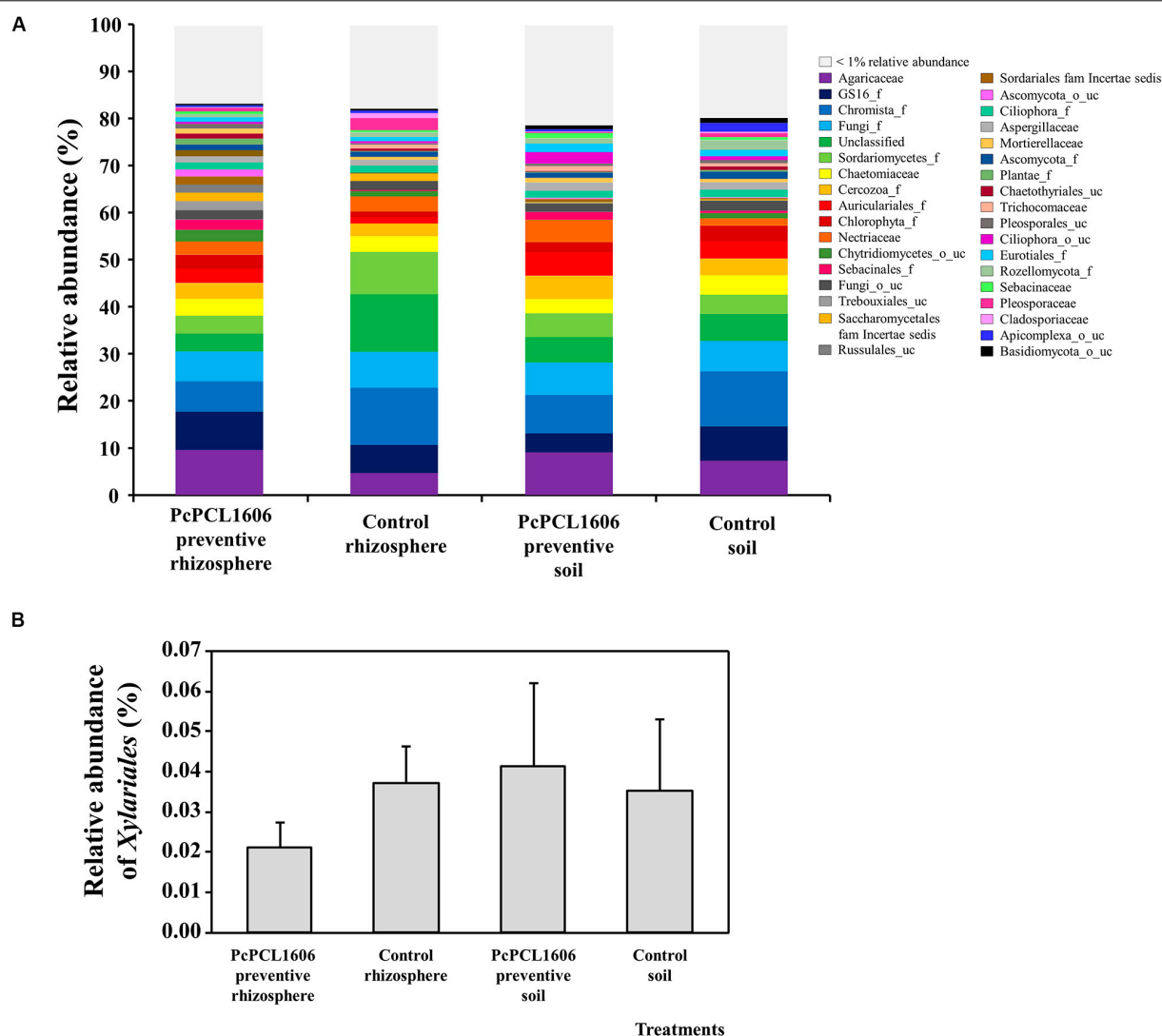


FIGURE 5 | Analysis of eukaryotic communities present in samples of soil and rhizosphere taken from avocado plants non-inoculated with *R. necatrix* during “assay 1” at T2. Negative control of soil (Control soil) and rhizosphere (Control rhizosphere) and samples from formulated PcPCL1606 preventive treatment of soil (PcPCL1606 preventive soil) and rhizosphere (PcPCL1606 preventive rhizosphere) were showed. **(A)** Relative abundance (percentage) of different eukaryotic groups detected by internal transcribed sequences (ITS) sequencing analysis of soil and rhizosphere DNA at family level. The group with < 1% relative abundance was represented in gray. **(B)** Relative abundance of the family *Xylariaceae* at different treatments.

soil treated and untreated with PcPCL1606, were tested. The highest fungal growth inhibition was displayed by the fresh soil amended with composted almond shell and the combinations including a preventive treatment with PcPCL1606, which had a significantly lower area (ANOVA, $P < 0.05$) than the untreated control soil and the rest of the soil combinations (Figure 9). To reveal the microbial nature from suppressiveness, suppressive soil after PcPCL1606 preventive treatment was heat-treated, which abolished its protective phenotype. To assign the protective effect to PcPCL1606, the heat-treated soil was complemented with PcPCL1606, which significantly recovered the suppressiveness. On the other hand, heat-treated soil complemented with the non-antagonistic HPR-defective strain $\Delta darB$ (Table 1) displayed a clear failure of soil suppressiveness, showing higher fungal colony

area of growth (ANOVA, $P < 0.05$) very similar to the growth in heat-treated soil and control soil without any treatment.

DISCUSSION

Pseudomonas chlororaphis PCL1606 (PcPCL1606) has emerged as a potential biocontrol agent in previous works (Pliego et al., 2011). PcPCL1606 has a strong antagonistic and biocontrol activity against several phytopathogenic fungi, among them *R. necatrix* (Cazorla et al., 2006), mainly due to the production of the antifungal compound HPR (Calderón et al., 2013). Previous studies have shown the biocontrol efficacy of PcPCL1606 at different levels (Cazorla et al., 2006;

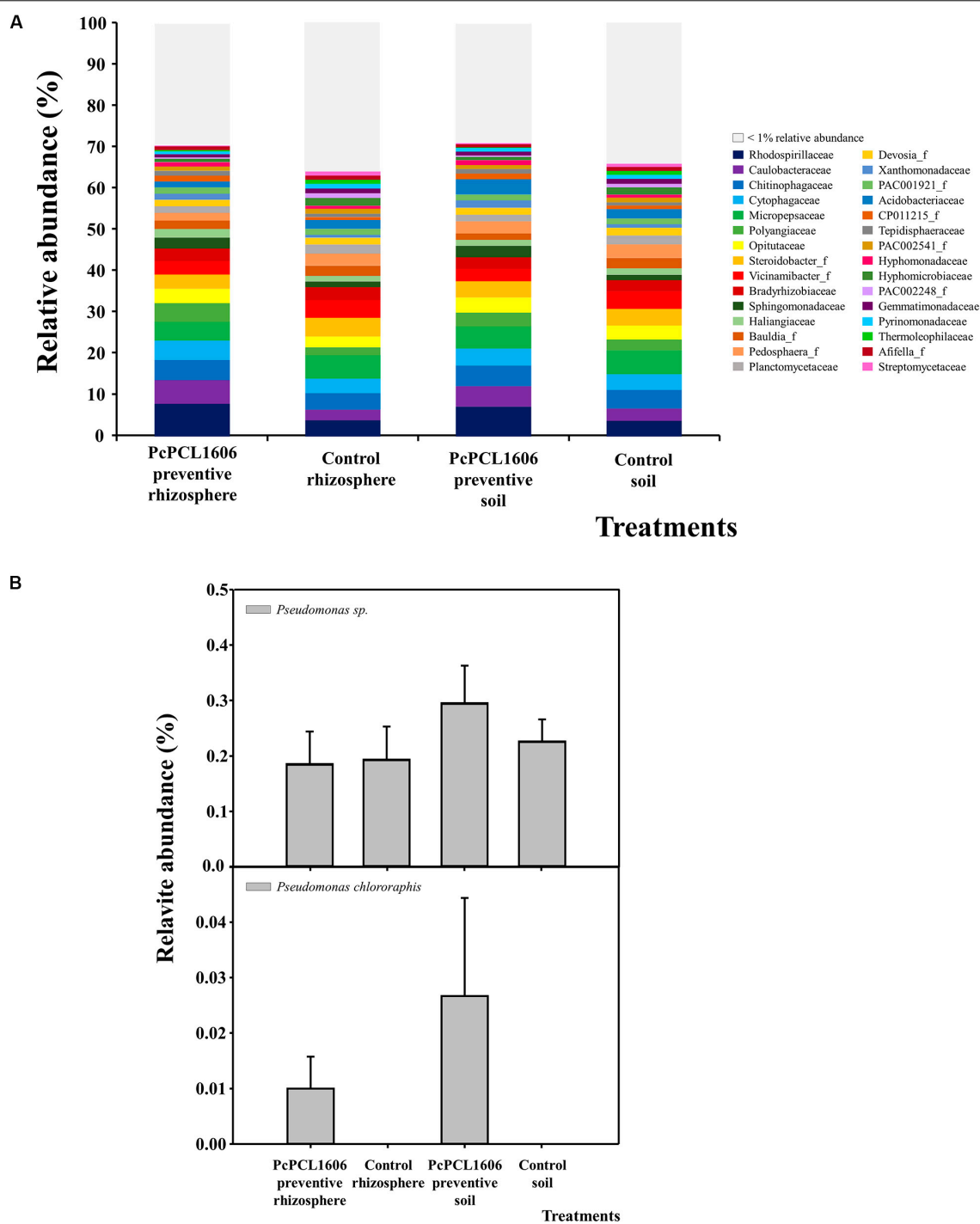
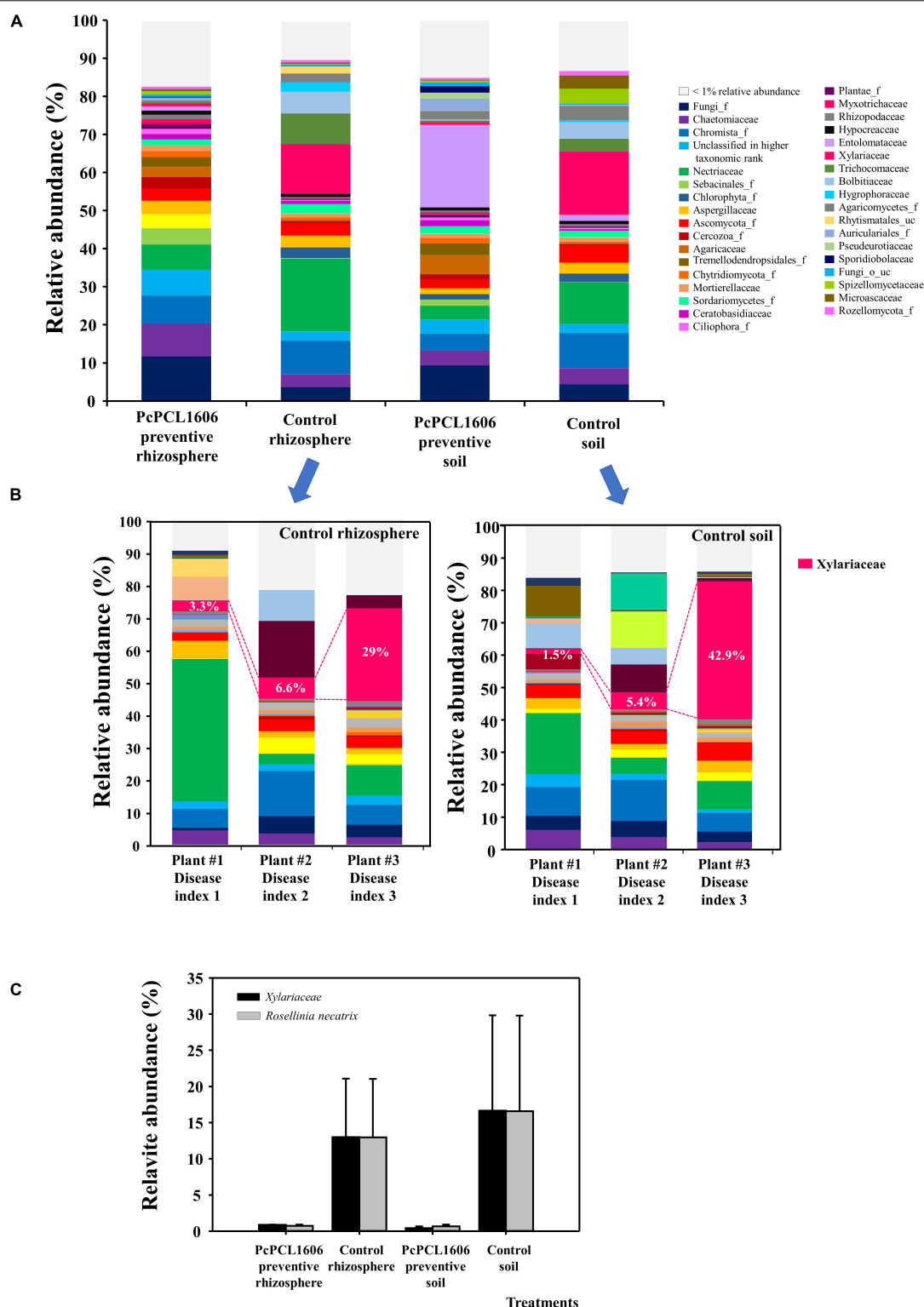


FIGURE 6 | Analysis of prokaryotic communities present in samples of soil and rhizosphere taken from avocado plants inoculated with *R. necatrix* during “assay 2” at T3. Negative control of soil (Control soil) and rhizosphere (Control rhizosphere) and samples from formulated PcPCL1606 preventive treatment of soil (PcPCL1606 preventive soil) and rhizosphere (PcPCL1606 preventive rhizosphere) were showed. **(A)** Relative abundance (percentage) of different prokaryotic groups detected by 16S rDNA gene sequencing analysis of soil and rhizosphere DNA at family level. The group with <1% relative abundance was represented in gray. **(B)** Relative abundance of *Pseudomonas* genus and *Pseudomonas chlororaphis* species at different treatments.

González-Sánchez et al., 2013), and recently, the potential of this strain as a biocontrol agent was also demonstrated in the integrated control against *R. necatrix* in avocado plants;

the strain was combined with low concentrations of fungicide, which had a higher plant protection, leading to a reduction in chemical residues and appearance of fungal resistance



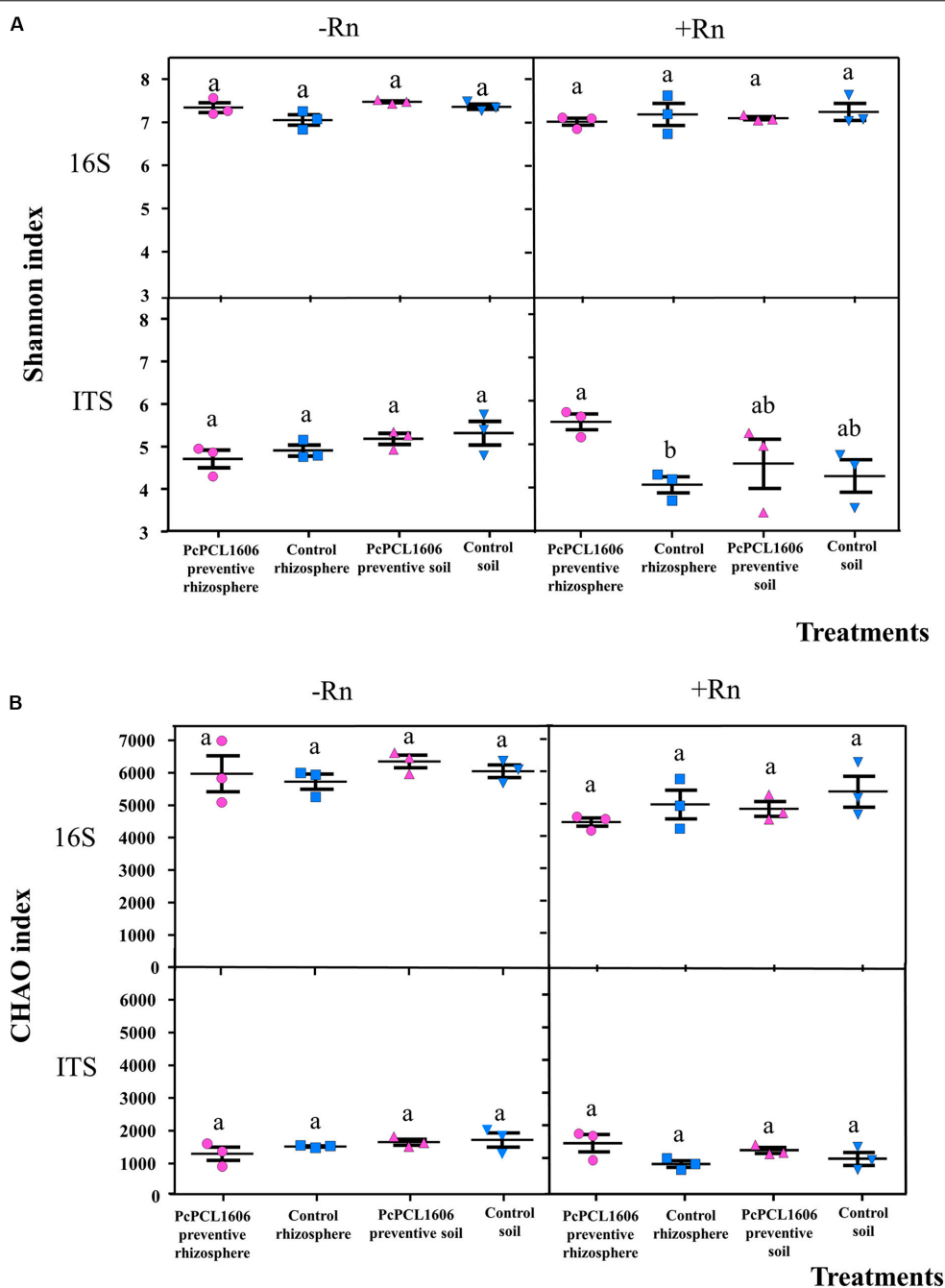


FIGURE 8 | Analysis of diversity with Shannon **(A)** and CHAO **(B)** index of 16S rRNA and ITS sequences from samples of soil/rhizosphere the avocado plant during biocontrol against *R. necatrix*. Samples analyzed were obtained from the negative control of soil (Control soil) and rhizosphere (Control rhizosphere), and samples from formulated PcPCL1606 preventive treatment of soil (PcPCL1606 preventive soil) and rhizosphere (PcPCL1606 preventive rhizosphere). -Rn, uninoculated plants; +Rn, plants inoculated with *R. necatrix*. Values of bars with same letter indications denote a non-statistically significant difference.

(Arjona-López et al., 2019). However, one of the final steps to propose PcPCL1606 as a useful and safe biocontrol agent against soilborne fungal pathogens includes the assessment of the presence and abundance of the applied biocontrol agent in soil, testing the efficacy and possible impact on autochthonous soil microbial communities. It is worth noting that this

analysis is required previously to the registration in Europe of any plant protection products (Commission Regulation No. 544/2011, L155/66).

Survival of inoculated *Pseudomonas* sp. in the soil and plant rhizosphere would be dependent on many factors, such as inoculate formulation, soil conditions, and physiological

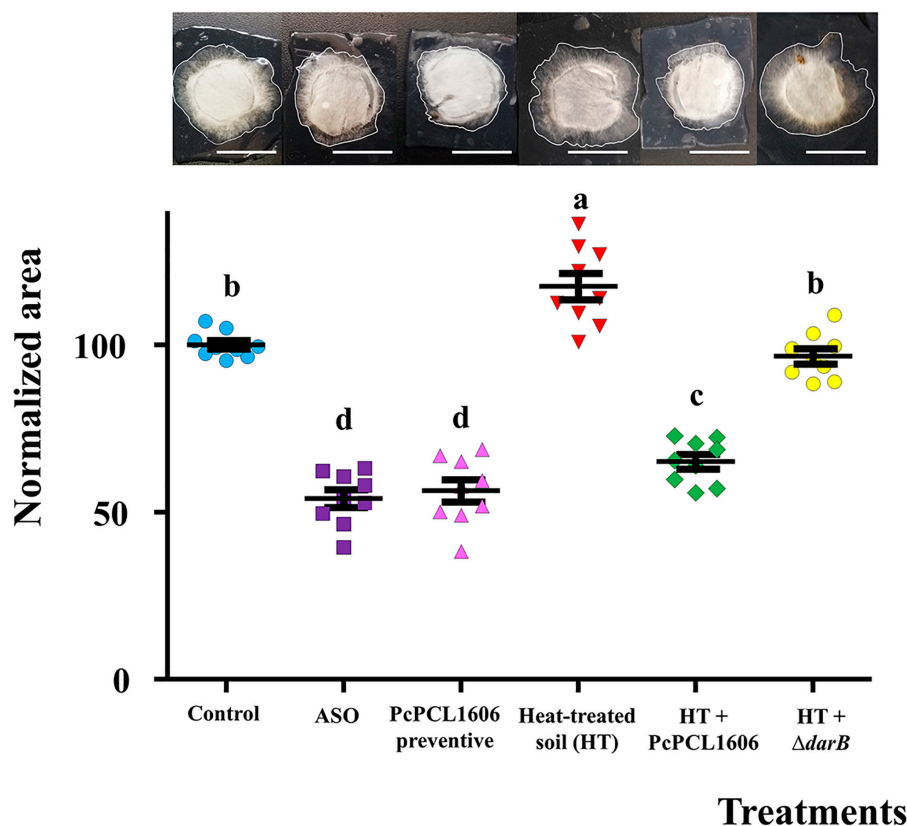


FIGURE 9 | Soil suppressivity assay against *R. necatrix* in diffusion chambers. *R. necatrix* growth area of different soil samples from “assay 2” at T3, used in mesocosm assays. Treatment assayed were Unamended/negative control (Control), composted almond shell (ASO) amended soil was used as positive control of suppressiveness, soil where PcPCL1606 formulated had been applied (PcPCL1606 preventive), heat-treated soil from PcPCL1606 preventive (HT), HT supplemented with PcPCL1606 (HT + PcPCL1606) and HT supplemented with $\Delta darB$ mutant, non-HPR-producing (HT + $\Delta darB$). Values of bars with different letter indications denote a statistically significant difference.

status of the plant (Wessendorf and Lingens, 1989; van Elsas et al., 1992). In our study, PcPCL1606 was experimentally formulated by Koppert B.V. (Netherlands) and applied by watering the soil around *R. necatrix*-infested plants to mimic the commercial conditions for this treatment. Formulated PcPCL1606 was previously tested in the laboratory and displayed equal antagonism, HPR production, biocontrol and specific amplification by PCR compared with the wild-type strain (data not shown).

It is worth mentioning that PcPCL1606 was previously isolated from avocado roots (Cazorla et al., 2006) in this same area, so it was expected that this bacterium would be very well adapted to this environment and could easily establish its interaction with natural avocado soils and avocado rhizosphere. The PcPCL1606 strain was found in both soil and rhizosphere samples, indicating that this strain can actively move and colonize avocado roots. Survival of PcPCL1606 was confirmed in the soil and rhizosphere at least 160 days after a single inoculation and under environmental conditions. This survival feature has also been shown by other previously reported *Pseudomonas* sp. (e.g., Gao et al., 2012), suggesting that the PcPCL1606 population stabilizes quickly after inoculating into soils and can persist for

several months. At the end of the experiment, the survival of PcPCL1606 ranged from 10^3 to 10^4 cfu/g, correlating the counts obtained in both media used, TPG-Gm and PSM, indicating that the bacterial counts of PcPCL1606-treated samples in PSM could correspond mainly to the originally formulated PcPCL1606 strain.

It was observed that with only one preventive application (50 days prior to *R. necatrix* inoculation), PcPCL1606 was able to significantly reduce the disease index (25–30%) compared with the untreated plants at the end of two independent experiments, confirming the previously observed biocontrol activity for this strain (Cazorla et al., 2006; González-Sánchez et al., 2013). The results of bacterial counts on the soil and rhizosphere of mesocosms under different treatments provided a first indication that the presence of *R. necatrix* has an impact on total heterotrophic bacteria and *Pseudomonas*-like bacterial counts. It was observed that the presence of the fungus stimulated the *Pseudomonas*-like counts but reduced the total heterotrophic bacteria counts. Similar results have been previously reported for culturable bacterial populations when interacting with other soil fungi. Some bacteria could use fungal-derived substrates and establish different bacteria-fungi relations

ranging from mutualistic exudate-consuming to mycophagous interactions (de Boer et al., 2005). This could help to explain the higher survival of PcPCL1606 in the rhizosphere and soil when *R. necatrix* was introduced. Thus, the survival of PcPCL1606 over time would be due to the increase in the available fungal metabolites in the nearby surroundings, which could be easily used by the bacterium. It has been reported that PcPCL1606 is strongly chemotactically attracted by avocado root exudates (Polonio et al., 2017). Once on the root surface, PcPCL1606 can establish microcolonies along the avocado root with the help of exudate compounds in the rhizosphere (Lugtenberg et al., 2001; Calderón et al., 2014). Since PcPCL1606 occupies the same root niches where *R. necatrix* initiates plant infection, the probability that both microorganisms will meet on the avocado root surface and compete for available nutrients is high (Lugtenberg et al., 2001). However, *R. necatrix* also produces exudates that strongly attract PCL1606 (Polonio et al., 2017), finally leading PcPCL1606 to contact the *R. necatrix* hyphae. As a result of such interactions, the bacterial production of antifungal compounds and enzymes would result in a deleterious effect on the fungus, favoring the increase in the *Pseudomonas*-like counts in the soil and rhizosphere.

The effect of PcPCL1606 applications on the microbial communities when no *R. necatrix* was present was elucidated from natural soil and rhizosphere samples (results from assay 1). In those analyses, no relevant differences in the prokaryotic community structure and composition were observed, independent of whether the samples were taken from rhizosphere or bulk soil or from samples with or without the preventive treatment with PcPCL1606. In these samples, soil and rhizosphere communities were predominated by the phyla *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, *Verrucomicrobia* and *Bacteroidetes*, which are the major phyla observed in soils with moderate inputs of organic matter (Lladó et al., 2017). The classes *Alpha*-, *Beta*- and *Gammaproteobacteria*, and *Acidobacteria* were, in proportion, the dominant bacterial taxa in the avocado soil and rhizosphere samples. These bacterial classes are the most frequently found in high C:N soil (Hermans et al., 2017) and are easily found in soil with the presence of organic matter in decomposition (Johnston et al., 2019). This is the case of the avocado soils of southern Spain, where attempts have been made to increase the low levels of organic matter by leaving leaf litter and chopped pruning waste on the top layer of soil every year (Bonilla et al., 2012) or where organic matter are currently used as amendments (Vida et al., 2016). The effect of PcPCL1606 on eukaryotic communities also revealed minor changes when formulated PcPCL1606 was applied to samples without *R. necatrix*. The clear majority of the eukaryotic communities were composed of fungi, with less than 10% of the eukaryotic ITS sequences belonging to organisms different than fungi (*Cercozoa*_f, *Chlororphyta*_f, *Ciliophora*_f, *Plantae*_f, etc.). It is worthy to note the presence of members from the family *Chromista*_f in all analyzed natural samples. This taxonomic group is widespread (Messenger et al., 2000) but is mainly associated with soils poorly drained, particularly clay soils, were the fundamental factor for the dissemination of spores

is water, as observed in the experimental area of this work (Pérez-Jiménez, 2008). Regarding the typical composition of the fungal communities of these samples, members belonging to the saprophytic families commonly associated with organic matter decomposition, such as *Agaricaceae*, *Chytridiomycetes*_f and *Sordariomycetes*_f, were also reported; these families are typically found in environments where leaf litter is decomposed (Kerekes et al., 2013), which occurs with avocado crops. No relevant changes in fungal communities could be observed among the analyzed samples, and the presence of *Xylariaceae* (where the pathogenic fungi *R. necatrix* belongs) was almost not detected, with very low relative abundance. These results were also supported by the absence of significant differences among the diversity and richness indexes. Other studies have reported similar results indicating no relevant changes in natural microbial populations after the application of a biocontrol microorganism. For example, repetitive applications of a soil *P. putida* strain within a citrus orchard showed no effect on the resident microbial community (Steddom et al., 2002) or the treatment with *P. fluorescens* 2P24 and CPF10; after 8 weeks of application in cucumber, the differences in bacterial population structure compared with the control disappeared (Yin et al., 2013). The same have also been observed for Gram-positive biocontrol agents, such as *B. subtilis* B579, which was applied in cucumber plants, with a minimal and transient effect on the rhizosphere bacterial population 4 and 9 weeks after treatment (Chen et al., 2015), and *Bacillus amyloliquefaciens* FZB42, where no taxonomic differences were observed in the rhizosphere microbiota of lettuce 2 and 5 weeks after treatment (Kröber et al., 2014).

When the fungal soilborne pathogen *R. necatrix* was introduced in the mesocosm experiments, the microbial communities of soil and rhizosphere were strongly impacted in different ways. This fungus can attack the plant roots, multiply and expand its hyphae inside the roots, necrotizing the plant living tissues, and finally survive in the decomposing organic matter overwinter (Pliego et al., 2009, 2012). It has been previously described that the presence of a dominant soil fungus can influence the soil and rhizosphere microbial communities (de Boer et al., 2015; Johnston et al., 2019), and in our study, the presence of *R. necatrix* impacted the microbial communities, mainly because they are dependent on the ecological strategy of the dominant fungus (Johnston et al., 2019). The prokaryotic populations of soil and rhizosphere samples showed a slight response to this new biological factor; mainly, the relative abundance of families containing chitinolytic bacteria increased (*Chitinophagaceae* and *Cytophagaceae*). The relative abundance of these groups of chitinolytic bacteria increased in response to the presence of this new and available substrate, resulted by the presence of a soilborne fungal infection (Carrión et al., 2019). The relative abundance of the genus *Pseudomonas* was still low, and under this condition, the species *P. chlororaphis* was only detected in samples taken from plants treated with PcPCL1606, which indicates the strong adaptation of the bacterial biocontrol agent to this specific environment, as previously mentioned.

On the other hand, the eukaryotic community was more impacted by the presence of *R. necatrix*, especially in the

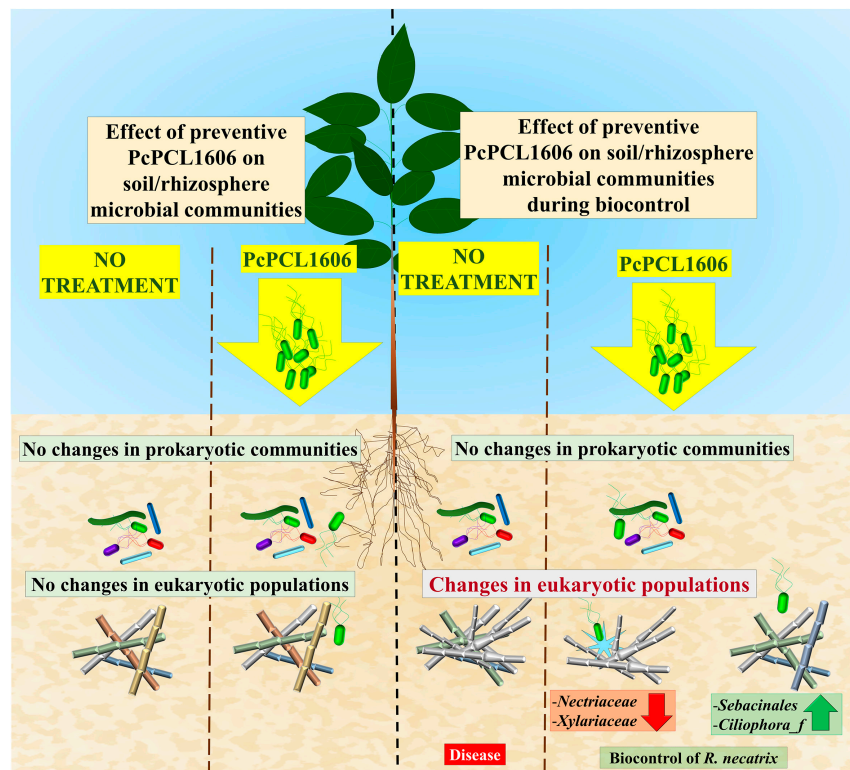


FIGURE 10 | Schematic model for the effect of the preventive application of PcPCL1606. The preventive application of the biocontrol PcPCL1606 strain has no effect on natural prokaryotic and eukaryotic populations in the absence of the pathogenic fungus *R. necatrix*. If *R. necatrix* was inoculated in the model, it causes the white root rot disease. The presence of *R. necatrix* disturbed the natural populations and became the predominant fungus. However, when the preventive application of PcPCL1606 take place, resulted in biocontrol of the fungus, reducing its presence and the modification of the microbial communities. A slight shift in the prokaryotic population was observed, appearing members with potential antifungal activity. And for the eukaryotic communities, reduced the relative abundance of *R. necatrix*. Allowing the development of other fast-growing well adapted fungi likely to be natural competitors.

taxonomic groups, with relative abundances of approximately 1–2%, which were completely different from samples collected from plants not inoculated. The rhizosphere and soil samples from untreated control plants showed the most evident impact, with 4 taxonomic groups (*Nectriaceae*, *Chromista-f*, *Xylariaceae* and *Trichocomaceae*) responsible for more than 50% of the relative abundance of eukaryotic communities. These observations agree with the obtained results for Beta-diversity, where the main impact observed in the analyzed samples was observed in those infested with *R. necatrix*. These impacts on eukaryotic organisms also resulted in a significant difference in the Shannon diversity index for the eukaryotic community, but no differences were observed when analyzing the Chao richness index. The dominant presence of *R. necatrix* in these soils resulted in white root rot disease, which can be considered conducive for this fungus because it shaped the surrounding environment, promoting the appearance of different fungi, mainly saprobes with aggressive colonization strategies and related to the degradation of wood and organic matter (Lasota et al., 2019). In those samples, the relative abundance of *Xylariaceae* and especially the species *R. necatrix* was almost the same in both control soil and rhizosphere samples, with average values above 12%. Since the avocado infection by *R. necatrix* is very aggressive (Pliego et al.,

2012), samples to be analyzed were taken from 3 plants showing different disease indexes at the moment of the sampling time. Individual analysis revealed the increase and predominance of *R. necatrix* according to aerial symptoms and the important differences among the relative abundance of fungal families. Although these results were not conclusive, it seems that a succession of different fungi that change in relative abundance takes place during the infection process, with a progressive increase in *R. necatrix*.

However, in these same samples but treated with PcPCL1606, almost no *Xylariaceae* and/or *R. necatrix* were detected (<1%). This preventive PcPCL1606 treatment keeps the microbial communities of the rhizosphere more stabilized resulting in less changes compared with the fungal population from non-inoculated samples treated with PcPCL1606. Notably, the family *Nectriaceae* also increased with the treatment with *R. necatrix*, and several of the species contained in this family are saprobes or weak to virulent, facultative or obligate plant pathogens (Lombard et al., 2015). It is interesting to highlight the increase in relative abundance of the family *Sebacinales* in the rhizospheric and soil samples where formulated PcPCL1606 was applied. *Sebacinales* are highly diverse root symbionts that form various mycorrhizae and endophytic interactions and promote beneficial

effects on host plants at diverse levels (Nautiyal et al., 2010; Franken, 2012; Kumar et al., 2012), enhancing abiotic stress resistance (Waller et al., 2005; Baltruschat et al., 2008; Ghimire and Craven, 2011) and resistance to pathogens (Waller et al., 2005; Serfling et al., 2007; Fakhro et al., 2010; Harrach et al., 2013). Interestingly, in soil samples treated with PcPCL1606, a clear impact was observed in fungal communities because the family *Entolomataceae* became predominant (>20% relative abundance). This fungal family is very species-rich, with most of them saprophytic on soil, wood or moss, but some members could be parasitic on plants or even ectomycorrhizal (Co-David et al., 2009). Since no symptoms were observed, these families are more likely to be related to organic matter decomposition or could be in a non-active form.

The suppressiveness displayed by the samples treated with PcPCL1606 can be inferred by the biocontrol experiments, but a more direct analysis showed that the application of PcPCL1606 can confer suppressiveness to the soil 130 days (T3) after the single treatment, similar to the positive control of suppressiveness-induced soil amended with composted almond shells (Vida et al., 2016). This suppressiveness is microbial-based since it completely disappeared after the soil samples were heat treated and can be recovered with a low-dose inoculation of PcPCL1606. Additionally, since HPR was described as the main factor involved in antagonism and biocontrol, soil complementation with the derivative mutant $\Delta darB$ confirmed the direct involvement of HPR in soil suppressiveness by PcPCL1606.

A conclusion of this work is represented in **Figure 10**. The application of a formulated PcPCL1606 treatment to the commercial soil of avocado plants did not impact the soil and rhizosphere natural microbial populations (prokaryotic or eukaryotic). However, in a situation under *R. necatrix* infection, the application of PcPCL1606 reduced the symptom development of white root rot disease. Interestingly, the single preventive application allowed us to determine the survival of PcPCL1606 in the soil and avocado rhizosphere for at least 160 days, also conferring biocontrol to the avocado plants against *R. necatrix* infection. During biocontrol, *R. necatrix* impacted the microbial communities; however, that impact was reduced by the preventive application of PcPCL1606. The bacterial populations were poorly influenced by *R. necatrix* introduction and by PcPCL1606 treatment. On the other hand, the severe impact of *R. necatrix* introduction on fungal communities was partially restored by the preventive PcPCL1606 treatment, which inhibited the development of *R. necatrix* and other saprophytic families of fungi that finally led to suppressiveness against this fungus. The basis for this protection is directly related to the production of the compound HPR, which confers the suppressive phenotype to the treated soil.

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://doi.org/10.6084/m9.figshare.12310253.v1>, <https://doi.org/10.6084/m9.figshare.12309788.v1>, and <https://www.ebi.ac.uk/ena/data/view/ERS4551058-ERS4551081>.

6084/m9.figshare.12310253.v1, <https://doi.org/10.6084/m9.figshare.12309788.v1>, and <https://www.ebi.ac.uk/ena/data/view/ERS4551058-ERS4551081>.

AUTHOR CONTRIBUTIONS

ST and FC designed the experiments. EL and SW formulated the bacterium. ST, CV, IL, EG, and JG-F performed the experiments. ST, AV, and FC analyzed the results and wrote the manuscript. All the 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/fmicb.2020.01874/full#supplementary-material>

FIGURE S1 | Disease index of white root rot on 2-years old avocado plants during the microcosms assays. 0, healthy plant; 1, plant with first symptoms of wilt; 2, overall wilted plant; 3, wilted plant with first symptoms of leaf desiccation; and 4, completely dried plant (dead plant).

FIGURE S2 | Climatic data during the biocontrol experiments (seasons 2017/18 and 2018/19). Blue bars indicate average relative humidity (HR), and red line indicated average temperature. Data are taken every ten days. Dotted lines indicated season average HR (blue) and temperature (red).

FIGURE S3 | Regression analysis of the bacterial counts of PcPCL1606-GFP growing in *Pseudomonas* selective medium and in TPG amended with gentamicin.

FIGURE S4 | Effect of formulated PCL1606 application on culturable microbial populations during the biocontrol, taken at T3 during the “assay 2” microcosms experiments. The population densities of fast-growing heterotrophic bacteria and pseudomonads-like were assessed by plate counts at different times (T0, T1, T2, and T3). Bacterial counts from samples inoculated with *R. necatrix* were showed as +Rn.

FIGURE S5 | Analysis of structure using the Bray-Curtis index of 16S rRNA (A) and ITS (B) sequences from samples of soil/rhizosphere the avocado plant during biocontrol against *R. necatrix*. Samples analyzed were obtained from the negative control of soil (Control soil) and rhizosphere (Control rhizosphere), and samples from formulated PcPCL1606 preventive treatment of soil (PcPCL1606 preventive soil) and rhizosphere (PcPCL1606 preventive rhizosphere). +Rn: plants inoculated with *R. necatrix*.

TABLE S1 | Specific primers for specific amplification of *Pseudomonas chlororaphis* PCL1606 (PcPCL1606) and *R. necatrix* from soil and rhizosphere DNA.

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Sub-Lethal Effects of Pesticides on the DNA of Soil Organisms as Early Ecotoxicological Biomarkers

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This review describes the researches performed in the last years to assess the impact of pesticide sub-lethal doses on soil microorganisms and non-target organisms in agricultural soil ecosystems. The overview was developed through the careful description and a critical analysis of three methodologies based on culture-independent approaches involving DNA extraction and sequencing (denaturing gradient gel electrophoresis, DGGE; next-generation sequencing, NGS) to characterize the microbial population and DNA damage assessment (comet assay) to determine the effect on soil invertebrates. The examination of the related published articles showed a continuous improvement of the possibility to detect the detrimental effect of the pesticides on soil microorganisms and non-target organisms at sub-lethal doses, i.e., doses which have no lethal effect on the organisms. Considering the overall critical discussion on microbial soil monitoring in the function of pesticide treatments, we can confirm the usefulness of PCR-DGGE as a screening technique to assess the genetic diversity of microbial communities. Nowadays, DGGE remains a preliminary technique to highlight rapidly the main differences in microbial community composition, which is able to give further information if coupled with culture-dependent microbiological approaches, while thorough assessments must be gained by high-throughput techniques such as NGS. The comet assay represents an elective technique for assessing genotoxicity in environmental biomonitoring, being mature after decades of implementation and widely used worldwide for its direct, simple, and affordable implementation. Nonetheless, in order to promote the consistency and reliability of results, regulatory bodies should provide guidelines on the optimal use of this tool, strongly indicating the most reliable indicators of DNA damage. This review may help the European Regulation Authority in deriving new ecotoxicological endpoints to be included in the Registration Procedure of new pesticides.

Keywords: pesticides, DNA, soil microorganisms, earthworms, ecotoxicological biomarkers, denaturing gradient gel electrophoresis, comet assay, next-generation sequencing

INTRODUCTION

Anthropogenic activities are associated with the massive disposal of contaminants that, in many cases, could be potentially genotoxic and carcinogenic. This poses a major challenge for regulatory authorities and environmental managers to protect the quality and the services provided by natural resources. Simple detection/quantification of xenobiotics in abiotic and biotic compartments has limited relevance, particularly when they occur as complex mixtures, unless their biological or ecological effects are properly evaluated. Biological systems indeed provide important information, which is not readily available from direct chemical analyses of the environmental samples and, thus, are increasingly used as diagnostic tools for integrated environmental management (Sarkar et al., 2006).

The effects of agrochemicals on the soil ecosystem components represented by microorganisms and macroinvertebrates deserve particular attention. The relationship between plant root apparatus and soil microbial communities is strictly linked with soil fertility at a chemical, biochemical, and microbiological level, and several studies on the interactions between root exudates and soil microbial biomass growth, structure, and activity were carried out in detail during the last 20 years (Mergel et al., 1998; Nannipieri et al., 2008; Steinauer et al., 2016; de Vries et al., 2019). Rhizobacteria and fungi can stimulate plant growth through the production of phytoestrogens (auxins, gibberellins), increase nutrient uptake, and induce tolerance to plants against abiotic stress or by suppressing biotic stressors like plant diseases or pests (Vryzas, 2016): any change and/or disturbance to the delicate regulation of these interactions can thus result in an impairment of soil fertility.

The use of pesticides against plants pests, weeds, and pathogens was proven to affect the chemical and biological fertility of soils in several cases, including a number of potential adverse effects versus soil microorganisms and/or non-target organisms. During the past, classical studies were performed evidencing the effects of pesticides on the whole soil microbial biomass and/or on the soil biochemical and enzymatic activities (Perucci et al., 2000; Vischetti et al., 2000, 2002; Puglisi et al., 2005, 2012; Nannipieri et al., 2012; Sofo et al., 2012; Suci et al., 2019), and contrasting results were derived, highlighting the detrimental effect in the major part of the studies but, in some cases, also stimulating effects due to pesticides acting as a carbon source (Puglisi, 2012).

Pesticide ecotoxicology is a relatively new branch of toxicology, being the study of the adverse effect of pesticides versus non-target organisms, including different species living in the ecosystems. Ecotoxicological indexes relative to different commercial pesticides are included in the Dossier for the Registration Procedure. To authorize a pesticide, risk assessment in the EU, the United States of America (USA), and most other Countries requires that the predicted environmental exposure concentration is below a concentration considered safe for non-target organisms (Boivin and Poulsen, 2017). In the EU, in a first tier of risk assessment, this safe concentration is established by the European Food Safety Authority (EFSA) in cooperation

with national agencies of the EU member states through a combination of standard toxicity tests, i.e., tests performed with single chemicals and single species under laboratory conditions without additional stressors, and safety factors that account for uncertainties in the extrapolation to real ecosystems [European Parliament, 2009; European Union [EU] (2011)]. The current methodologies to assess the toxicity of pesticides for terrestrial biota by the European Chemical Agency (ECHA) take into account the mortality, the reproduction activity, and morphological and behavioral changes of earthworms, collembola and predatory mites (OECD, 2004, 2016a,b). At the same time, for microorganisms, ECHA evaluates nitrogen and carbon transformation activity (OECD, 2000a,b). In the last years, unexpected negative effects of pesticide residues on non-target organisms were detected in different ecosystems and under different pesticide exposure levels (Desneux et al., 2007; Beketov et al., 2013; Brühl et al., 2013; Wood and Goulson, 2017), and the assumptions of the Regulation Authorities have been often contradicted. In this context, the novel introduction of proper methodologies to ascertain the damage effect of pesticides on the soil living organisms is a decisive step to ascertain any undesired effect at a high tier of safety (Schäfer et al., 2019). Recently, EFSA has issued a scientific opinion on the risk assessment of plant protection products on soil organisms (Ockleford et al., 2017), where microorganisms are also included. While acknowledging the limitations of assays based on single microbial species, EFSA recommends the use of molecular methodologies addressing whole communities but highlights the difficulties of interpreting complex outcomes for regulatory purposes.

Additional endpoints were introduced in the last years as improved estimators of the sub-lethal effects of pesticides, and in this respect, genotoxicity represents a critical marker of xenobiotic exposure having important repercussions not only on the viability of non-target species but also on the ecological fitness of the organisms. Indeed, a clear link between genotoxicity and lowered reproductive performance and embryotoxicity has been highlighted. Therefore, new methodologies have been introduced to study the effects of sub-lethal doses, i.e., doses which have no lethal effects on the organisms, versus soil biota: comet assay, denaturing gradient gel electrophoresis (DGGE), and more recently next-generation sequencing (NGS). The comet assay addresses DNA damage on invertebrates, while DGGE and NGS use DNA and RNA biomarker genes as molecular tools to evaluate changes in the microbial community.

The review describes the researches performed in the last years on the effect of pesticides on soil microorganisms and non-target organisms, using new approaches involving DNA extraction and sequencing (DGGE; NGS) to characterize the microbial population and DNA damage assessment (comet assay) to determine the effect on soil invertebrates.

The aim was to detect the impact of pesticide sub-lethal doses in agricultural soil ecosystems and help European Registration Authorities to derive ecotoxicological parameters useful for the pesticide Registration Procedure at a high tier of risk assessment, taking into account the difficulties of interpreting complex outcomes for regulatory purposes.

ARTICLE TYPES

Reviews and experimental papers dealing with the effects of pesticides on soil organisms as assessed by means of DGGE, NGS, or comet assay.

CRITICAL ANALYSIS OF THE SCIENTIFIC LITERATURE

Denaturing Gradient Gel Electrophoresis

Over the last decade, PCR-based molecular fingerprinting techniques, giving a direct comparative overview of the composition and diversity of soil microbiota, replaced most other post-PCR analytical methods (van Elsas and Boersma, 2011). Specific examples of direct molecular monitoring approaches in soil microbiology are represented by DGGE, temperature gradient gel electrophoresis (TGGE) (Heuer and Smalla, 1997; Muyzer and Smalla, 1998), terminal restriction fragment length polymorphism (T-RFLP) (Kuske et al., 2002), single-strand conformational polymorphism (SSCP) (Schwieger and Tebbe, 1998), ribosomal internal spacer analysis (RISA) (Ranjard et al., 2000), and length heterogeneity-PCR (LH-PCR) (Ritchie et al., 2000). Among culture-independent fingerprinting methods, the DGGE, firstly theoretically described in the early 80s by Fischer and Lerman (1979), has been widely applied (Hoshino and Matsumoto, 2007; Coppola et al., 2011; Umar et al., 2017). To obtain optimal results in DGGE analysis, the first practical aspect concerns the high-quality extraction of total DNA from samples and then the control of amplification via PCR selecting universal primers targeting part of the 16S or 18S rRNA sequences for bacteria and fungi, respectively. Subsequently, the separation is based on the differences in mobility of partially melted DNA (with the same length but different sequences) in polyacrylamide gels containing a linear urea and formamide gradient of DNA, properly set (Fakruddin and Mannan, 2013). The identification of the microbial species can be obtained by sequencing the band excised by the polyacrylamide gel (Salles et al., 2004).

Denaturing gradient gel electrophoresis was originally developed to detect small mutation changes in DNA sequences, but quickly the technique has been applied for transversal microbial analyses, thanks to evident advantages such as rapidness, high reproducibility, and low costs (Fakruddin and Mannan, 2013). Currently, PCR-DGGE is a routine and widely used method in soil ecosystem studies. Nevertheless, the technique has some limitations such as PCR biases, variable DNA extraction efficiency, difficult sample handling (Fakruddin and Mannan, 2013), and limited sensitivity (Nocker et al., 2007). Other problems in DGGE analysis could be the formation of heteroduplex molecules, which can alter the distribution of the bands in the acrylamide gel (Ercolini, 2004).

As discussed below, DGGE is also suffering from a very limited resolution power as compared to NGS, with the latter replacing it in several studies. Nevertheless, DGGE is still commonly employed for soil microbial ecology studies in order to monitor population structure and dynamics during that time, especially because of its ability to provide a representative profile of the

dominant microbial diversity from specific environments such as soils (Ercolini, 2004; Munaut et al., 2011). In particular, DGGE is a useful tool for the rapid evaluation of microbial profiles in complex ecosystems (Kurtzman et al., 2011; Ng et al., 2014; Di Lenola et al., 2017; Zhang C. et al., 2017), allowing a rapid and efficient separation of the DNA fragments (Umar et al., 2017).

In common with the NGS methods discussed below, different primer sets can be used in PCR-DGGE, thus addressing microbial communities at the phylogenetic level (e.g., 16S primers for bacteria and archaea, ITS for fungi) or at the functional level, depending on functional selected genes. PCR-DGGE protocols optimized for use with soil DNA constitute a consolidated and reliable method that can be used in addition to culture-dependent methods to obtain a complete picture of microbial diversity and dynamics. It can also be as a possible alternative to the most modern NGS techniques since it does not require complex bioinformatics for the analyses of results (Shokralla et al., 2012; da Silva Barros et al., 2019; Hemmat-Jou et al., 2019; Ruanpanun and Nimnoi, 2020).

The analysis of the relevant articles published in the last years on the effects of pesticides upon the soil microorganisms, determined through the DGGE technique, is summarized in **Table 1**.

Recently, Di Lenola et al. (2017) compared different molecular methods to assess soil microbial diversity considered as a complex habitat. Delgado-Baquerizo et al. (2016) already established that anthropogenic pressures such as chemical pollution or agriculture practices strongly affect microbial composition and identified that PCR-DGGE as a rapid method to highlight this shift is promising.

Pesticides are defined as bioactive and toxic substances that can influence, directly or indirectly, soil productivity, and agroecosystem quality (Joergensen and Emmerling, 2006). Their principal function is the growth inhibition of target organisms even if their effects can be extended to non-target microorganisms, causing an alteration in the microbial community structure (Tortella et al., 2013a,b). The evaluation of pesticide impact on non-target organisms in soils, including microorganisms, could be a useful tool to monitor soil health and evaluating their quality. Some years ago, Imfeld and Vuilleumier (2012) already made an extensive investigation on industrially produced pesticides in agriculture in relation to the contamination of soil ecosystems. The authors considered a large variety of cultivation-dependent and -independent methods potentially applied to measure and interpret the effects of pesticide exposure, expanding the study in the specific context of the responses of the soil microflora to pesticide exposure. They established a systematic combination of microbial culture-based and molecular culture-independent methods that will comprehensive contribute to this field.

Gao et al. (2012) compared DGGE and T-RFLP techniques for monitoring the effect of a natural pesticide, *Pseudomonas fluorescens* 2P24, on the soil fungal community in the cucumber rhizosphere. In this case, DGGE results indicated that the fungal community was shocked at the beginning of the trial,

TABLE 1 | Summary of the relevant articles on the application of denaturing gradient gel electrophoresis technique to the pesticide effect on soil microorganisms.

References	Substance	Type	Study	Structure impact	Comments
Hoshino and Matsumoto (2007)	Chloropicrin and 1,3-Dichloropropene	Fumigants	Field	Significant effect	Chloropicrin changed DGGE profiles radically and no recovery was found after 1 year.
Coppola et al. (2011)	Penconazole, Dimethomorph, Metalaxyl, Azoxystrobin, Cyprodinil, and Fludioxonil	Fungicides	Lab	Transitory effects	Evident variation of microbial population after pesticides treatments; no significant differences at the end of the experiment.
Gao et al. (2012)	<i>Pseudomonas fluorescens</i> 2P24	Biological control	Field	Transitory effects	Fungal community was significantly shocked at first, but it improved gradually after 1 month.
Gupta et al. (2013)	Chlorpyrifos, endosulfan and azadirachtin	Chemical and natural insecticides	Lab	Dose- dependent significant	High doses of azadirachtin simulated the effects of chemical pesticides
Chen et al. (2013)	<i>B. subtilis</i> B579	Biological control	Lab	Minimal and transient effects	Only minimal and temporary changes in rhizobacterial population structure were detected.
Tortella et al. (2013a)	Atrazine	Herbicide	Lab	Transitory effects	Robustness of microbial community toward the treatments.
Tortella et al. (2013b)	Carbendazim	Fungicide	Lab	Transitory effects	Microbial population remained stable over the time when compared to the untreated control.
Lin et al. (2016)	Pentachlorophenol	Herbicide	Lab	Positive effects vs. earthworms	The microbial population was changed by the earthworm treatments.
Huang et al. (2016)	Chlorpyrifos	Insecticides	Lab	Significant effects	The insecticide inhibits the fungal abundance significantly.
Diez et al. (2017)	Atrazine, chlorpyrifos, and iprodione	Herbicide, insecticide, and fungicide	Lab	Transitory effects	Evident variation of microbial community detected only at first treatment.
Wang et al. (2019)	Metalaxyl	Fungicides	Field	Significant effects	Metalaxyl inhibits the growth of fungi.

but it improved gradually after 1 month, with the decline of *P. fluorescens* 2P24. This study revealed the transient effect of biological control agents against the microbial populations.

Analogously, Chen et al. (2013) studied the effect of *Bacillus subtilis* B579, another natural biological pesticide, on rhizobacteria community structure, using cultivation-based analysis coupled with DGGE to profile the changes of bacterial community structure. As expected, also in this case, the analysis revealed a minimal and transitory effect on microbial populations.

Gupta et al. (2013) applied a DGGE approach to compare the effects of two chemical pesticides (chlorpyrifos and endosulfan) and a biopesticide (azadirachtin) on the bacterial community in rhizospheric soil. In this case, results showed that high doses of azadirachtin simulated the effects of chemical pesticides on bacterial communities showing a significant dose-dependent effect.

The efficacy of natural biopesticides emphasizes the need to widely investigate their effect in agriculture before accepting them as safe alternatives to chemical pesticides. Tortella et al. (2013a; 2013b), studied the effects of chemical pesticides (atrazine and carbendazim) on the microbial community of a biopurification system, revealing a transient reduction of the microbial population after each treatment. Indeed, the DGGE analysis confirmed that microbial structure remained stable for a long time.

More recently, Diez et al. (2017) evaluated the role of the rhizosphere in pesticide dissipation and consequential microbial community changes in a biopurification system. Analogous to the results obtained by Coppola et al. (2011) and Marinozzi et al. (2013), they found that microbial communities were

immediately modified 1 day after the fungicides treatments, but the community structure was recovered at the end of the experiment. The shift in the composition was thus only transitory and, at the end of the trial the populations, returned to their initial robustness.

Umar et al. (2017) recently applied DGGE fingerprinting of 16S rDNA to study the bacterial profile of Nigerian agricultural soil. This study confirmed the complex nature of this matrix, and the PCR-DGGE approach gave the advantage of not requiring previous knowledge on this habitat, providing an immediate picture of specific microbial population constituents in both a qualitative and a semi-quantitative way.

In contrast with these critically discussed results, other researchers demonstrate the irreversible effect of chemical pesticides on non-target microbial soil populations. Among them, Hoshino and Matsumoto (2007) revealed that the fumigants chloropicrin and 1,3-dichloropropene (1,3-D) have an adverse effect on fungal community structure. They showed that the chloropicrin treatment changed soil DGGE profiles significantly after 2 months of treatment. The DGGE profiles were not completely recovered even after a period of 12 months. On the other hand, the DGGE profiles of 1,3-D-treated soils showed a small change after 2 months of fumigation, but then after 6 months the treated DGGE profiles became indistinguishable from the controls.

The effects of chlorpyrifos on fungal abundance and community structure as revealed by DGGE were assayed by Huang et al. (2016). A significant inhibition of fungal abundance was induced by chlorpyrifos, a persistent insecticide that is widely used in agriculture despite being very potentially dangerous to non-target environmental organisms. Analogously,

Chu et al. (2008) observed the inhibitory effect on the fungal community by chlorpyrifos.

Wang et al. (2019) studied the ecological toxicity of metalaxyl applications on soil microorganisms; the study of T-RFLP and DGGE revealed that metalaxyl inhibits the growth of fungi.

Lin et al. (2016) studied the impact on the soil microbial community and enzyme activity of two earthworm species during the bioremediation of pentachlorophenol-contaminated soils, evaluating the community structure on day 42 by DGGE with the 16S rRNA amplification of the V3 region. This study addressed the roles and mechanisms by which two earthworm species (epigeic *Eisenia fetida* and endogeic *Amyntas robustus*) affect the soil microbial community and enzyme activity during the bioremediation of PCP-contaminated soils. The results obtained confirmed that the soil microbial community was changed by the earthworm treatments.

Considering the overall critical discussion on microbial soil monitoring in the function of pesticide treatments, we can confirm the usefulness of PCR-DGGE as a screening technique to assess the genetic diversity of microbial communities. Nowadays, DGGE remains a preliminary technique to fast highlight the main differences in microbial community composition and is able to give further information if coupled with culture-dependent microbiological approaches, while thorough assessments can be gained by high-throughput techniques such as NGS.

Next-Generation Sequencing

Next-generation sequencing is an advanced sequencing method that allows the high-throughput nucleotide sequencing of millions of DNA strands in parallel, resulting in reads that are analyzed through bioinformatics, thanks to the availability of reference sequence libraries. Among the available NGS technologies, Illumina is nowadays mostly applied because of throughput, data quality, and costs per sequenced nucleotide. The use of NGS in recent years has increased in many scientific fields (e.g., Clinical Research, Food Science, Agricultural Science, Toxicology) as it became cheaper and therefore accessible to most labs all over the world (Beedanagari and John, 2014; Rockmann et al., 2019; Wiedmann and Carroll, 2019). The technology provides a thorough depiction of soil microbiology, paving the way for the discovery of previously unexplored compositions and diversities of both culturable and unculturable soil microorganisms (Simon and Daniel, 2011; Thompson et al., 2017). Current approaches used to study the diversity of soil microorganisms by NGS are based on phylogenetic and functional marker genes to address the two main questions: “who is there?” and “what are they doing?” This PCR-based approach often uses the amplification of targeted genomic regions of the 16S rRNA marker gene for bacterial diversity and 18S or ITS (internal transcribed spacer) for fungal diversity to answer the first question of “who is there?” (Vasileiadis et al., 2013). Despite the fact that the method itself is highly sensitive and is often the representative of microbial diversity, the presence of relic DNA (DNA of extracellular origin/from the cells that are no longer intact) is its main drawback (Carini et al., 2016). Moreover, the use of metagenomic analysis in combination with other methods (e.g., metatranscriptomics, metabolomics) to reveal the

impact of active members on the results thus answering the latter question of “what are they doing?” has been recently adopted by many (Jansson and Baker, 2016; Jansson and Hofmockel, 2018; Shakya et al., 2019). The method is thus a useful approach to understand the impacts of pesticides on soil microorganisms from rhizosphere to bulk soil, both in the short and long term. A review by Imfeld and Vuilleumier (2012) suggested that up until that year of publication, the use of NGS to investigate the impact of pesticides on the soil bacterial populations has not been reported. However, our current review shows that it is almost of common use nowadays, making precedent approaches almost obsolete, mainly because of its unprecedented sensitivity. Indeed, NGS, besides revealing the effectiveness of the applied pesticide on its target, may also reveal the unintended impact of pesticide through screening of non-target groups present in soil (e.g., impact of insecticide on bacteria, herbicide, on fungi) (Kollah et al., 2019; Mallet et al., 2019). In addition, it also has the potential to reveal crucial information regarding the presence and activity of microbial degraders of pesticides and other contaminants in the soil for remediation purposes (Jeffries et al., 2018).

The studies evaluated in this review include works that were focusing on the impact of various types of pesticides (i.e., herbicides, fungicides, insecticides, fumigants, nematocides, and one novel bactericide) either separately on bacteria and fungi or on bacteria and fungi altogether. Interestingly, as shown in **Table 2**, most of the works focused only on the impact of one to three pesticides on either bacterial or fungal communities. Present literature review shows that, while some of the earlier works investigated biodiversity of soil microbial communities often focusing on commonly used regions of the DNA, some of the most recent works, on the other hand, focused on specific communities focusing on various roles and specific genes to correctly evaluate the impact of the treatments on the functionality of soil fertility's key players (Gallego et al., 2019; Tang et al., 2019; Fang et al., 2020). Only two studies, Armalyte et al. (2019) and Panelli et al. (2017), focused on the overall impact of the specialized farming systems and their relative pesticides' overall impact on either bacterial or fungal communities.

Particularly, in the work of Armalyte et al. (2019), the comparison of soil bacterial communities revealed no major differences among the main phyla of bacteria between the two farming systems with similar soil structure and pH, suggesting the important role played by these factors. However, slight differences and minor shifts of lower taxa were observed following the treatments and fertilization regimes, and these were interpreted as minor shifts in which the soil community is responding and adjusting itself to handle these treatments and re-stabilize its balance. The study of Panelli et al. (2017), on the other hand, revealed the alteration of fungal consortia starting from the first year of the study, due to various management conditions. It was found that Ascomycota always predominated, with the exception of conventional farming in which high abundance of Basidiomycete species was detected.

The field study of Storck et al. (2018) was the only one in which an insecticide, a herbicide, and a fungicide (namely chlorpyrifos, isoproturon, and tebuconazole) were evaluated

TABLE 2 | Summary of the relevant articles on the application of next-generation sequencing to the pesticide effect on soil microorganisms.

References	Substance	Type	Study	Comments
Wang et al. (2020)	Azoxystrobin	Fungicide	Lab	Significant dose dependent impact of the substance on the bacterial community diversity and changes in its composition following exposure to specialized degraders.
Farthing et al. (2020)	Glyphosate and imidazolinone	Herbicide	Field	Transient significant and overall little impact following treatments. Changes mostly attributed to the field properties.
Fang et al. (2020)	Chloropicrin, dazomet, dimethyl disulfide, allyl isothiocyanate and 1,3-dichloropropene	Fumigant	Lab	Transient significant changes with initial diversity decline and brief stimulation. Fumigant type dependent various responses from soil bacterial community and its denitrifiers.
Zhang D. L. et al. (2019)	1,3-dichloropropene	Fumigant	Field	Bacterial community composition remained unaffected by 1,3-D fumigation whereas its impact was detrimental to biodiversity of bacteria, AOA-amoA and AOB-amoA genes.
Zhang C. et al. (2019)	Pyraclostrobin	Fungicide	Lab	Significant impact in the abundances of genera with important roles in soil fertility and pollutant biodegradation.
Tang et al. (2019)	Glufosinate (glyphosate)	Herbicide	Lab	Some variations in bacterial diversity of rhizosphere caused only by plant growth stages. These changes were not attributed to treatments.
Mallet et al. (2019)	Leptospermone	Herbicide	Lab	Significant shifts in community structure and diversity in fungal communities of soils caused by natural weed killer leptospermone. Recovery was only possible for the soil in which indigenous fungal community prior to experiments was already diverse and rich.
Hu et al. (2019)	Fomesafen	Herbicide	Field	A dose dependent effect impact on diversity. Long-lasting significant impact on the soil microbial community and changes toward specialized microorganisms that are able to degrade Fomesafen.
Gallego et al. (2019)	Oxamyl	Nematicide	Field	Increased abundance of the specialized fraction and transient changes in the composition total bacterial community.
Feng et al. (2019)	Lindane	Insecticide	Field	Bacteria were than fungi but stable community structure exhibited in the hybrid rice under lindane stress.
Storck et al. (2018)	Chlorpyrifos, isoproturon, and tebuconazole	Insecticide; herbicide; fungicide	Field	Diversity and composition varied over time more in mesocosms than field. Overall, all pesticides referred as low-risk.
Jeffries et al. (2018)	Chlorpyrifos	Insecticide	Field	Legacy effect after 13 years, community that is able to adapt and degrade OP is still reflected.
Zhang S. T. et al. (2017)	Chloropicrin	Fumigant	Field	Richness and diversity after 3 years continuous fumigation were the lowest. Increase of fumigation years reduced the incidence of bacterial wilt.
Jiang et al. (2017)	Acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide)	Herbicide	Field	No significant impact on soil microbial community composition after 9 years of treatments. Changes between 8th and 9th year were found to be not related to herbicide but to a seasonality.
Chen et al. (2017)	Paichongding neonicotinoid	Insecticide	Lab	Significant, both positive and negative, soil type dependent impact on soil bacterial community. Diversity was found to be gradually increasing in control group while it was decreasing in the IPP group. The inoculation of an IPP degrading strain positively affected the microbial species diversity in contaminated soil.
Panelli et al. (2017)	Farming systems analysis	Various	Field	Fungal and bacterial community alterations caused by various treatments, exact comparison of two systems were not possible.

altogether. The authors found that the α -diversity of soil bacteria varied more in microcosms as compared to fields. In the field conditions, significant differences in OTUs (operational taxonomic units) were observed for all pesticides by the end of a 70-day-long experiment. Pesticides, regardless of doses implemented, failed to have an impact on the α -diversity indices analyzed in this study. Similarly, also β -diversity was not affected by any of the pesticides nor the doses as the composition of soil bacterial community did not change significantly over time. However, the insecticide chlorpyrifos caused a slight but significant temporary impact at the microcosm level, whereas the fungicide tebuconazole caused a slight but significant temporary impact at the field level only. The herbicide isoproturon, on the other hand, did not cause significant changes in the β -diversity of soil bacteria neither in microcosms nor in the field. These

small and transient changes were mainly attributed to various degradation dynamics and transformation products of pesticides. However, as the β -diversity of soil bacteria eventually recovered, all of the pesticides were conclusively referred to as low-risk pesticides.

Fungicides

Wang et al. (2020) assessed the impact of the fungicide azoxystrobin on soil microbial communities, revealing a significant dose-dependent impact. Particularly, the OTUs richness and biodiversity, according to the Shannon index, decreased. Furthermore, *Streptomyces* and *Sphingomonas* were found to be dominating genera in most of the treated soils. This particular result was attributed to the use of the pesticide as a carbon source for growth because both *Streptomyces*

and *Sphingomonas* hold a potential as bioremediation agents in the soils contaminated with pesticides. Furthermore, in a study carried out by Zhang C. et al. (2019), it was found that a commonly used fungicide pyraclostrobin also caused a significant impact in the abundances of genera with important roles in soil fertility and pollutant biodegradation. However, this time in contrast to azoxystrobin, the fungicide pyraclostrobin decreased the relative abundance of the *Sphingomonas* genus regardless of exposure levels. *Cupriavidus*, *Methylobacillus*, and *Methylophilus*, genera that include degraders of pollutants and methane oxidators with some roles in salt stress tolerance and denitrification were also affected negatively.

Insecticides

Feng et al. (2019) studied the impact of lindane on root-associated microbiomes of rice and found that root-associated bacteria were more sensitive to the presence of this insecticide as compared to fungi. The α -diversity analyzed through the Shannon index revealed that the higher insecticide levels had significantly decreased the bacterial and fungal diversity of both rhizosphere and bulk soils. It was also found that regardless of rice cultivars used, lindane significantly decreased the α -diversity also in the endosphere. Furthermore, their results showed that bacteria and fungi were both affected by lindane, respectively, in the phylum and class levels. Chloroflexi, Proteobacteria, and Actinobacteria were found to be dominant phyla, while contrasting responses to lindane from Acidobacteria and Firmicutes were obtained. In the case of fungal community, Basidiomycota and Ascomycota were found to be dominant phyla with several classes of both dominating most of the rhizosphere. However, lindane at various doses did not significantly change the composition of the fungal community.

The longest-term field study considered in this review was that of Jeffries et al. (2018), in which the legacy effect of the Chlorpyrifos insecticide was revealed. It was found that the degradation of pesticide was maintained even after its discontinued use 13 years ago, and the soil microbial community was able to adapt and degrade the pesticide but was still reflected in the latest samplings. Authors found that α -diversity was negatively related to degradation rates, which explained the slower degradation of chlorpyrifos in highly diverse soils. Microbial abundance, on the other hand, together with metabolic function abundances, was found to be positively related to degradation. Results related to a higher abundance of both microbes and pathway mechanisms employed by these microbes were further explained by taxonomic analysis and degradation assays.

Furthermore, Chen et al. (2017) investigated the impact of a novel neonicotinoid Paichongding (IPP) on soil bacterial community in a study in which pyrosequencing was used as one of the early studies of NGS in the present review. Degradation of IPP and its four variants were also investigated. A significant impact of IPP in this study on soil bacterial community, both positive and negative, was found to be closely connected to soil type. During the course of the experiment, α -diversity was found to be gradually increasing in the control group while it was decreasing in the IPP group. This contrast

eventually caused significant differences between control and IPP-treated soils. Chen et al. (2017) further investigated the impact of the inoculation of an IPP degrading strain into soil in their experiment and found that the inclusion of IPP degrading strain positively affected the microbial species diversity in contaminated soil. Furthermore, IPP also caused significant changes in the composition of the microbial communities after spraying. Differences were found both at the phylum and genus levels in both inoculated and non-inoculated soils due to accumulation of IPP metabolites.

Herbicides

Farthing et al. (2020) found that commonly used weed suppression techniques of repeated glyphosate application, repeated glyphosate application + imidazolinone herbicide use, and repeated glyphosate application + mechanical above-ground biomass removal had only little impact on bacteria and archaea. In particular, none of these weed-suppressing techniques had a significant impact over controls in terms of α -diversity indexes (species richness and Shannon diversity). The only significant difference was observed between communities of different locations, but this difference was found to be related only to the field differences rather than treatment impact. Although overall changes and compositions generally suggested the absence of overlap following treatments with herbicides, Farthing et al. (2020) found that changes in a number of OTUs were quite similar regardless of the experimental site. This phenomenon was still observed a year following the treatment. The authors also highlighted the necessity of different field site inclusions in the trials, as various responses eventually led to minimal changes that were attributed to pre-existing local microbial communities of soils in these fields.

Tang et al. (2019) investigated the use of glyphosate in the cultivation of transgenic herbicide-resistant plants and found that there was no significant impact on the diversity and structures of rhizosphere bacterial communities in one growing season. The differences in rhizosphere bacteria were only caused by plant growth stages. These results indicated that the growth stage was the most important factor influencing rhizosphere bacterial communities' diversity, in contrast to the hypothesized influence of cultivar and herbicide application.

Mallet et al. (2019) studied the impact of leptospermone, a natural b-triketone weed killer, on the fungal community in a microcosms study. It was found that leptospermone caused significant shifts in community structure and diversity in fungal communities of soils used in their experiment. Starting from the beginning of the experiments, significant differences were found in the α -diversity of the fungal community according to Chao1, Shannon, and Simpson between soil types and controls. During the experiments, the impact of herbicide treatment resulted in further significant differences both between soil types and controls. Authors found significantly decreased observed richness even after only 4 days together with changes in fungal community composition in one of the soils used. Even though the impact of the herbicide on α -diversity was different on different soils used in this experiment, fungal community β -diversity changed regardless of soil type. Mallet et al. (2019) also showed

that even after the complete degradation of the used herbicide, the recovery of the fungal community was possible. However, recovery was only possible for the soil in which indigenous fungal community prior to experiments was already diverse and rich. The findings indicate how important it is to assess the impact of the various soil types not only from the physicochemical point of view but also from the indigenous community point of view for their resilience.

Fomesafen, a diphenyl ether broadleaf weed killer widely used in soybeans and other legumes, was tested for its impact on the microbial community composition of rhizosphere bacteria by Hu et al. (2019). Depending on the dose of its application, fomesafen had an impact on both α and β diversity. Particularly for α -diversity, according to Shannon index, negative impacts on rhizosphere bacteria were proportional to the application dose, regardless of sampling times. This impact was related to the direct toxicity of fomesafen and competitive changes adapted by some taxa in community. The authors concluded that functional impacts were long-lasting on the soil microbial community despite the rapid degradation rate of this herbicide in the rhizosphere. Finally, Jiang et al. (2017) revealed that the long-term application of acetochlor (2-chloro-N-(ethoxymethyl)-N-(2-ethyl-6-methylphenyl) acetamide) had no significant impact on overall soil microbial community composition after 9 years of treatment. Particularly for α -diversity, observed species, the Chao1, Shannon, and Simpson indexes indicated various alternation between manual weeding and pesticides. This result eventually interpreted as a reduction on the biodiversity of soil bacteria caused by manual weeding was more significant than the one caused by herbicide application.

Fumigants

The impact of fumigants chloropicrin (CP), dazomet (DZ), dimethyl disulfide (DMDS), allyl isothiocyanate (AITC), and 1,3-D on the total bacterial community and on denitrifiers were studied by Fang et al. (2020). According to the diversity indexes of Shannon, Chao1, and ACE, the α -diversity of soil bacteria significantly decreased for the whole experiment following the fumigation with CP. However, 24 days after fumigation, diversity in the soils of the remaining fumigants were found to be significantly higher than the controls. Later, sampling revealed that biodiversity of the bacterial community in the soils of DZ, DMDS, AITC, and 1,3-D eventually were not significantly different from controls. The stimulation effect of this fumigant, therefore, remained only as a short-term and transient one. Fang et al. (2020) also found that although initially affected negatively by fumigation, denitrifiers eventually recovered. Relative abundances eventually increased significantly in relation with stimulated microbial denitrification caused by fumigation of soil.

Zhang D. L. et al. (2019) also investigated the impact of 1,3-D fumigation on soil bacterial community. The authors also paid particular attention to the abundance of ammonia oxidation genes of the bacterial community in an attempt to evaluate again the unintended impact that a fumigant could have on soil fertility. It was found that soil fumigation with 1,3-D reduced the abundance of total bacteria and AOA-amoA and AOB-amoA

genes. It was found that 1,3-D fumigation significantly reduced the total number of species according to the Ace and Chao diversity indexes. The bacterial community recovered from this reduction as the experiment continued.

Finally, in a multi-year field study by Zhang S. T. et al. (2017), the impact of chloropicrin fumigation on the bacterial community of soil was investigated for a 3-year-long continuous fumigation. Continuous fumigation for 3 consecutive years was found to be detrimental for the soil bacterial community in terms of microbial richness and diversity. In detail, α -diversity, as measured by the Chao and Shannon Index, was found to be significantly lower after 3 years of fumigation when compared to control and year-long fumigation. Although slightly to a lesser extent than 3 years, fumigation after 1 year was still detrimental to both microbiota and species richness. Differences were further investigated in the phyla and genus levels to evaluate the impact the duration of fumigation had on the bacterial community. Differences between a year-long and 3-year-long fumigation significantly increased only at the genus level. Authors further implemented the least discriminant effect size analysis to be able to identify the main phylotypes behind the differences obtained. It was found that Nitrospirae and Saccharibacteria were two most prominent phyla in, respectively, no-fumigation and 3-year-long fumigation samples. However, identifying a biomarker for a year-long fumigation at the phylum level was not possible. Therefore, the authors identified *Nocardiopsis* at the genus level as a biomarker for a year-long fumigation sample. Authors also found that increased fumigation years reduced the incidences of bacterial wilt disease.

Nematicides

The study of Gallego et al. (2019) was the only one dealing with the impact of a nematicide on soil bacteria: the molecule studied was oxamyl, and particular attention was also devoted to degradation kinetics. Soil bacterial community was not significantly affected by oxamyl in terms of α - and β -diversity. However, the abundance of a specialized fraction of oxamyl degraders increased in agreement with mineralization of the nematicide. The nematicide use also increased the abundance of the specialized fraction of the soil bacterial community carrying the *cehA* gene, which means that oxamyl induced changes in the abundance of oxamyl-degrading microorganisms.

Comet Assay

Detection of DNA damage and its extent is of paramount importance in different fields of basic and applied medical and health sciences, including environmental studies for verifying the toxicity of xenobiotics or other chemicals released in the environment. Among the different methods developed to quantify DNA damage, the single-cell electrophoresis or comet assay is prominent, being a simple, rapid, and sensitive method for measuring DNA breaks in small numbers of cells (Glei et al., 2016). When standardized and validated, the comet assay can provide valuable information for hazard identification and risk assessment of environmental exposure to environmental pollution in humans, in sentinel organisms, or *in vitro* toxicity studies (de Lapuente et al., 2015).

The method was originally developed in the 80s by Ostling and Johanson (1984) who used the technique to quantify DNA damage in mammalian cells exposed to gamma rays. The technique was further improved by Singh et al. (1988), who developed an alkaline version of the assay, the most commonly used, that enables the detection of alkali labile sites in addition to single- and double-strand breaks visualized using the original neutral version. The assay involves very limited processing steps, not requiring DNA isolation and purification. On the contrary, cells are directly embedded in a low melting agarose matrix at temperatures compatible with cellular viability and stratified on a microscopy slide. After gel solidification microgels on slides are subjected to a lysis step in a saline solution containing detergents that promote cellular membrane degradation and protein precipitation leaving on the slide nucleoids constituted simply by DNA. Subsequently, slides are transferred into an electrophoretic chamber, and DNA is allowed to unwind in electrophoresis buffer, that in the version developed by Singh is at pH > 13, and electrophoresed in the same buffer. Finally, cells are neutralized and either stained with DNA intercalating dyes, mainly fluorescent such as DAPI, ethidium bromide or SYBR Gold and analyzed directly or dehydrated for further analysis. Analysis is conducted using a fluorescent microscope; the image of the comet produced by electrophoresis is represented by a head of intact DNA and a tail of damaged DNA streaming away from it in the direction of electrophoresis. Classification of comet according to their DNA damage can be either done using visual scores but more commonly is supported by an image analysis software that is capable of extrapolating numerical indexes of damage, the most common of them being the length (Tail Length) of the comet and the percentage of DNA present in the comet (Tail Intensity%). A third index of reference widely used is a product of the previously mentioned indexes and is known as Tail Moment. New promising techniques in the classification of comet cells is provided by artificial intelligence algorithms applied to image analysis that will improve and accelerate the classification process in the near future (Bernardini et al., 2019). Despite its popularity, the comet assay still has some shortcomings mainly due to a high inter-operator as well as inter-laboratory variability and the limited use of calibrators. Indeed, although the process is very straightforward, limited variations in each one of the methodological steps described may complicate the comparison of data from different laboratories, in particular when the starting material is not constituted by cultured cell lines as in the case of reference organisms used in the ecotoxicological assessment. One of the purposes of the present review is to summarize not only the application but also some critical methodological details in order to describe the most suitable methodological approaches in line with the paper published in the past 5 years in the field of soil environmental ecotoxicology.

The analysis of the relevant articles published in the last years on the effects of pesticides upon the soil earthworms, determined through comet assay, is summarized in **Table 3**.

Among the techniques developed to assess DNA damage, the comet assay has received remarkable interest for its easy and cost-effective implementation. Although it was originally designed mainly for human and cultured cells, ecotoxicological

applications widened the analysis to a broad set of invertebrate species. Within ecotoxicological applications, the technique was primarily used for genotoxicity assessment in marine and freshwater invertebrates, and subsequently, it was extended to terrestrial invertebrates. Soil invertebrates are known to be efficient accumulators of xenobiotics and to respond to their exposure in a sensitive and measurable manner, hence their popular use as bioindicators of soil contamination. The comet assay has been applied to various annelids including polychaetes, oligochaetes, and leeches, although the majority of studies were carried out on selected species of earthworms (*Eisenia* spp.) that are recognized models to assess soil quality and environmental impacts of cropping systems and pollutants. In fact, among soil organisms, earthworms deserve particular interest because of their ecological role in soil biocenosis representing 60–80% of the total biomass; by ingesting soil particles, they represent extremely pertinent bio-indicators (Sanchez-Hernandez, 2006).

Sub-lethal markers such as DNA damage are pivotal to identify potential environmental risks, taking into account the chronicity of exposure of non-target organisms to pollutants widely used in agricultural practices (e.g., insecticides, fungicides, and herbicides) (Di Marzio et al., 2005).

The bioavailability of pesticides in soils is affected by the amount and quality of pesticide-absorbing soil colloids and microbial growth and activity, which results in a different extent of pesticide biodegradation (Castillo et al., 2008; Katagi, 2013).

According to the relevance of earthworms in soil ecotoxicology studies, in the present review, we revised the application of the comet assay in the investigation of environmental pesticide sub-lethal effects in these non-target species. The literature of the last 5 years on the topic consists of 16 articles: only one among them was conducted in field conditions related to the effects of persistent organic pollutants (POPs), including those introduced as pesticides in the environment (Espinosa-Reyes et al., 2019). In particular, in this study, genotoxicity was tested in wild earthworms in soils at different levels of urbanization (industrial, urban, and rural areas). The analysis unambiguously identified the highest concentration of POPs and the highest rate of DNA damage in industrial areas. Urban and rural areas had a different composition of POPs with the more prominent influence of agrochemistry in rural areas; however, the latter seemed to have a lower impact, compared to urban organic pollutants in triggering DNA damage in earthworm coelomocytes.

With the exception of this paper, all other screened articles were conducted in standardized laboratory conditions through the application of OECD guidelines. Moreover, these studies did not use wild earthworms; on the contrary, they mainly used as a reference organism *E. fetida*, with the exception of three articles (Fouché et al., 2016; Chevillot et al., 2017; Mincarelli et al., 2019) that analyzed the effects of pesticides on the other commonly used earthworm reference species *Eisenia andrei*. As reported in **Table 3**, most of the studies used three organisms as an experimental unit for experimental conditions, and exceptionally few studies increased the observational sample to up to 10 or 18 samples (Wang et al., 2015; Chen et al., 2018; Espinosa-Reyes et al., 2019). In all studies, DNA damage assessment was

TABLE 3 | Summary of the relevant articles on the application of comet assay to the pesticide effect on soil earthworms.

References	Substance	Type	Study	Organism	Comment
Ma et al. (2019)	Pyraclostrobin	Fungicide	Lab	<i>E. fetida</i>	DNA damage increases at 7 and 14 days.
Espinosa-Reyes et al. (2019)	Persistent organic pollutants	"Pesticide"	Field	Not specified	DNA damage correlated to POP accumulation in soil.
Qiao et al. (2019)	Cyantraniliprole	Insecticide	Lab	<i>E. fetida</i>	Increase oxidative stress damage alters antioxidant cellular status inducing DNA damage.
Zhang et al. (2018)	Fluoxastrobin	Fungicide	Lab	<i>E. fetida</i>	Oxidative stress parameters correlated to DNA damage.
Li B. et al. (2018)	Acetamiprid	Insecticide	Lab	<i>E. fetida</i>	Maximum of DNA damage after 14 days. Recovery phase started after 21 days.
Chen et al. (2018)	Tribenuron methyl Tebuconazole	Herbicide and fungicide	Lab	<i>E. fetida</i>	Not significance changes in OTM and TL at all pesticides concentrations alone and combined.
Li X. et al. (2018)	Mesotrione	Herbicide	Lab	<i>E. fetida</i>	Maximal OTM values at highest dose at day 28.
Chevillat et al. (2017)	Neonicotinoids	Insecticide	Lab	<i>E. Andrei</i>	The low NEOs concentrations were not lethal but induce significant increase in class 4 (extremely DNA damage).
Duan et al. (2017)	Polychlorinated biphenyls	Pesticide	Lab	<i>E. fetida</i>	PCB treatment increase DNA damage in both type of soil, even at lowest concentration tested.
Wang et al. (2016)	Imidacloprid	Insecticide	Lab	<i>E. fetida</i>	OTM and tail DNA% increased at 7 days increasing doses. At 21 and 28 days DNA cross- links were observed.
Fouché et al. (2016)	Biofumigants, glucosinolates	Natural toxins	Lab	<i>E. Andrei</i>	Broccoli and oilseed radish DNA damage level similar to ctrl. Only Mustard significantly different to ctrl.
Shen et al. (2015)	Deltamethrin	Pesticide	Lab	<i>E. fetida</i>	Nickel produces more DNA damage compared to Deltamethrin. Synergistic effect in increasing DNA damage.
Zhang et al. (2015)	Spirotetramat	Insecticide	Lab	<i>E. fetida</i>	Extent of DNA damage reveal 90% worms have low DNA damage at 0.25 mg/kg.
Feng et al. (2015)	Thiacloprid	Insecticide	Lab	<i>E. fetida</i>	1 and 3 mg/kg reached higher DNA damage at 28 and 35 days.
Mincarelli et al. (2019)	Copper sulfide	Pesticide	Lab	<i>E. andrei</i>	DNA damage at concentrations lower than those found in most agricultural soils worldwide after 9 days of exposure.
Wang et al. (2015)	Guadipyr	Insecticide	Lab	<i>E. fetida</i>	Guadipyr has no effect on OTM, TM, Tail DNA%.

conducted on coelomocytes – hemocytes of the annelids that can be recovered from the coelomic fluid with non-invasive techniques by submerging the organism in ethanol 5% saline solution that might be integrated with the chelating agent EDTA and the mucolytic agent guaiaicol or similar. The studies explored the potential genotoxic effect of a broad set of chemicals including as insecticides Cyantraniliprole (Qiao et al., 2019), Acetamiprid (Li B. et al., 2018), Neonicotinoids (Chevillat et al., 2017), Imidacloprid (Wang et al., 2016), Spirotetramat (Zhang et al., 2015), Thiacloprid (Feng et al., 2015), and Guadipyr (Wang et al., 2015) that represented the most numerous, followed by fungicides Pyraclostrobin (Ma et al., 2019), Fluoxastrobin (Zhang et al., 2018), Tebuconazole (Chen et al., 2018), and a smaller set referred to herbicides Tribenuron (Chen et al., 2018) and Mesotrione (Li X. et al., 2018) or general pesticides including Polychlorinated biphenyls (Duan et al., 2017) and natural toxins used as biofumigants (Fouché et al., 2016) and copper sulfide (Mincarelli et al., 2019) that is one of the few chemical treatments allowed in organic farming. In all studies, synthetic compounds were studied at increasing doses of exposure normally below 10 mg/kg of soil, with the exception of the insecticide Guadipyr (Wang et al., 2015) that was used between 10 and 100 mg/kg of soil. Furthermore, typically, DNA damage was evaluated at specific time points, most commonly once a week up to 1 month. Studies by Shen et al. (2015) and Feng et al. (2015) reported in **Table 2** investigated the effect of pollutant exposure up to 6 weeks or 2 months. Interestingly in both studies, it was observed

that prolonged exposure led to a significant decrease in the rate of DNA damage highlighting a potential adaptation to the environmental stress that is compatible with the ecology of *Eisenia* spp. that is quite resistant and therefore also useful as a sentinel organism for sub-lethal toxicity.

Most of the studies screened used as a reference index Olive Tail Moment (OTM), which is a widely used index of DNA damage that essentially represents the product of the percentage of total DNA in the tail and the distance between the centers of the mass of head and tail regions [Olive moment = (tail mean-head mean) × % of DNA in the tail]. This is quite surprising considering that tail intensity DNA% has been mainly used in recent investigations due to its robustness (Collins, 2004; Kumaravel and Jha, 2006).

DNA damage was often related to markers of oxidative stress that exhibited a similar trend in response to pollutants. This is central in describing the mechanism of genotoxicity of pesticides that often is mediated by an increase in cellular reactive oxygen species formation due to cellular impairment (Zhang et al., 2015, 2018; Duan et al., 2017; Li B. et al., 2018; Li X. et al., 2018; Qiao et al., 2019). Interestingly despite the different nature and target of the pesticides tested, a common pattern of response is often evincible in the biological response of *Eisenia* spp. with a dose-dependent response to the chemical at each time point analyzed; however, when they are observed on a longitudinal time scale during the typical month of exposure (28 days) at the lowest dose of chemicals used, often a decrease in DNA damage

is observed from the second week of exposure highlighting an adaptive response; at intermediate dosage, DNA damage tends to stabilize during the last 2 weeks of exposure while at the highest concentration DNA damage is able to increase in comparison to the previous time points even after 1 month of exposure (Li X. et al., 2018; Zhang et al., 2018; Ma et al., 2019). Mincarelli et al. (2019) also showed an adaptive response at the level of gene expression of metallothionein as well as proteins involved in the immunological response (antimicrobial peptide fetidin and toll-like receptors).

The decrease in the extent of DNA damage might be the result of an adaptive response to low doses of chemicals but is also known to be potentially due to the artifactual decrease in the mobility of damaged DNA associated to the DNA-DNA or DNA-protein crosslink. This seemed to be the case of the insecticide Imidacloprid (Wang et al., 2016) that differs from the other pesticides that showed a dose-dependent decrease of DNA damage already after 1 week of exposure despite a significant increase in markers of oxidative stress and damage. Also, the data relative to the genotoxicity of the neonicotinoid insecticide Guadipyr presented by Wang et al. (2015) are in antithesis with other reports showing very low toxicity in general, and also at the DNA integrity level, following exposure at a concentration 10 times higher compared to other pesticides considered in this review. The compatibility of the high doses of chemicals nominally used with the reproduction and viability of earthworms seems to suggest that Guadipyr is remarkably safer compared to other insecticides; however, the lack of positive control of toxicity in the same set of experiments and bioaccumulation data at the organism level might suggest the need for further investigation to better classify the toxicity of this compound.

Finally, a particular mention should be devoted to innovative approaches of the use of the comet assay among the selected articles. Chevillot et al. (2017) studied the biological exposure of earthworms to complex chemical mixture exposure that mimics environmental complex matrixes. Despite the exciting experimental design, however, the section on DNA damage is quite limited and indicates a slight but significant increase in DNA damage of earthworm coelomocytes following exposure to neonicotinoids.

Another application of the comet assay for ecotoxicological soil studies is to investigate the use of particular amendments, such as organic material, and verify whether they could affect the pesticide concentrations and its toxic potential. The study by Shen et al. (2015) developed along this line demonstrating that humic acid alleviated the damage to DNA, proteins, and lipid membranes caused by nickel and deltamethrin spiked in the soil.

Despite the shortcomings mainly associated with operator dependent and interlaboratory variability, the comet assay remains an elective technique for assessing genotoxicity in environmental biomonitoring, being mature after decades of implementation and widely used worldwide for its direct, simple, and affordable implementation. Nonetheless, in order to promote the consistency and reliability of results, regulatory bodies should provide guidelines on the optimal use of this tool, strongly indicating the most reliable indicators of DNA damage. In this

respect, most of the articles reported in the review use OTM, while in the last decades, many evidences seem to highlight DNA tail intensity% as the most informative index characterized by a broader dynamic range. Moreover, the use of computer-aided image analysis software rather than visual scoring should be strongly suggested as well as the use of appropriate reference of damage such as negative and positive controls treated with DNA damaging agents or gamma radiation, ideally at least at 2 doses. Reference cells should be used as standard and to verify the reproducibility of the techniques and do not necessarily have to be of the same type of analytical samples; indeed an optimal reproducibility could be achieved with cultured cells.

CONCLUSION

The examination of the published articles showed a continuous improvement of the possibility to detect the detrimental effect of the pesticides on soil microorganisms and non-target organisms at a sub-lethal level. In the present review, we considered the methodological aspects considering the most promising DNA-based methodologies used to generate data for biodiversity and biomonitoring studies; moreover, we described the specific outcomes of research papers using these techniques in describing the effect of pesticides on soil. The critical analysis of the papers examined can help registration authorities to derive ecotoxicological parameters useful for a high-tier risk assessment. Regarding microbial communities, soil microorganisms play key roles in significant ecological processes such as bioremediation, recycling of elements, soil structure establishment, and degradation of organic matter and chemical xenobiotics (Umar et al., 2017; Walvekar et al., 2017). Among them, pesticides are the most common contaminants in the agricultural field (Wołejko et al., 2020), and their persistence in the soil can alter the physico-chemical structure and microbiota composition causing a negative impact on soil biodiversity (Diez et al., 2017). Indeed, the extensive use of pesticides has gradually led to soil contamination with proven damages to environmental health (Fernandes et al., 2020; Kafaei et al., 2020). Reported data using DGGE-PCR studies confirmed that the chemical nature and the doses used play a role in the disturbance of microbiological complexity of the soil.

The use of biopesticides, differently from synthetic ones, does not affect the microbial community reversibly (Reali and Fiuza, 2016; Umar et al., 2017). In this respect, several studies have shown that the pesticide-induced microbial changes are mainly transient and the structure of the microbial population can improve gradually (Gao et al., 2012; Chen et al., 2013). However, this advantage is lost at high concentrations. Gupta et al. (2013) found that the treatments of rhizosphere with high doses of biopesticide azadirachtin had an adverse effect on the microbial population. They were suggesting that concerning the environmental impact, the lower toxicity of biopesticide is lost at high concentrations.

Regarding synthetic pesticide impact on microbial soil, some PCR-DGGE studies suggest that also in this case in the long term, they seem not to affect the rhizosphere population negatively.

For example, Coppola et al. (2011); Marinozzi et al. (2013), and more recently Diez et al. (2017) show that the acute soil population modification is reversible since over a short time the soil community was able to return to the initial composition as reported by Tortella et al. (2013b).

However, these studies are in contrast with previous evidence (Hoshino and Matsumoto, 2007; Chu et al., 2008; Huang et al., 2016; Wang et al., 2019) showing that synthetic pesticides significantly affect the abundance of soil microorganisms, causing irreparable damage. The evidence shows the ecological toxicity of several pesticides such as metalaxil and chloropicrin on the fungal community (Hoshino and Matsumoto, 2007; Wang et al., 2019).

Further studies using novel high-throughput techniques such as NGS has shown that in laboratory experiments the majority of the pesticides used had a significant impact on the microbial communities with the sole exception of the observations by Tang et al. (2019) in which the changes were attributed to plant growth stages rather than the Glyphosate. In the field experiments, on the other hand, significant changes were mostly depending on the dose, period, and frequency of application, and the characteristics of the soil, including its indigenous microbial community properties. It is therefore not possible to pinpoint exactly one type of pesticide or substrate as the source of major impacts. Several studies indicated that the rapid shift of communities in favor of pesticide-degrading groups already presents in the microbial community. The presence and activity of pesticide-degrading groups before the application of tested pesticides may be considered as preliminary indicators of synergies among molecules and of the possible outcome of pesticide application. Pesticides used had relatively lower impact, as expected, in the experiments where soil microbial communities were already highly diverse and rich from the beginning. Several studies cited in this work underlined the importance of carrying out various samplings throughout the cropping season. This was because changes in microbial composition happen during the growing season via plant-microbes interaction, and some transient changes may also be explained by the presence of the crops rather than pesticides. Furthermore, the replication of the experiments in different fields is also necessary in order to comprehend the real impact of pesticide(s), as different fields have different physical characteristics and microbial diversity. These factors can indeed affect the response and recovery of the microbial communities. Using the comet assay, the reported study analyzed the toxicity of pesticides in non-target organisms, in this case, referring to soil macroinvertebrates, in terms of DNA damage. Remarkably the main effect on soil biocenosis is also confirmed in this studies that support a high level of adaptation and overtime to the pesticides, suggesting that dose and time of observation are critical aspects to take into consideration in environmental risk assessment. Although this

aspect may seem quite obvious, this consideration should be addressed in particular in relation to natural compounds that have limited constraint in terms of dosage to be used, whereas the discussed data suggests that their toxicity at high concentrations should be carefully evaluated. In the light of the above cited findings, we can suggest that the way forward for studies on the impact of pesticides on soil ecosystem lies in the inclusion of more than one soil type/field in the studies; samplings at various intervals during the experiment in order to better assess the shifts and recovery of the microbial community; and coupling the overall biodiversity and functionality with that of key microorganisms of agricultural and soil fertility relevance such as denitrifiers, saprophytic fungi, and archaea. The inclusion of these factors into studies would, therefore, pave the way to understanding that also the unintended impact of a secondary nature on the terrestrial ecosystems besides the main purpose of the pesticide should be considered, which is a very important point for both regulatory authorities and researchers. DNA-based methodological tools provide a robust and efficient technique for the evaluation of microbiological biodiversity as well as early biomarkers of genomic damage in soil organisms that represent an early reporter of stress and potential risk for biota survival and reproduction. The major advantage of these techniques is related to the uniformity of protocols and high-throughput capabilities that enable rapid and comprehensive assessment, addressing major challenges in ecological risk assessment (i.e., provide information at higher levels of biological organization moving from individual organisms to communities and population) and the ability to consider multiple stressors and context dependencies, allowing recovery analysis. On the other hand, at the interlaboratory level, care should be taken in order to standardize the methodologies and gain reproducible results using appropriate reference markers. Moreover, information should be handed in an integrated manner with other biological and environmental indicators using multivariate predictive models and multimetric approaches.

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

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From Microbiome to Traits: Designing Synthetic Microbial Communities for Improved Crop Resiliency

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Plants teem with microorganisms, whose tremendous diversity and role in plant–microbe interactions are being increasingly explored. Microbial communities create a functional bond with their hosts and express beneficial traits capable of enhancing plant performance. Therefore, a significant task of microbiome research has been identifying novel beneficial microbial traits that can contribute to crop productivity, particularly under adverse environmental conditions. However, although knowledge has exponentially accumulated in recent years, few novel methods regarding the process of designing inoculants for agriculture have been presented. A recently introduced approach is the use of synthetic microbial communities (SynComs), which involves applying concepts from both microbial ecology and genetics to design inoculants. Here, we discuss how to translate this rationale for delivering stable and effective inoculants for agriculture by tailoring SynComs with microorganisms possessing traits for robust colonization, prevalence throughout plant development and specific beneficial functions for plants. Computational methods, including machine learning and artificial intelligence, will leverage the approaches of screening and identifying beneficial microbes while improving the process of determining the best combination of microbes for a desired plant phenotype. We focus on recent advances that deepen our knowledge of plant–microbe interactions and critically discuss the prospect of using microbes to create SynComs capable of enhancing crop resiliency against stressful conditions.

Keywords: synthetic microbial community (SynCom), plant microbiome, inoculants, metagenomics, plant growth-promoting (PGP)

INTRODUCTION

In recent years, significant steps have been taken towards understanding many facets of the plant microbiome. With advances in sequencing technologies and analytical tools, we have learned that a functionally diverse microbiota is recruited from the environment and assembled into a defined structure that is dependent on soil type, host genotype and environmental changes (Walters et al., 2018; Xu L. et al., 2018; Liu et al., 2019; Zhang et al., 2019). These studies profoundly affected our

perception of the complexity and dynamics of plant–microbe interactions. More importantly, they allowed the establishment of a link between microbial diversity and plant traits, such as resiliency to biotic and abiotic stresses (Gómez Expósito et al., 2017; Jacoby et al., 2017; Lemanceau et al., 2017; Compant et al., 2019). However, while the field of plant microbiome research has rapidly evolved, few if any of these novel concepts have been considered in the selection of beneficial microbes for agricultural applications.

Conventional inoculants used in current agricultural practices are generally composed of a single strain isolated by *in vitro* screening assays for plant growth-promoting (PGP) activities or inoculation experiments under controlled conditions. Despite being broadly adopted, these strategies fail to capture important aspects of plant–microbe interactions. Recent studies have shown that the plant microbiome is composed of a highly diverse and complex community, often sustained by multiple interactions between microbes and their host. Moreover, the beneficial effects of the microbiota are frequently described as being provided by synergistic interactions between microbes (Timm et al., 2016; Niu et al., 2017).

More recently, synthetic microbial communities (SynComs) have received a great deal of interest. SynComs are small consortia of microorganisms designed to mimic, at some scale, the observed function and structure of the microbiome in natural conditions. The rationale is to reduce the complexity of the microbial community while still preserving some of the original interactions between microbes and hosts, providing a repertoire of functions that would not be achievable by a single microbe (Qin et al., 2016; Niu et al., 2017; Vorholt et al., 2017; Kaminsky et al., 2019). In addition to broadening the scope of features and metabolites, SynComs may also increase community stability through synergistic interactions between their members (McCarty and Ledesma-Amaro, 2019). Notably, the major advantage of SynComs lies in the possibility of tailoring communities using concepts from microbial ecology and genetics with defined and predictable traits. In this sense, the concept of SynComs can be expanded to include the rationale of designing communities that incorporate a desired set of microbial traits for agriculture.

Tailoring SynComs has become a valuable approach for uncovering plant–microbe interactions. By adding, removing or replacing microorganisms in a SynCom formulation, the role of each microbial member can be further investigated, as well as the factors governing community assembly (Vorholt et al., 2017). In maize, for instance, removal of a single strain of *Enterobacter cloacae* dismantled a microbial community capable of reducing the severity of blight disease (Niu et al., 2017). In another example, by comparing *indica* and *japonica* rice varieties, Zhang et al. (2019) observed that the recruitment of a larger proportion of nitrogen cycle-related bacteria in *indica* was associated with NRT1.1B, a plant nitrate transporter. A SynCom containing *indica*-enriched microorganisms had a greater effect on rice growth than a *japonica*-enriched SynCom. These studies highlight that factors governing microbial community assembly should be considered when designing inoculants for agricultural applications.

Here, we argue in favor of using the SynCom concept to create consortia of microbes that can enhance plant production and

resiliency against biotic and abiotic stress in agriculture. Microbiome data, such as genome and metagenome sequences, along with microbial profiling, could help design SynComs that confer stable plant phenotypes and promote robustness in terms of both plant colonization and persistence throughout plant development. We explored relevant bottlenecks, functional gaps, and underexploited tools in the plant microbiome that may help develop novel strategies for bridging microbial ecology and screening procedures associated with microbial functions towards developing microbiome technologies for agricultural sustainability.

IDENTIFYING RELEVANT MICROBES WITH KEY TRAITS FOR STABLE AND EFFECTIVE SYNCOMS

Traditionally, the selection of microbes for agricultural application has essentially involved *in vitro* screening for well-known taxa or PGP activities such as nitrogen fixation, phytohormone production, and 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity (Glick, 2012). However, except in some extensively investigated cases, such as rhizobium–legume interactions and mycorrhizal fungi, there is still no clear correlation between these traits and their effectiveness in plant growth promotion or their contribution to sustaining stable plant–microbe associations (Finkel et al., 2017; de Souza et al., 2019). Furthermore, inoculants designed with these conventional approaches are often unable to establish and sustain associations with plants under field conditions, yielding unsatisfactory results (Nadeem et al., 2014; Zimmer et al., 2016).

Advances in microbial ecology, leveraged by high-throughput sequencing of metagenomes and molecular markers, helped to shed light onto the factors involving the successful establishment of the microbial community, as well as the reasons why some microbes used as inoculants fail to robustly colonize plants. The plant microbiome comprises highly diverse and complex microbial communities that are influenced by many factors, such as host genotype, environmental changes, and plant development (Coleman-Derr et al., 2016; de Souza et al., 2016; Liu et al., 2019). A successful inoculant must compete with indigenous microbes, efficiently colonize plant organs, and establish stable and resilient associations despite changes in the environment and soil microbial composition throughout the growing season. In this scenario, it is not surprising that common screening approaches for single traits fail to capture required traits for creating robust inoculants for applications in the field (de Souza et al., 2016; Finkel et al., 2017).

Since *in vitro* evidence of such traits *per se* is insufficient to ensure that microbes are capable of eliciting the desired phenotype in plants, the incorporation of additional variables for microbial selection is highly demanded. In this context, large sequencing datasets currently available in public databases comprise a promising alternative for identifying beneficial and efficient microbes. In contrast to selecting microbes based on single PGP

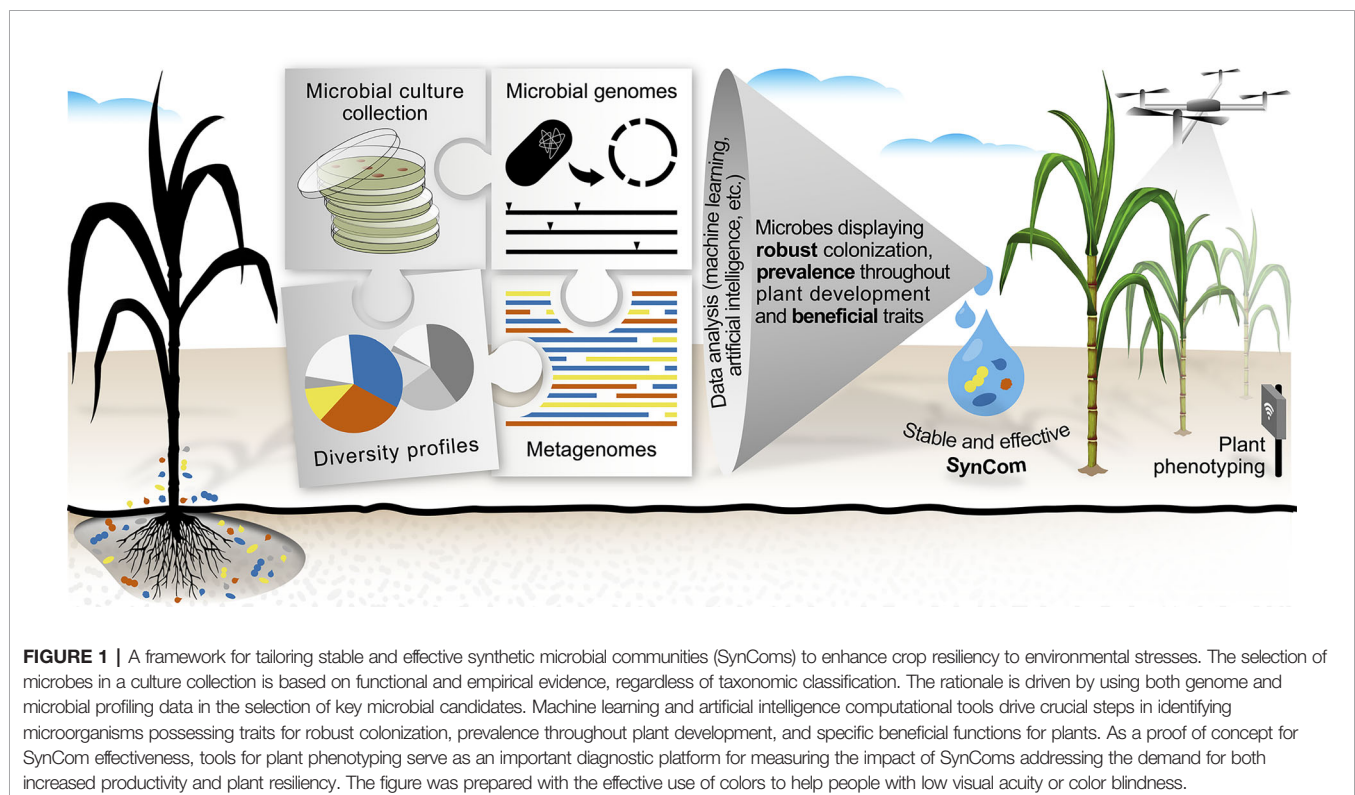
activities or taxonomy, genomic datasets can be used to design SynComs harboring multiple traits, such as robust colonization (high abundance in plant organs), prevalence (consistency across plant developmental stages) and specific beneficial functions (Figure 1).

One strategy to overcome gaps in current inoculants is to select microbes based on the diversity profile of plant microbiota. Deep-sequencing analyses of the 16S rRNA gene have revealed that certain groups of microbes are able to robustly colonize, consistently establish and sustain association with plants regardless of changes in the environment or plant developmental stages (de Souza et al., 2016; Müller et al., 2016; Xu J. et al., 2018). Members of these dominant groups, named the core microbiome, can be incorporated in SynComs, thus preventing the lack of efficiency and prevalence observed in situations where strains are outcompeted by naturally occurring microbiota. Notably, studies using both abundant and prevalent core microbes in SynComs have shown efficient colonization and beneficial effects such as plant defense against pathogens and root growth (Niu et al., 2017; Armanhi et al., 2018). These studies confirm that core microbial groups are extremely efficient at colonizing plants, highlighting that dominant groups are likely involved in functions important for plant growth and survival. Overall, incorporating both concepts into the design of SynComs is a fundamental step towards ensuring inoculum stability.

Designing SynComs containing microbes compatible with different plant genotypes and resiliency through different

environments is challenging. Recent examples have shown that robust colonizing microbes from sugarcane are capable of colonizing maize and benefiting its growth (Armanhi et al., 2018). Bacterial strains isolated from lodgepole pine significantly improve maize plant biomass accumulation (Puri et al., 2015). The genome sequences of core microbiome members isolated from sugarcane shows that robust-colonizing strains are enriched in genes coding for carbon metabolism when compared with non-core microbiome strains (de Souza et al., 2019). As the genome sequences of plant microbiomes become increasingly available, comparative genomics would help to identify specific genomic markers for key traits, which will guide the selection of beneficial microbes (Finkel et al., 2017; Toju et al., 2018).

In addition to microbial profiling data, the expanding number of reference genomes and metagenomes in public databases is an important foundation for identifying microbes with desired traits. This rationale is driven by using genomic information and gene expression profiles to select microbes containing plant-beneficial functional traits or metabolic capabilities that will help in designing the best microbial combination for inoculants (Vorholt et al., 2017; Toju et al., 2018). Because important traits such as colonization efficiency and prevalence are likely associated with multiple genes, genome surveys for multiple gene markers will be key to identifying relevant microbes (Cole et al., 2017; Levy et al., 2018; de Souza et al., 2019). Ultimately, genomics-available datasets will make it easier to screen microbial candidates based on genomic markers as the use of these datasets tend to be less laborious than traditional procedures (Finkel et al., 2017). Identifying microbial candidates that contain multiple plant



beneficial traits will assist to more precisely design SynComs containing microbes with synergistic traits.

In light of these massive amounts of data, computational tools such as machine learning and artificial intelligence (AI) will be critical for identifying microbial candidates from large datasets and culture collections. In biomedical science, these tools have already proven to be effective in discovering novel antibiotics (Stokes et al., 2020). In plant science, however, there are few reports employing these tools to address questions related to plant–microbe associations (Levy et al., 2018). Machine learning and AI will be critical for predicting the outcome of SynComs based on microbiomes and will likely take the field to a new level (Figure 1).

MAGNIFYING MICROBIAL CULTIVABILITY

Building microbial culture collections is key to manipulating plant-associated microorganisms and designing SynComs with agronomic functional properties (Schlaeppli and Bulgarelli, 2015; Finkel et al., 2017; Vorholt et al., 2017). However, culture-independent data have shown that a sizeable amount of microbial diversity may remain unexplored given cultivability limitations (Lundberg et al., 2013; Turner et al., 2013). Thus, the lack of better approaches for microbe cultivation reduces our capability to design inoculants impacting plant performance and represents a major hurdle to exploring novel microbes.

Novel strategies have been proposed to maximize access to microbial strains while maintaining their viability (Kehe et al., 2019). Recently, the use of microfluidic platforms has been shown to be a promising method to cultivate hitherto-uncultured microorganisms in complex communities (Alekkett et al., 2018). By facilitating microbial interactions in a microenvironment reflective of natural conditions, microbe–microbe interactions are preserved, and microbial survival is dramatically increased (Nichols et al., 2010). In accordance, high-throughput droplet-based systems for manipulating core microbiomes have allowed the screening and isolation of sets of microorganisms based on cell sorting and encapsulation (Hosokawa et al., 2015).

Since many microbial groups required defined growing conditions, several studies have suggested different approaches to increase microbial cultivability. An example is coculturing microbial mixtures of low richness, introduced by the concept of community-based culture collection (CBC) (Armanhi et al., 2018). By picking non-confluent colonies from primary platings, regardless of whether they represent single or multiple microorganisms, this approach allows culturing communities instead of solely axenic colonies and greatly increases microbial cultivability. Instead of employing purification procedures in search of axenic cultures of all colonies, one can later target only those of interest including, for instance, robust colonizing microorganisms (Armanhi et al., 2016). Additionally, many cross-feeding compounds have already been found in microbe–microbe interactions (Kosina et al., 2016; Lubbe et al., 2017). Cocultivation empowers metabolic interactions between microbes and enables more efficient microbes to thrive. Also, simple and convenient approaches like supplementing the

culture media with extracts from their environmental origin have helped to increase retrieval of microbial groups (Stewart, 2012; Armanhi et al., 2018).

Undeniably, when seeking robust colonizing microbes to compose a SynCom, the culture and identification of microorganisms have little impact unless their relevance for plants is considered. Therefore, an equally important but often neglected step is the cross-referencing of isolated microbes and the plant microbiome profile. Based on that, recent studies verified that previously obtained microbial culture collections comprise substantial proportions of the host microbiome (Bai et al., 2015; Armanhi et al., 2018). Such a strategy is built on the rationale of selecting microbes in a culture collection based on empirical evidence from microbial surveys, regardless of taxonomic classification or preselected traits (Figure 1). By using this approach, microbes are targeted based on relevant traits, such as robust colonization and prevalence (Armanhi et al., 2018; de Souza et al., 2019).

DESIGNING SYNCOMS FOR CROP RESILIENCY

Crop development is known to be strongly influenced by adverse environmental conditions. For instance, drought is considered one of the most severe weather events that directly reduces crop yield (Bartels and Sunkar, 2005; Yamaguchi and Blumwald, 2005). Another major constraint for crop production refers to biotic stress, which includes those caused by bacterial, fungal, and viral pathogens (Boyd et al., 2013). In addition, limited bioavailability of nutrients for plant metabolic processes is also a critical concern in arable lands, as in the case of nitrogen (Oldroyd and Dixon, 2014) and phosphorus (Sharma et al., 2013), among other macro- and micronutrients essential for crop growth.

In the last few years, a flurry of reports has supported the beneficial impact of microbes on the alleviation of detrimental effects caused by climatic events. In many of those reports, however, beneficial microorganisms were individually investigated. Notably, studies on the microbiome are gradually considering the synergistic and cumulative effects of SynComs on different microbial groups (Qin et al., 2016; Kong et al., 2018; Toju et al., 2018; Arif et al., 2020). For example, by inoculating poplar with many bacterial consortia composed of diazotrophs, Knoth et al. (2014) observed a dramatic increase in plant biomass. Additionally, very recently, Carrión et al. (2019) elegantly demonstrated that *Flavobacterium* and *Chitinophaga* together provide more consistent disease protection to sugar beet than when individually inoculated.

Under stressful environmental conditions, plants recruit sets of microorganisms with the ability to alleviate specific detrimental effects (Compant et al., 2019). This phenomenon was investigated by Naylor et al. (2017), who interestingly observed in C4 grasses under drought stress significant enrichment of Actinobacteria, a bacterial class previously reported as being related to plant growth under stress (Anwar et al., 2016). With such knowledge, it seems reasonable that traits incorporated by the SynComs should also be considered to strategically ensure plant homeostasis in unfavorable

environments, thus mitigating losses in plant productivity, rather than solely targeting increases in plant performance under normal circumstances.

It is well accepted that the plant microbiome is shaped through a process of coevolution with its host under adverse environmental conditions (Theis et al., 2016). Thus, further studies have suggested that plants in stressful ecosystems may harbor microorganisms capable of electing traits of tolerance to that unfavored condition (Woodward et al., 2012; Camargo et al., 2019). A successful strategy of microbial selection aiming to further application in the field should consider microbiome origin as the first clue to microbial capabilities in an environmentally guided selection of traits. For example, microbes with a significant impact on crop stress resiliency have been isolated from saline habitats. These halotolerant bacteria are capable of mitigating salt stress in wheat (Ramadoss et al., 2013). In another significant example, bacterial strains isolated from zinc-polluted soil decreased the concentration of zinc in clover (Vivas et al., 2006).

ASSESSMENT OF SYNCOM INFLUENCE ON PLANT PRODUCTIVITY AND PHYSIOLOGY

The emerging interests in designing and applying beneficial SynComs for agricultural sustainability are challenged by the demand for assessing microbial impacts on plant physiology. The assessment of plant traits conferred by SynComs requires methodologies capable of quantifying microorganisms in terms of their robustness of colonization and preferred organs in plants, as well as their capability to outcompete pathogenic resident microbiota. Such further validation should consider both adverse environmental conditions and heterogeneity in farmlands (Toju et al., 2018). Overall, beyond a deep investigation of microbial composition, the assessment of plant development and productivity is a fundamental step as a proof of concept for SynCom effectiveness. That stated, as microbes significantly affect host physiological status, phenotyping might serve as an important diagnostic platform for measuring the impact of SynComs or even detecting an imbalance in the microbiota (Figure 1).

Invasive and punctual approaches of plant phenotyping have conventionally been employed in a time-consuming manner. The development of automated and noninvasive techniques for measuring plants has increased for small-, medium-, and large-scale setups, especially with regard to imaging (Fahlgren et al., 2015; Rouphael et al., 2018). Optical techniques (such as RGB, infrared and hyperspectral imagery) are routinely applied in plant disease detection and crop breeding (Mahlein, 2016; Mohanty et al., 2016; Shakoor et al., 2017). As environmental parameters intrinsically face continuous fluctuations, a recent discussion pointed out the need for continuously measuring plant traits under experimental conditions (Halperin et al., 2017). Nevertheless, the lack of tools for continuous phenotyping still remains an important gap in the functional analysis of plant-microbe interactions and their application in agriculture.

Integration of phenotyping and *omics* data through machine learning and AI algorithms will be an important step towards

data-driven optimization and monitoring of SynCom efficiency. By constantly monitoring and integrating multiple genomic and phenomic datasets throughout different growing seasons, the analysis platform will become increasingly robust in determining the best combination of microbes as well as predicting the outcome of SynCom inoculation. While predictive pipelines and algorithms are becoming popular, devising solutions for integrating data from different *omics* fields remains challenging.

CONCLUSION AND FUTURE PERSPECTIVES

As the microbiome is extensively reported as playing fundamental roles in plant processes, the application of microorganisms in agriculture has emerged as a promising and sustainable alternative for improving crop performance, especially with regard to enhancing plant resiliency to environmental stresses. However, developing stable and effective SynComs for agriculture will require novel approaches that incorporate recent advances in microbiome research, such as the rational use of both genome and microbial profiling data in the selection of key microbial candidates.

We argued that two major factors to be considered are microbial robustness in terms of colonization and their prevalence through plant development. Identifying and incorporating robust and prevalent plant colonizers, such as those belonging to core microbiomes, has the potential to increase SynCom stability throughout the growing season and to prevent the inoculated community from being overcome by naturally occurring microbes. Additionally, the selection of microbial candidates should consider screening approaches based on the microbial genome in search of traits related to functions beneficial to plants and traits that enhance SynCom stability. A combination of these strategies will likely be leveraged by computational methods, including machine learning and AI, for the design of SynComs with predictable and successful impacts on plants.

When designing SynComs for agriculture, some constraints should also be considered. Since scaling up microbial growth in industrial processes is still a bottleneck, the ability to use a minimal number of microbes is urgently needed to reduce costs and simplify procedures, a requirement that can be achieved by tailoring SynComs whose members display synergistic and cumulative effects. The validation of SynCom stability, effectiveness and robustness of colonization is supported by sequencing techniques applied to small-scale proof-of-concept trials. Additionally, the application of SynComs to address the demand for both increased productivity and increased plant resiliency faces a further limitation regarding approaches for measuring and assessing plant performance under field conditions. Recently, efforts have been made towards developing tools capable of providing a comprehensive picture through plant phenotyping, with an emphasis on imagery. Data from such platforms will greatly contribute to quantifying SynCom efficiency and improving the SynCom design process thereafter. Although multidisciplinary approaches for integrating different dimensions of

omics data are still lacking, the rational design of SynComs for agricultural purposes will undoubtedly create novel opportunities for sustainable production.

AUTHOR CONTRIBUTIONS

RdS, JA, and PA equally contributed to writing and revising the manuscript.

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Cell-Free Fermentation Broth of *Bacillus velezensis* Strain S3-1 Improves Pak Choi Nutritional Quality and Changes the Bacterial Community Structure of the Rhizosphere Soil

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Bacillus velezensis is a plant growth-promoting rhizobacteria (PGPR) that has long been proven to improve the growth of plants, and it has been widely used in agriculture. However, in many reports, we observed that during the application of bacterial fluids, it appeared that the effect of the cell-free fermentation broth (CFB) was ignored. The purpose of this study is to compare the effect of the no inoculation treatment (CK), the *B. velezensis* strain S3-1 treatment (S), the CFB treatment in the Pak choi, soil bacterial community structure, soil enzyme activity, and field soil properties. The results have shown that, compared to the inoculation *B. velezensis* strain S3-1 treatment and the no-inoculation treatment; the inoculation of the CFB treatment can significantly enhance the soluble protein, soluble solids, ascorbic acid of Pak choi and increase the total phosphorus content and electrical conductivity (EC) in the soil. Based on high-throughput sequencing data, our analysis of soil microbial communities used R, NETWORK, and PICRUST showed that the CFB treatment can enhance the relative abundance of *Acidobacteria* in the soil, decrease the abundance of native *Bacillus* in the soil, change the microbial community structure of the top 50 operational taxonomic units (OTUs), and improve soil microbial carbon metabolism and nitrogen metabolism. Overall, we observed that CFB treatment can also improve plant nutrition and change soil microbial communities. This study provides new insights for the application of microbial fertilizers in agricultural production.

Keywords: *Bacillus velezensis* strain S3-1, Pak choi, soil properties, bacterial community, soil enzyme activity

INTRODUCTION

Pak choi (*Brassica campestris* L. ssp. *chinensis* Makino) is one of the most widely used and commercially essential crops in China. It has high nutritional value as it provides an intake of cellulose, vitamins, and minerals (Han et al., 2019). Therefore, there is great interest in improving Pak choi nutritional quality by using rhizosphere-promoting bacteria.

The use of the plant growth-promoting rhizobacteria (PGPR) as a substitute minimizes the adverse effects of artificial fertilizers and pesticides on the environment. Compared to the use of chemical fertilizers, bio-fertilizers made from rhizosphere bacteria can also provide better yield and nutritional value for organic fruits and Pak choi (Al-Farsi and Lee, 2008).

PGPR can be used as bio-fertilizers. They are applied to the soil to support the availability and absorption of nutrients, especially under adverse conditions (Hamad et al., 2015). In the soil, PGPR inoculants have been reported to participate in nutrient cycling and increase crop productivity (Singh et al., 2011). *Bacillus velezensis* is widely reported to be in the soil and plants as an essential plant rhizosphere-promoting bacteria (Mateus et al., 2019; Rabbee et al., 2019). *B. velezensis* can produce volatile organic compounds (VOCs), iturin, fengycin, surfactin, and antibiotics, among others, to inhibit fungal growth (Jin et al., 2017). Bacteria can also inhibit the growth of fungi by altering the gene expression of the fungus (Kang et al., 2019). At the same time, bacteria can produce indole acetic acid (IAA), siderophore, and phosphorus solubilization to promote plant growth (Kim et al., 2017). For example, *B. velezensis* GF267 is capable of producing siderophore that increases the chlorophyll content of tomatoes (Paula et al., 2019), and *B. velezensis* also promotes soybean and *Brassica* growth (Kanjnamaneesathian et al., 2013; Hassan et al., 2019). However, the survival of the bio-fertilizers in the soil tends to be poor due to the complexity of the soil environment. Furthermore, the role played by cell-free fermentation broth (CFB) is unclear (Wang et al., 2019).

Soil rhizosphere microbial communities play an important role in plant growth. For instance, different fertilization treatments change soil microbial communities and affect apples growth (Thompson et al., 2019). Moreover, soil microbial communities improve the resistance to the invasion of fungi (Li et al., 2019; Wei et al., 2019). Nevertheless, few studies have focused on the link between CFB and soil microbial communities.

Here, we studied the properties of *B. velezensis* S3-1, a strain isolated from the cucumber rhizosphere soil. The whole-genome sequencing results of this strain indicate that it has the ability to produce substances, such as IAA and 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and has the potential to promote plant growth (Jin, 2019). In the present study, we aimed to understand (i) how CFB affects Pak choi nutritional quality; (ii) the relationship between the effects of CFB on soil properties and Pak choi nutritional quality; and (iii) the relationship between the effects of CFB on the rhizosphere soil microbial community.

MATERIALS AND METHODS

Preparation of Inoculum and Identification of Fermentation Products

B. velezensis S3-1 (CCTCC AB 2014337) was grown in Luria-Bertani (LB) medium (10 g/L Tryptone, 5 g/L yeast extract, and 5 g/L NaCl) at 28°C (with shaking at 200 rpm) until the number of 5×10^8 CFU ml⁻¹. Then the CFB was clarified by centrifugation at 10,000 g for 20 min at 4°C.

The bacterial inoculum was prepared by centrifugation at 3,000 g for 20 min at 4°C and resuspension of the pellet in equal volumes of water.

Take 200 µl of CFB and add 500 µl of methanol: acetonitrile = 2:1 (containing 5% of internal standard L-2-chlorophenylalanine), vortex for 30 s; after vortex mixing, ice-water bath ultrasonic extraction 30 min; let the sample stand at -20°C, 30 min; centrifuge at 4°C 13,000 rcf for 15 min; take the supernatant, put it into a glass derivatization bottle, and blow dry with nitrogen; add 80 µl of methoxyamine hydrochloride to the glass derivation vial Pyridine solution (15 mg/ml), vortex and shake for 2 min, then perform oxime reaction in a shaking incubator at 37°C for 90 min; remove the sample and add 80 µl of BSTFA (containing 1% TMCS) derivative reagent, vortex and shake for 2 min, and react at 70°C for 60 min; and after removing the sample, leave it at room temperature for 30 min for GC-MS metabolomics analysis.

After derivatization, the sample was injected into the GC-MS system in splitless mode for analysis, and the injection volume was 1 µl. The sample was separated by HP-5MS UI capillary column (30 m × 0.25 mm × 0.25 µm, Agilent J&W Scientific, Agilent 19091S-433), and then subjected to mass spectrometry. The inlet temperature is 260°C, high-purity helium is used as the carrier gas, the carrier gas flow rate is 1 ml/min, the septum purge flow rate is 3 ml/min, and the solvent is delayed by 5 min. Heating program: initial temperature 60°C, equilibration for 0.5 min, then increase to 310°C at a rate of 8°C/min, and maintain for 6 min. The electron bombards the ion source (EI), the transmission line temperature is 310°C, the ion source temperature is 230°C, the quadrupole temperature is 150°C, and the electron energy is 70 eV. Scanning mode is full scan mode (SCAN), mass scanning range: m/z 50–500, scanning frequency is 3.2 scan/s.

Characterization of Bacteria

One milliliter the bacterial suspension (5×10^8 CFU ml⁻¹) was inoculated into 100 ml of LB broth containing L-tryptophan (100 µg ml⁻¹), and then the mix was incubated at 28°C for 7 days, while estimating the concentration of IAA in the culture supernatant every day as described in the literature (Bano and Musarrat, 2003). The enzymatic activity of ACC deaminase was measured as described previously (Penrose and Glick, 2003). The phosphate solubilizing activity of the bacterial isolate was determined using Pikovskaya agar containing precipitated tricalcium phosphate (Pikovskaya, 1948). If a transparent area is observed, bacteria are considered to have the ability to dissolve phosphorus. Chitinase was detected by the colloidal chitin medium using the method of Frändberg and Schnürer (Emma and Johan, 1998). Detection of proteases was determined as described in the literature (Dutta et al., 2015). The cellulose degradation ability of the bacterial isolate was analyzed by streaking on a cellulose Congo red agar medium (Gupta et al., 2012). The lipase activity of the bacterial isolate was determined using a Tween lipase indicator assay. The appearance of a transparent circle is considered to be an indication of lipase activity (Howe and Ward, 1976). Chrome azurol S (CAS) agar media were used for the evaluation of siderophores production (Alexander and Zuberer, 1991).

Plant Growth Stimulation With *B. velezensis* S3-1

To test the plant growth-promoting ability and optimal concentration of *B. velezensis* strain S3-1, the seeds of Pak choi were surface-sterilized with a 1% NaClO solution for 5 min, sterilized with 95% ethanol for 3 min, washed five times with sterile distilled water (Egamberdieva et al., 2017), placed on a sterile filter paper in a Petri dish, inoculated with 10 ml of different concentrations of *B. velezensis* strain S3-1 fermentation broth (based on dilution of one culture), $100(5 \times 10^8 \text{ CFU ml}^{-1})$, $10(5 \times 10^7 \text{ CFU ml}^{-1})$, $4(2 \times 10^7 \text{ CFU ml}^{-1})$, $2(1 \times 10^7 \text{ CFU ml}^{-1})$, and $1(5 \times 10^6 \text{ CFU ml}^{-1})$, with 10 seeds in each treatment, in triplicate. The seeds were grown in a plant growth chamber for 8 h at 28°C and then 16 h at 22°C in the dark. The root length and stem length of the plants were measured after 7 days.

Field Experimental Design

The field trials were carried out in the area of Shanghai city. The mean temperature of the growing season in 2018 was 13–19°C (October to November) and 19–25°C (October to November). The pH of the soil was 7.37 and EC = 1,143. The experimental plots (1.5 m × 3.5 m) were arranged in a randomized block design with three replicates per treatment. This experiment lasted 49 days. Seeds were sown by hand (about 10 g per field). The three treatments were as follows: (i) no inoculation control (CK), (ii) plant inoculated with the bacterial inoculum mixed with water at a ratio of 1:100 (S), and (iii) plant inoculated with CFB mixed with water at a ratio of 1:100. The above treatments were used to water the fields twice a week, each field receiving about 10 L.

Plant Physiology Analysis

The hydrogen peroxide (H_2O_2) content in Pak choi was evaluated as described by Mukherjee and Choudhuri (1983). Pre-cooled acetone at 4°C was used to extract the leaf samples (g: V = 1:1), and 1 ml of the supernatant was mixed with 0.1 ml of 5% TiSO_4 and 0.2 ml of NH_4OH (20%). The supernatant was removed by centrifugation. The precipitate was mixed with 5 ml of 2 mol H_2SO_4 , centrifuged at 6,000 g for 10 min, and the supernatant was then read at 415 nm.

The titratable acid was titrated with 0.1 mol/L NaOH (Khaliq et al., 2019). Ascorbic acid was titrated with 0.1 g/L of 2,6-Dichlorophenol (Khaliq et al., 2019). For the soluble solids determination, an Abbe refractometer was used. Soluble proteins were determined using Coomassie Brilliant Blue G-250. Briefly, 1 ml of the supernatant was mixed with 5 ml of Coomassie Brilliant Blue G-250, incubated for 2 min, and read at 595 nm (7,504 UV/VIS Spectrometer, China; Bradford, 1976). The soluble sugar was determined using anthrone. A 0.5 ml sample extract was pipetted and mixed with 1 ml of distilled water, then 0.5 ml of anthrone-ethyl acetate and 5 ml of concentrated sulfuric acid were added, heated in 100°C water for 1 min, and naturally cooled and read at 630 nm (7,504 UV/VIS Spectrometer, China; Roman, 2002).

Plant roots were measured using a root scanner. Plant biomass was evaluated dry for constant weight at 105°C.

Soil Sample Properties Analysis

The bulk soil was collected by shaking the roots of the plants, while the rhizosphere soil was collected by brushing (Chen et al., 2016). Six Pak choi were taken from each plot to collect rhizosphere soil for mixing. The rhizosphere soil was placed in coolers with ice packs and transported to the lab, where samples were kept at −80°C until further analysis.

One gram of rhizosphere soil was dried at 40–50°C, ground into a powder, passed through a 100-mesh sieve, and then analyzed for nitrogen (N) and carbon (C) with an elemental analyzer (ECS 8020, Italy; Howard et al., 2014). The dried soil was digested with aqua regia, and the elemental contents were analyzed using inductively coupled plasma optical emission spectrometry (ICP-OES; AcmeLabs, Canada; Zhang et al., 2017).

The bulk soil amylase and sucrase activities were detected using 3,5-dinitrosalicylic acid. For example, 5 g of soil were mixed with 15 ml of an 8% sucrose solution, 5 ml of phosphate buffer (pH = 5.5), and five drops of toluene, and then placed at 37°C for 24 h, heated in a boiling water bath for 5 min, and after cooling, it was read at 508 nm (Ge et al., 2017). The soil peroxidase and polyphenol oxidase activities were detected using 3,5-dinitrosalicylic acid. Briefly, 1 g of soil was mixed with 10 ml of 1% pyrogallol and 2 ml of 0.5% H_2O_2 , and incubated in a 30°C incubator for 2 h. Then, 4 ml of citrate phosphate buffer (pH = 4.5) and 35 ml of ether were added. After an extraction period of 30 min, the samples were read at 430 nm (7504 UV/VIS Spectrometer, China). A standard colorimetric assay determined the soil urease activity (Kizilkaya, 2009).

DNA Extraction and MiSeq Sequencing of 16S Amplicons

The rhizosphere soil DNA was extracted with the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, United States). The DNA extract was checked on a 1% agarose gel, and the DNA concentration and purity were determined with NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, United States). The hypervariable region V3-V4 of the bacterial 16S ribosomal RNA (rRNA) gene was amplified with primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') using an ABI GeneAmp® 9,700 PCR thermocycler (ABI, CA, United States). The PCR amplification of the 16S rRNA gene was performed as follows: initial denaturation at 95°C for 3 min, followed by 27 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 45 s, and then a single extension at 72°C for 10 min. The PCR mixtures contained 5 × TransStart FastPfu buffer 4 µl, 2.5 mM dNTPs 2 µl, forward primer (5 µM) 0.8 µl, reverse primer (5 µM) 0.8 µl, TransStart FastPfu DNA Polymerase 0.4 µl, template DNA 10 ng, and finally ddH₂O up to 20 µl. PCR reactions were performed in triplicate. The PCR product was extracted from a 2% agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) according to manufacturer's instructions and quantified using Quantus™ Fluorometer (Promega,

United States). In total, nine samples were prepared for sequencing with the MiSeq PE300 platform. The raw reads were deposited in the NCBI Sequence Read Archive (SRA:PRJNA534410) database.

Data Analysis

The raw 16S rRNA gene sequencing reads were demultiplexed, quality-filtered by Trimmomatic, and merged by FLASH with the following criteria: (i) the 300 bp reads were truncated at any site receiving an average quality score of <20 over a 50 bp sliding window, and the truncated reads shorter than 50 bp were discarded; reads containing ambiguous characters were also discarded; (ii) only overlapping sequences longer than 10 bp were assembled according to their overlapped sequence. The maximum mismatch ratio of the overlap region was set to be 0.2. Reads that could not be assembled were discarded; and (iii) samples were distinguished according to the barcode (exact barcode matching) and primers (up to two nucleotide mismatches allowed), and the sequence direction was adjusted.

Operational taxonomic units (OTUs) with 97% similarity cutoff were clustered using UPARSE (version 7.1¹), and chimeric sequences were identified and removed. The taxonomy of each OTU representative sequence was analyzed by RDP Classifier² against the 16S rRNA database (e.g., Silva SSU128) using a confidence threshold of 0.7.

All of the statistical analyses were performed using R packages (V3.5.1), in addition to using Networkx to analyze the mesh and constructing a phylogenetic tree using interactive tree of life (ITOL; Beckers et al., 2017). The variance analysis was performed using an LSD in SPSS 24.0 (SPSS Institute, USA). Values of $p < 0.05$ were considered to be significant.

The 16S function prediction was employed to standardize the OTU abundance table by PICRUSt, which was used to remove the effect of the number of copies of the 16S marker gene in the species genome. Manually screen out genes related to the C and N cycle, and the abundance of each gene could be calculated according to the OTU abundance.

RESULTS

Identification of Fermentation Products and Characterization of the Bacteria

B. velezensis S3-1 could produce IAA (19.156 g/ml), ACC deaminase, phosphate solubilizing activity, and siderophores, but it was unable to produce lipase, chitinase, and cellulase (Table 1).

¹<http://drive5.com/uparse/>

²<http://rdp.cme.msu.edu/>

Based on the NIST database for comparison, the identification results with a matching degree higher than 85 were considered to be reliable. A total of 192 substances were identified, which were divided into 20 types of substances others (18.99%), O-methylatedisoflavonoids (1.27%), phenethylamines (1.27%), tryptamines and derivatives (1.27%), phenols and derivatives (1.27%), pterins and derivatives (1.27%), sesquiterpenoids (1.27%), hydroxycinnamic acids and derivatives (1.27%), alcohols and polyols (2.53%), tricarboxylic acids and derivatives (2.53%), benzoic acids and derivatives (2.53%), pyrimidines and pyrimidine derivatives (2.53%), pyridinecarboxylic acids and derivatives (2.53%), flavones, fatty alcohols (2.53%), fatty acids and conjugates (3.80%), purines and purine derivatives (5.06%), dicarboxylic acids and derivatives (5.06%), amines (5.06%), carbohydrates and carbohydrate conjugates (8.86%), and amino acids, peptides, and analogues (26.58%; Figure 1, Supplementary Table S1).

Plant Growth Stimulation With *B. velezensis* S3-1

The Pak choi seeds did not germinate under the condition of 100% concentration. Compared with no inoculation, the shoot length of Pak choi did not change significantly at the 10% concentration, but the root length was severely inhibited and grew only one-fifth of the control. There was no significant difference in root length and shoot length of inoculated bacteria at the 4 and 2% concentrations compared with the non-inoculated bacteria. The difference in shoot length between the 1% concentration and the non-inoculated bacteria was not significant, but it could effectively promote the root length of the plant and increase the root length by about 56% (Supplementary Figure S1). This indicates that *B. velezensis* S3-1 had the ability to promote plant root growth and the potential to promote plant growth (Supplementary Figure S1). In view of these results, the 1% bacterial concentration was used for subsequent experiments.

Plant Physiology and Soil Sample Analysis

ANOVA tests showed that the CFB treatment significantly decreased the hydrogen peroxide content ($p < 0.05$) compared with the S treatment, and significantly increased the soluble solid content and ascorbic acid of Pak choi ($p < 0.05$) compared with the CK treatment (Table 2). CFB treatment could significantly increase the soluble protein and ascorbic acid content relative to the CK and S treatments ($p < 0.05$), but the results showed that there was no significant difference in soluble sugar, nitrate content, and plant biomass between the CK, S, and CFB treatments (Table 2).

TABLE 1 | Characterization of *B. velezensis* strain S3-1.

Strain	IAA (μg/ml)	Lipase	Protease	Chitinase	ACC deaminase	Phosphorus	Siderophore	Cellulase
S3-1	19.156	–	+	–	+	+	+	–

+, positive; –, negative.

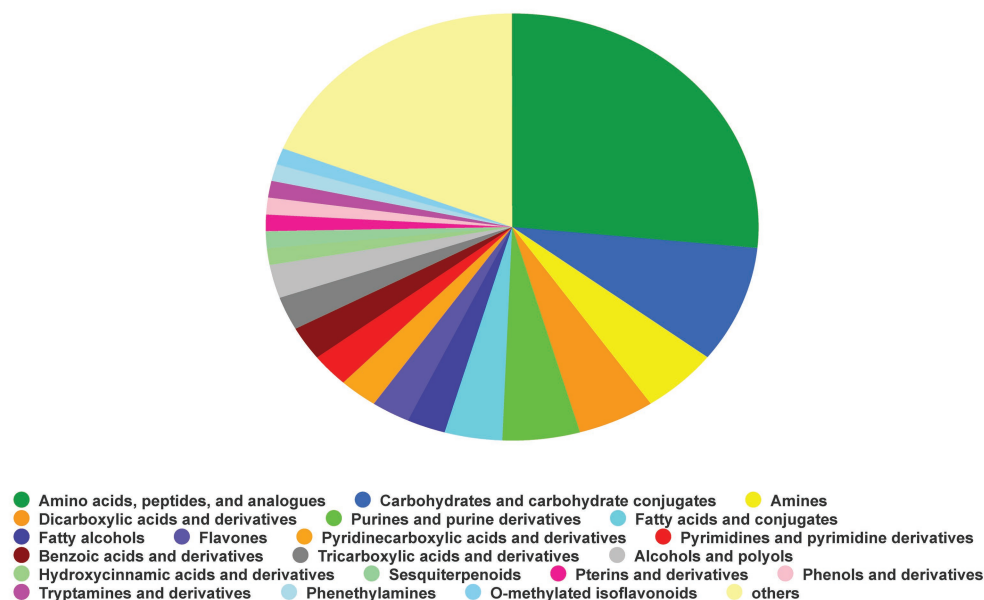


FIGURE 1 | Compound classification based on HDBM.

Inoculation with CFB significantly decreased the pH and Fe content of the soil, and significantly increased EC and the P content ($p < 0.05$). However, inoculation with S treatment significantly decreased TN and sob ($p < 0.05$). Furthermore, inoculation with CFB or S treatment could significantly improve the length, tips, and forks of the plant roots ($p < 0.05$; **Table 3**).

Variations in Bacterial Composition

At the OTU level, the effects of environmental factors can be divided into four groups; EC and P, pH and Fe, TC and Mg, and TN and Na. The effects of the EC and P concentrations were the most significant, and the effects of pH and Fe came second. TC, TN, Mg, and Na had no significant effect (**Figure 2**). Among the OTUs, the relative abundances of 13 OTUs affiliated of *Rhodobiaceae* (OTU1739), *Rhodospirillaceae* (OTU168), *Acidobacteria* (OTU1278 and OTU580), *Sphingomonas* (OTU1434, OTU889, and OTU240), *Nocardioides* (OTU1075), *Rhizobiales* (OTU341), *Microvirga* (OTU1483), *Gaiella* (OTU19), *Hyphomonadaceae* (OTU1789), and *Acidimicrobiaceae* (OTU599) were positively correlated with EC in a significant way. On the contrary, the relative abundance of *Bacillus* (OTU2009 and OTU139) was significantly negatively correlated with EC. The relative abundance of *Rhodospirillaceae* (OTU168), *Gemmatimonadetes* (OTU1769), *Rhizobiales* (OTU341), *Microvirga* (OTU1483), *Gaiella* (OTU19), and *Acidimicrobiaceae* (OTU599) was significantly positively correlated with P content. In contrast, the relative abundance of *FictiBacillus* (OTU1384) and *Bacillus* (OTU139, OTU2009, and OTU1994) was negatively correlated with P content. In addition, the relative abundance of *Bacillus* (OTU139 and OTU2009) was significantly positively correlated with pH, while the relative abundance of *Acidobacteria* (OTU1278), *Sphingomonas* (OTU1434, OTU240, and OTU889),

Rhizobiales (OTU341), *Microvirga* (OTU1483), and *Hyphomonadaceae* (OTU1789) was negatively correlated with pH. In addition, the relative abundance of *Microvirga* (OTU1483) and *Sphingomonas* (OTU889 and OTU240) was significantly negatively correlated with the Fe content. In addition, the relative abundance of *Prokaryote* (OTU2120) was significantly positively correlated with the Na⁺ content (**Figure 2**).

The Overall Change of Bacterial Community

The purity, concentration, and integrity of DNA extraction could meet the requirements of high-throughput sequencing (**Supplementary Table S2**). The complete coverage of bacterial communities was confirmed by rarefaction curves (**Supplementary Figure S2**). Based on the OTU level, PLS-DA analysis found that S, CFB, and CK could be classified into one group. COMP1 explained 24.29% mutation rate, and COMP2 interpreted 17.83% mutation rate (**Figure 3A**). The phylogenetic breadth of the number of unique OTUs in the S and CFB treatments was lower than the CK treatment (**Figure 3B**). Fewer specific OTUs were found in the samples of the CFB treatments than the S treatments. It also showed that most OTUs were shared between samples (**Figure 3B**).

The main bacteria associated with the S and CFB treatments were the *Proteobacteria* and *Actinobacteria* phyla in the rhizosphere soil. The predominant of 16S rRNA sequences were clustered into *Proteobacteria* (30.9–31.56%), *Actinobacteria* (27.2–27.7%), and *Chloroflexi* (10.5–10.85%). The CFB treatment could decrease the relative abundance of *Firmicutes* and increase the relative abundance of *Acidobacteria* ($p < 0.05$). The S treatments could decrease the relative abundance of *Firmicutes* ($p < 0.05$; **Figure 3C**).

TABLE 2 | Differences in hydrogen peroxide, Pak choi quality, and plant biomass between S, CFB, and CK treatment.

Sample name	Hydrogen peroxide content ($\mu\text{mol/g}$)	Soluble protein content ($\mu\text{g/ml}$)	Soluble solid content ($^{\circ}\text{Bx}$)	Titrateable acidity content (%)	Soluble sugar content ($\mu\text{g/ml}$)	Ascorbic acid ($\text{mg}/100\text{ g}$)	Nitrite content ($\mu\text{g/ml}$)	Biomass (g/plant)
CK	0.0379 ± 0.0118^a	0.02848 ± 0.0000166^b	2.33333 ± 0.127^b	0.3 ± 0.130^b	0.00881 ± 0.0000632^a	18.7 ± 1.742^b	6.407 ± 5.527^a	6.88 ± 5.142^a
S	0.0185 ± 0.00124^b	0.0281 ± 0.0000691^c	2.9 ± 0.1^a	0.1125 ± 0.023^c	0.00926 ± 0.000270^a	$22.033 \pm 2.516^{a,b}$	5.0931 ± 4.224^a	7.00633 ± 2.822^a
CFB	0.0180 ± 0.00248^b	0.02873 ± 0.0000230^a	2.93333 ± 0.115^a	0.45 ± 0.0001^a	0.00885 ± 0.000157^a	26.7 ± 1.732^a	3.779 ± 1.202^a	6.45367 ± 1.076^a

Data are expressed as the average \pm SD. Different letters in the same column denote statistically significant differences at $p < 0.05$.

TABLE 3 | Differences in below-ground in S, CFB, and CK treatments.

Treatment	Soil							
	pH	EC ($\mu\text{S cm}^{-2}$)	TN (g kg^{-1})	TC (g kg^{-1})	Fe content (mg kg^{-1})	Mg content (mg kg^{-1})	Na content (mg kg^{-1})	P content (mg kg^{-1})
S	7.357 ± 0.163^a	985.333 ± 47.374^b	0.103 ± 0.0035^a	1.619 ± 0.057^b	155.105 ± 4.355^a	72.525 ± 4.243^a	66.740 ± 46.600259^a	133.913 ± 1.186^c
CFB	6.680 ± 0.213^b	1976.333 ± 47.962^a	0.108 ± 0.0068^a	1.940 ± 0.200^a	139.7486 ± 9.741^b	79.387 ± 17.776^a	135.149 ± 60.689^a	310.673 ± 21.102^a
CK	7.540 ± 0.125^a	932.667 ± 51.033^b	0.112 ± 0.0107^a	2.176 ± 0.184^a	148.699 ± 3.4652^a	68.919 ± 0.804^a	138.660 ± 40.293^a	195.517 ± 1.851^b
Treatment	Microbial diversity index				Plant root			
	SOB	Shannon	Simpson	Length(cm)	Root volume(cm^3)	Tips	Forks	
S	1919.667 ± 63.799^b	6.327 ± 0.0750^a	0.00550 ± 0.000572^a	113.086 ± 17.319^a	2.819 ± 1.567^a	1555.333 ± 812.775^a	4569.333 ± 1157.235^a	
CFB	2029.000 ± 6.083^a	6.463 ± 0.0925^a	0.00475 ± 0.00167^a	88.016 ± 16.193^a	1.613 ± 1.012^a	1121.333 ± 153.526^a	3312.333 ± 481.531^a	
CK	1964.667 ± 52.501^a	6.362 ± 0.122^a	0.00683 ± 0.00258^a	39.0160 ± 5.219^b	0.940 ± 0.191^a	99.681 ± 57.551^b	1632.000 ± 503.027^b	

Data are expressed as the average \pm SD. Different letters in the same column denote statistically significant differences at $p < 0.05$.

These results did not allow us to clearly understand which OTU changes lead to change at the phylum level, so we selected the top 50 OTUs to observe their distribution in the different treatment groups. They were divided into 11 genera, namely *Alphaproteobacteria* (24.46%), *Actinobacteria* (10.67%), *Clostridia* (1.51%), *Gammaproteobacteria* (3.94%), *Cyanobacteria* (1.31%), *Flavobacterium* (1.07%), *Thrmppmicrobia* (2.21%), *KD4-96* (3.49%), *Bacilli* (44.43%), *Nitrospira* (1.68%), and *Acidobacteria* (3.22%). The ANOVA model was (OTU) compartment which included all three treatments, followed by LSD. In the S treatment sample, OTU1384, OTU1495, and OTU2122 were significantly enriched, and OTU19 and OTU599 were significantly decreased ($p < 0.05$). In the CFB treatment, we observed a significant enrichment of OTU899, OTU240, OTU1769, OTU1789, OTU19, and OTU599, and a significant decrease of OTU1384 compared to the other treatments ($p < 0.05$). Compared with CK, S,

and CFB treatments significantly decreased OTU139 and increased OTU1278 and OUT1075 ($p < 0.05$; **Figure 3D**).

At the same time, we found that the relative abundance of OTU1061 in the rhizosphere soil of the S treatment was six times higher than that of the CK and CFB treatment, and the 16S rDNA sequence of OTU1061 and *B. velezensis* S3-1 had 100% similarity (**Supplementary Figure S3**).

All three networks of different treatments clearly showed different compositions. For example, the inoculation with S treatment of *Bacteroidetes* (OTU620) had more negative correlations with other OTUs compared to the CK treatment. Furthermore, *Bacteroidetes* (OTU620) disappeared when inoculated with CFB treatment (**Figures 4A–C**). In addition, the relative abundance of *Actinomycetes* treated with CFB inoculation was increased, but the correlation was weaker compared to those in the CK and S treatments (**Figures 4A–D**).

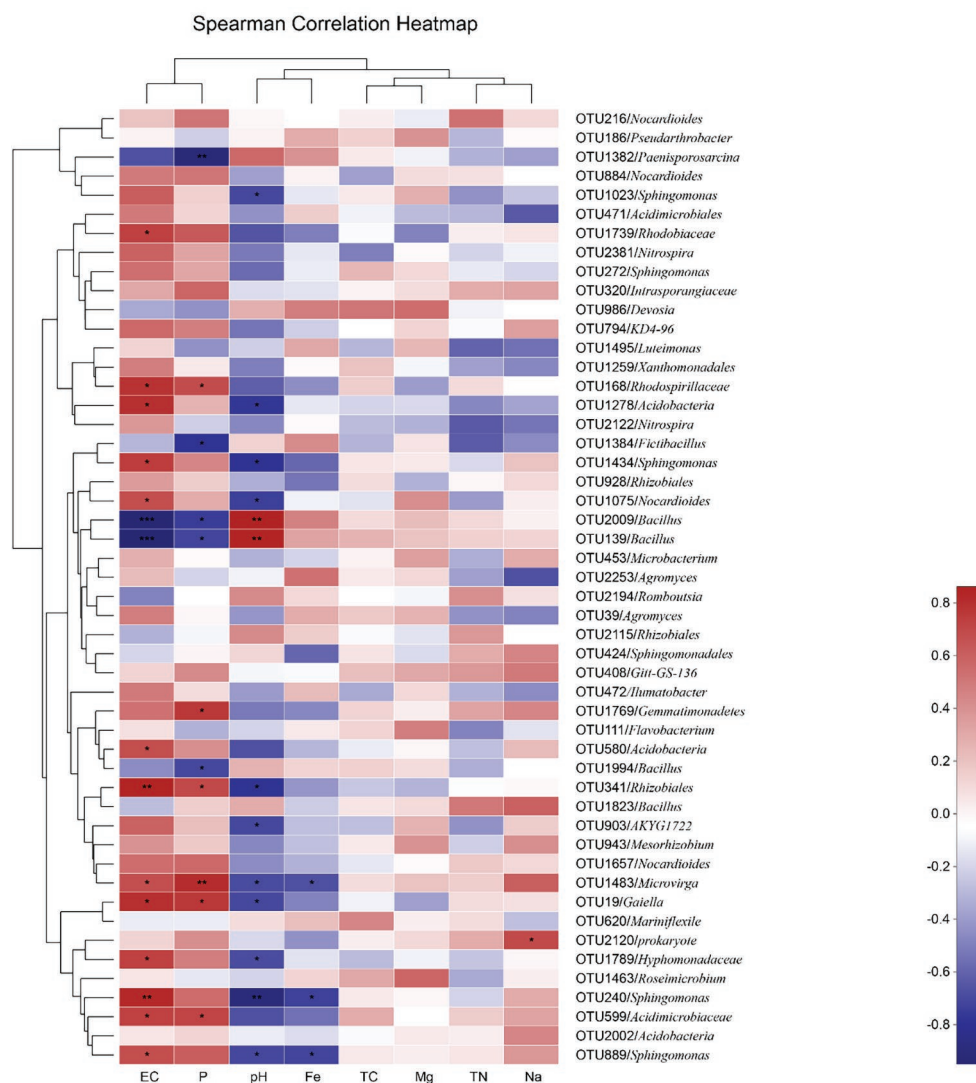


FIGURE 2 | The heatmap depicts the relative abundances of the top 50 operational taxonomic units (OTUs) and Spearman's correlations between OTU abundances and soil properties. The top 50 OTUs based on the values of mean decrease in the Gini index are selected and only the ones with average relative abundances of $>0.1\%$ are shown. EC, Electrical Conductivity; P, phosphorus; Fe, iron; TC, total carbon; Mg, magnesium; TN, total nitrogen; and Na, Sodium.

Moreover, compared with CK and S treatment, the number of *Acidobacteria* nodules inoculated with CFB treatment was higher (Figure 4E).

Soil Enzyme and 16S Functional Prediction Analysis

Our results showed a consistent trend in enzyme activity over time after inoculation with the S, CFB, and CK treatments. Compared with CK treatment, CFB treatment significantly increased the activities of amylase and sucrase on the 21st day, amylase, sucrase, and urease activities on the 35th day, and urease and sucrase activities on the 49th day (Figures 5A,B,D). However, the CFB treatment had no significant effect on the activity of polyphenol oxidase and peroxidase at any time (Figures 5C,D).

The gene abundance was calculated by 16S sequences function prediction, the predicted values were all less than 0.17, indicating that the results were credible (Supplementary Table S3; Langille et al., 2013). Here was an increase in the relative abundance of genes associated with the nitrogen metabolism in addition to *nreA* (nitrate regulatory element) after CFB treatment compared with the S and CK treatments (Figure 6A). In addition to *cmpB* (Calvin cycle) and *cmpC*, the relative abundance of genes associated with carbon metabolism was also increased compared with S and CK treatments. Notably, the relative abundance of

the *ccmL*, *ccmM*, *ccmN*, and *ccmO* genes was increased by more than 100% (Figure 6B).

DISCUSSION

In this study, we examined the effects of the S, CFB, and CK treatments on rhizosphere nutrient quality and soil properties (i.e., soil enzyme activity and soil microbial structure) of Pak choi.

Identification of Fermentation Products

After the fermentation of *B. velezensis* S3-1, the composition of LB medium changed significantly. A large number of acids, alcohols, and sugars appeared after fermentation. Among them, the acids could reduce the pH of the rhizosphere soil. The addition of sugars will increase the C metabolism capacity in the soil, change the root structure of the plant, as well as increase the soluble solids, ascorbic acid, and antioxidant capacity (Figure 1, Supplementary Table S1; Shen and Bartha, 1997; Ya-Jie et al., 2018). The fermentation broth of *B. velezensis* S3-1 contains a variety of plant growth promoting substances, such as putrescine, spermidine, 2,3-butanediol, trigonelline, melatonin, etc. (Supplementary Table S1). These substances can activate plant genes and regulate the distribution of ions

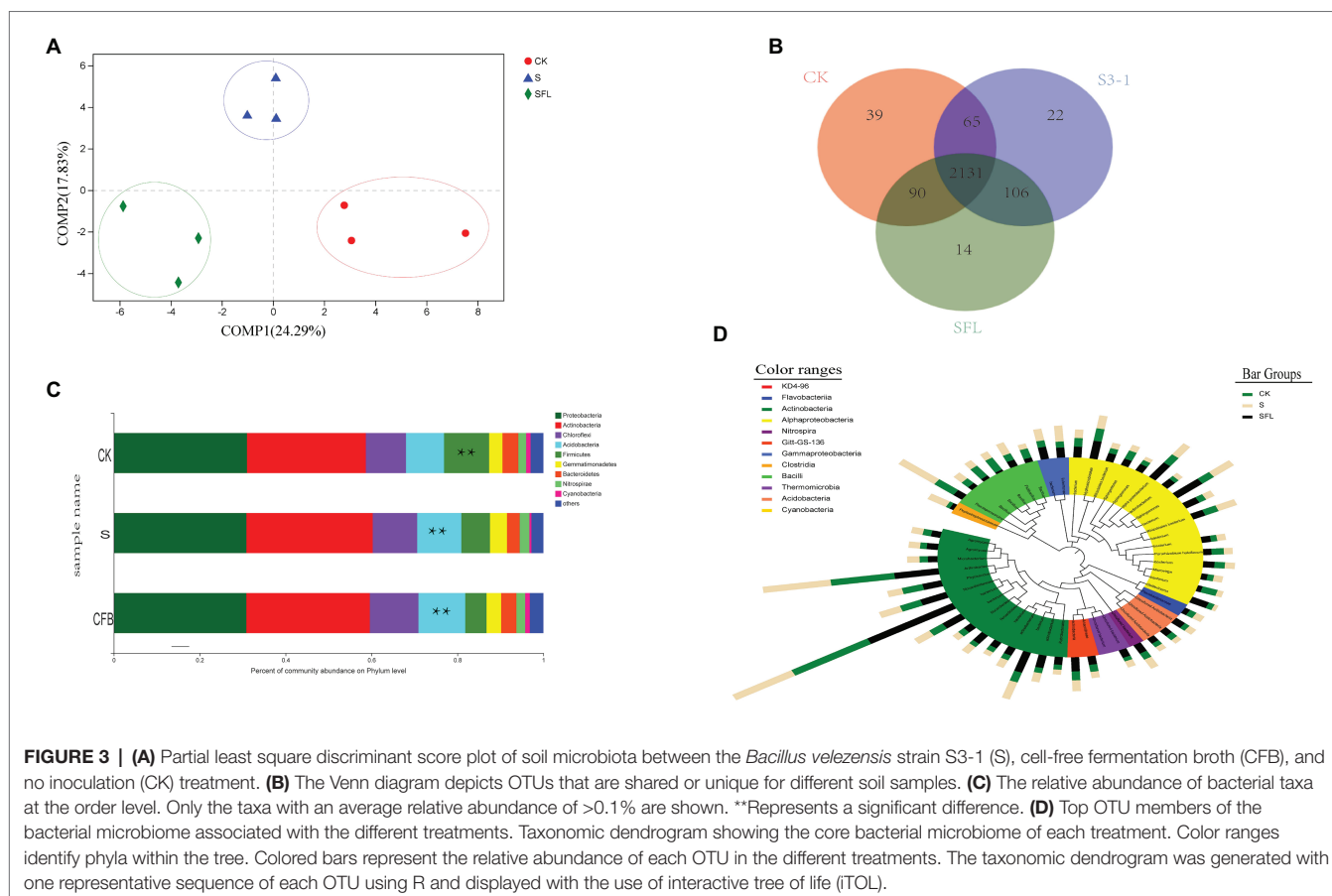


FIGURE 3 | (A) Partial least square discriminant score plot of soil microbiota between the *Bacillus velezensis* strain S3-1 (S), cell-free fermentation broth (CFB), and no inoculation (CK) treatment. **(B)** The Venn diagram depicts OTUs that are shared or unique for different soil samples. **(C)** The relative abundance of bacterial taxa at the order level. Only the taxa with an average relative abundance of >0.1% are shown. **Represents a significant difference. **(D)** Top OTU members of the bacterial microbiome associated with the different treatments. Taxonomic dendrogram showing the core bacterial microbiome of each treatment. Color ranges identify phyla within the tree. Colored bars represent the relative abundance of each OTU in the different treatments. The taxonomic dendrogram was generated with one representative sequence of each OTU using R and displayed with the use of interactive tree of life (iTOL).

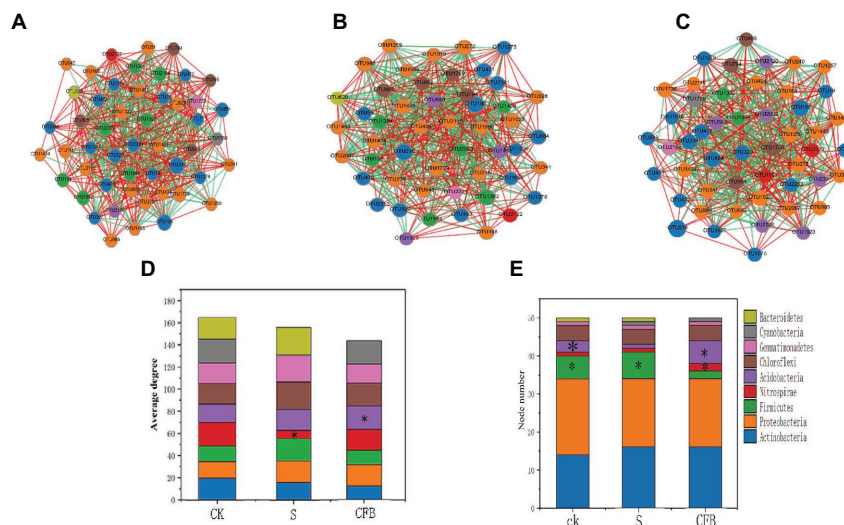


FIGURE 4 | Network analyses of bacterial communities at CK treatment (A), S treatment (B), and CFB treatment (C). Average degree between different treatment groups (D). Node number between different treatment groups (E). The nodes in the networks are colored by module class. *Represents a significant difference.

in plants, thus promoting plant growth (Seiler and Raul, 2005; Igarashi and Kashiwagi, 2010).

Characterization of Bacteria and Plant Growth Under Laboratory Conditions

Inoculation with high concentration of bacteria ($\geq 2\%$) did not promote the growth of plant roots and buds, while the inoculation with lower concentration (1%) promoted the growth of roots and shoots. This is due to the dual effects of IAA on inhibiting the growth of high concentration plants and promoting the growth of low concentration plants. ACC deaminase is an extracellular enzyme secreted by bacteria to promote plant growth. Siderophore and phosphorus-solubilizing activities, which are ensured by bacteria, also promote plant growth and improve plant quality (Podile and Kishore, 2006; Shaharoon et al., 2008; Hassen et al., 2018). However, the production of IAA requires the use of plant-derived tryptophan as a precursor for bacterial synthesis (Duca et al., 2014). S treatment can provide the above four substances, but the CFB treatment can only provide three other than IAA. This may explain why the S treatment is more likely to increase plant root length than the CFB treatment, although it is not significant (Table 2).

Effects of Different Treatments on the Quality and Physiology of Pak Choi

Soluble sugar, soluble protein, titratable acid, and soluble protein are important flavor substances in vegetables (Cao et al., 2007). Compared with the other treatments, the CFB treatment enhanced the soluble protein of Pak choi (Table 2). This may be due to the increase in the EC of the soil (Durian et al., 2016). Interestingly, the CFB treatment did not increase the hydrogen peroxide content of the plants, but we observed a significant increase in the ascorbic acid (ASA) content, which

may be resulted from the use by the plants of the ASA-GSH cycle pathway to scavenge hydrogen peroxide. Compared with other treatments, the CFB treatment resulted in the highest increases in titratable acid due to the Pak choi antioxidant properties (Table 2).

Compared with CK treatment, the number of root tips and forks of Pak choi were improved by CFB and S treatment, which were consistent with the research on the effect of PGPR inoculation on root structure, which helped plants enhance root tips, roots and absorb water and nutrients from the soil (Table 3; Moubayidin et al., 2009). In short, this means that the CFB treatment can effectively improve the nutritional quality of Pak choi, because it is resistant and could enhanced the absorption capacity of plants.

Relationship Between Soil Properties and Bacterial Communities

The CFB treatment significantly changed the soil properties, because compared with other treatments; it reduced soil pH and Fe content, and increased P content and EC content. According to published research, increasing soil nitrogen cycling will reduce soil pH. The abundance of genes related to N cycle increased significantly in the CFB treatment group, which was a factor in the decrease of soil pH (Guo et al., 2010). The plant roots can absorb P to release OH^- to neutralize H^+ in the soil to increase the soil pH. However, the accumulation of the P in the CFB-treated soil may be due to the absorption of P by plants. As a consequence, OH^- was not released, which was also responsible for the decrease in soil pH (Gardner and Boundy, 1983). It is believed that the reduction of soil pH will make the iron ions in the soil more easily absorbed by plants, which may be the reason for the decrease of iron ion content in the soil observed in the CFB treatment (Table 3; Guo et al., 2010).

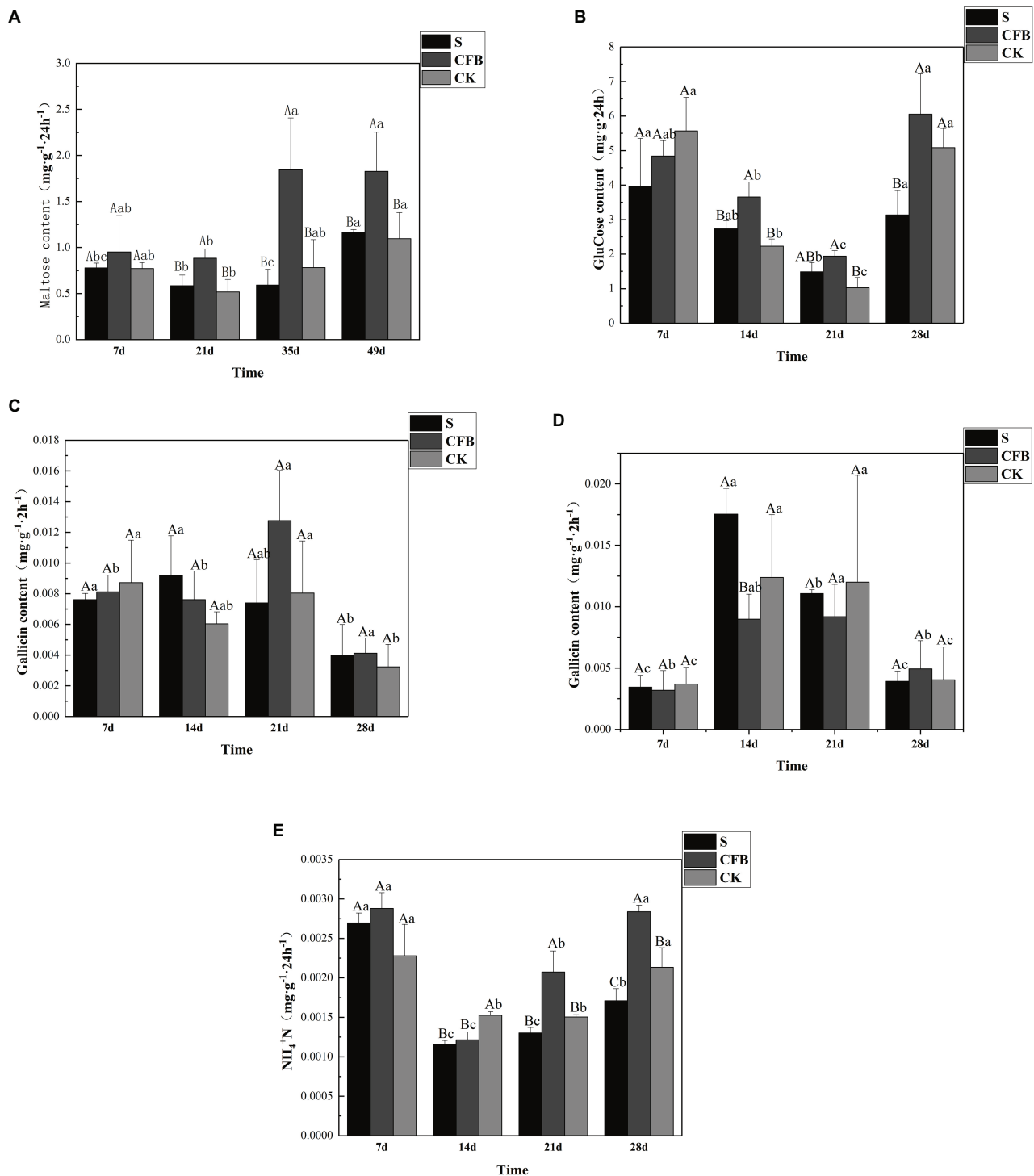


FIGURE 5 | Activities of (A) amylase, (B) surcuse, (C) polyphenol oxidase, (D) peroxidase, and (E) urease in the inoculated S, inoculated CFB, and CK treatment. The capital letter means different treatment at the same time; the lowercase letter means different treatment at different times. Data shown are mean \pm SE, $n = 3$ replicates. Different letters indicate significant differences for each enzyme among treatments (one-way ANOVA, $p < 0.05$).

In short, due to the change of soil properties, compared with S and CK treatments, CFB treatment can significantly improve the nutritional quality of Pak choi.

Among the top 50 of the relative abundance, we observed that the *Acidobacteria* and *Bacillus* were most closely related to environmental factors. EC and pH can significantly affect

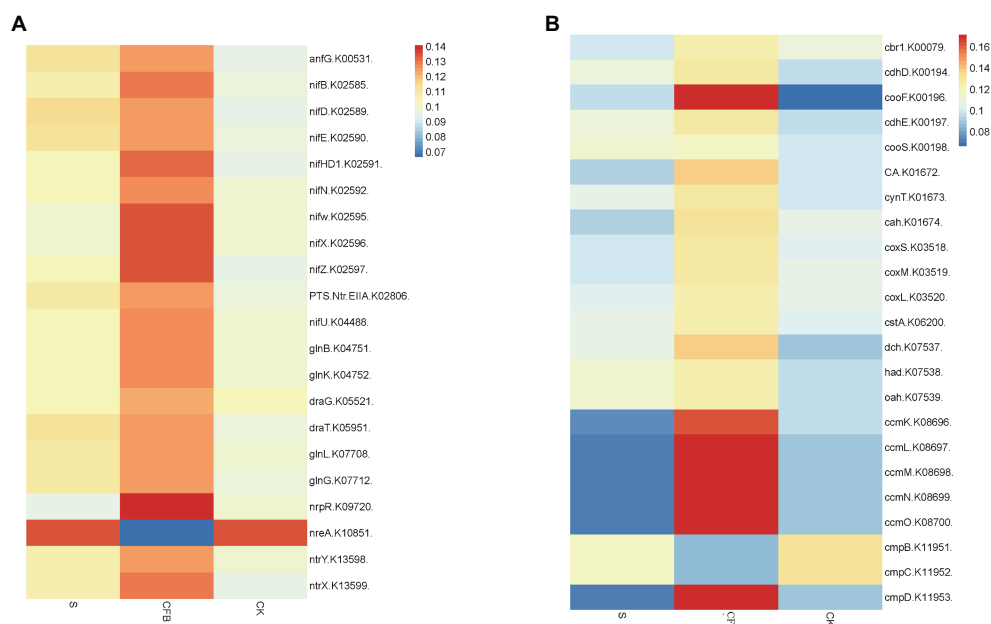


FIGURE 6 | Potential gene abundance and potential function as it is based on 16S sequences. **(A)** The relative gene abundance associated with N metabolism. **(B)** The relative gene abundance associated with C metabolism.

the distribution of *Acidobacteria* in the soil (Figure 2). This is consistent with the results, we observed at the Phylum level (Figure 3C). Notably, the relative abundance of *Acidobacteria* increased with the decrease of pH, which was consistent with the strong correlation between the abundance of these bacteria and soil pH (Jones et al., 2009). The distribution of *Bacillus* was affected by the contents of EC, pH, and P, which was consistent with the study of Fierer (2017). This indicated that soil properties can drive changes in rhizosphere soil microbial communities, which may be an essential way to improve the nutritional quality of Pak choi.

Soil Microbial Community Structure

B. velezensis S3-1 was capable of colonizing in rhizosphere soil (Supplementary Figure S3). Based on the PLS-DA, it ignored the random differences within the group and highlights the differences between the groups. We found that the S, CFB, and CK treatments could be clearly distinguished and divided into three groups (Figure 3A). This indicated that there were significant differences in soil microbial composition among the three treatments (Zhao et al., 2017). The results of the Venn diagram showed that compared with CK treatment, CFB treatment had fewer unique OTUs than S treatment (Figure 3B).

Our study found a significant increase in *Acidobacteria* under the CFB treatment (Figure 3C). *Acidobacteria* play a critical role in soil material circulation and ecological environment construction (Hugenholz et al., 1998; Barns et al., 1999). In previous studies, the enrichment in Fe also increased the abundance of *Acidobacteria*, but our study found that there was no significant correlation between the distribution of

Acidobacteria and Fe (Lauber et al., 2008; Da Rocha et al., 2010). Consistent with the results of S treatment, previous studies have shown that PGPR inoculation can increase the number of *Acidobacteria* in soil, thus promoting plant growth (Kalam et al., 2017). However, the CFB treatment could increase the abundance of *Acidobacteria* and promote plant growth compared to the S treatment (Figure 3D). The relative increase of *Acidobacteria* content also means that the ability to degrade residues in soil is enhanced, and the ability to metabolize carbon is enhanced (Pankratov et al., 2008; Pankratov and Ivanova, 2011). In short, the increase of *Acidobacteria* may be one of the key factors to promote the nutritional quality of Pak choi.

Since the main component of the *Firmicutes* is the *Bacillus* spp., the relative abundance of this group may be related to the reduction of *Bacillus* spp. The relative abundance of the *Firmicutes* in the CFB treatment was significantly lower than that in the S and CK treatments. In the phylogenetic trees of the top 50 OTUs of the three treatments, the relative abundance of *Bacillus* in the CFB treatment was lower than that in the CK and S treatments (Figure 3D). This meant that reducing natural soil *Bacillus* may help to improve the nutritional quality of Pak choi.

In the network, we observed that the node number of the *Acidobacteria* is increased in the CFB treatment. Compared with other treatments, the location of *Acidobacteria* tended to the core, which meant that *Acidobacteria* played an important role among bacterial communities (Figures 4A–C). At the same time, the number of nodes in *Firmicutes* decreased, which meant that the relative abundance of *Firmicutes* decreased, so it is more difficult to enter top 50 OTUs.

Prediction and Analysis of Soil Enzyme and 16S Function

C cycle and N cycle are very important in crop growth. The *cmp* gene family, *ccm* gene family, and *cox* gene family are responsible for carbon capture, carbon sequestration, and oxidation of CO to CO₂. Compared with other treatment groups, the CFB treatment group could increase the abundance of the *cmpD* (The low-CO₂ high affinity HCO₃⁻ transporter) gene, *ccm* gene family, and *cox* (carboxydophilic Oligotropha carboxidovorans) gene families. This means that the ability of soil microorganisms for carbon capture, carbon sequestration, and CO to CO₂ oxidation has been increased (Figure 4A; John et al., 2007; Heinrich et al., 2018; Willis et al., 2019).

AnfG (alternative nitrogenase) gene, *nif* (N₂-fixing) gene family, *gln* (PII signal transduction) gene family, *dra* (nitrogenase reductase ADP-ribosyl transferase) gene family, and *ntr* gene family are generally responsible for regulating nitrogenase activity, N metabolism, dinitrogenase reductase activity, and nitrate metabolism. Compared with the S and CK treatments, the CFB treatment increased the abundance of the above gene families, which means that the ability of soil microorganisms to fix nitrogen, nitrogen metabolism, and nitrate metabolism was enhanced (Figure 4B; Greco et al., 2012; Bonato et al., 2016; Moure et al., 2019).

The enzyme activity is an important indicator to measure soil fertility, especially invertase, amylase, catalase, peroxidase, and urease (Henry et al., 2005; Allison and Treseder, 2008). This was also supported by our research. The activities of invertase, amylase, and urease showed an up-down-up pattern in the three different treatment groups. Compared with the other treatments, the activities of invertase, amylase, and urease in CFB treatment were significantly increased ($p < 0.05$), but the peroxidase and catalase activities were not increased (Figures 5A–E). This indicated that compared with the CK and S treatment, the CFB treatment enhanced the C and N cycling in soil, which was consistent with our prediction results using PICRUS. Therefore, the change of enzyme activity was caused by the change of soil microbial community (Marumoto et al., 1982; Singh et al., 1989).

In summary, CFB can improve the quality of Pak choi, which may be explained by three main factors. Firstly, the CFB treatment has substances that can directly promote plant growth and improve the plant quality, such as ACC deaminase, siderophores, phosphorus-solubilizing activity, and sugar. Secondly, CFB treatment changed soil properties and indirectly improved the nutritional quality of Pak choi by increasing soil nitrogen cycling and acid production. Finally, soil characteristics driven the changes of rhizosphere

microbial community, that is, the content of acid bacteria increased greatly, and the number of protobacillus decreased, which further enhanced the nutritional quality of Pak choi.

However, our experiments still have some shortcomings. Due to the complexity of soil microbes, we were unable to effectively identify which microorganisms significantly enhance the N cycle and thus reduce the soil pH. At the same time, due to the complexity of bacterial metabolites, it is not possible to determine which substance can attract acidic bacteria or other bacterial communities in soil.

Considering that bio-fertilizers are often ineffective due to competition for resources and soil complexity with natural microbiota, it is encouraging to find that a CFB can also improve the quality of crops. This discovery provides a new idea for the future application of CFB in agriculture field.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the NCBI with accession numbers CP016371.1 and PRJNA534410.

AUTHOR CONTRIBUTIONS

KHL and QJ designed the experiment, and KHL completed most of the experiments. KHL and QJ co-authored the article. SSL, WWL, CHZ, JRJ, QJ and LCL did a small number of experiments and provided experimental methods. MX guided the experiment and modified the manuscript. All authors contributed to the article and approved the submitted version.

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The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2020.02043/full#supplementary-material>

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When Salt Meddles Between Plant, Soil, and Microorganisms

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In extreme environments, the relationships between species are often exclusive and based on complex mechanisms. This review aims to give an overview of the microbial ecology of saline soils, but in particular of what is known about the interaction between plants and their soil microbiome, and the mechanisms linked to higher resistance of some plants to harsh saline soil conditions. Agricultural soils affected by salinity is a matter of concern in many countries. Soil salinization is caused by readily soluble salts containing anions like chloride, sulphate and nitrate, as well as sodium and potassium cations. Salinity harms plants because it affects their photosynthesis, respiration, distribution of assimilates and causes wilting, drying, and death of entire organs. Despite these life-unfavorable conditions, saline soils are unique ecological niches inhabited by extremophilic microorganisms that have specific adaptation strategies. Important traits related to the resistance to salinity are also associated with the rhizosphere-microbiota and the endophytic compartments of plants. For some years now, there have been studies dedicated to the isolation and characterization of species of plants' endophytes living in extreme environments. The metabolic and biotechnological potential of some of these microorganisms is promising. However, the selection of microorganisms capable of living in association with host plants and promoting their survival under stressful conditions is only just beginning. Understanding the mechanisms of these processes and the specificity of such interactions will allow us to focus our efforts on species that can potentially be used as beneficial bioinoculants for crops.

Keywords: extreme environment, halophilic microorganisms, endophytes, PGPR—plant growth-promoting rhizobacteria, agriculture, microbial metabolism, salty soils

INTRODUCTION

Soil salinization is a process of localised accumulation of soluble salts. This phenomenon today is unanimously considered a severe threat to agricultural lands as it directly undermines the value and quality of soil (Ammari et al., 2013; Daliakopoulos et al., 2016). The soil is a complex system in continuous evolution and dynamic equilibrium with the other environmental components, sensitive

to the effects of climate change and human activities (Smith et al., 2012). Still, it constitutes a substantially non-renewable resource in the sense that the rate of its degradation is potentially rapid (Zewdu et al., 2017), while the soil formation and regeneration processes are extremely slow.

Globally it has been estimated that 33% of the irrigated agricultural land and over 20% of the total cultivated land of the world is saline. If the current trend of salinization is maintained, then by 2050 we will see an approximately 30% increase in the cultivated land salinity. This means that agricultural productivity will be reduced due to the decline in cultivable land, with a consequent increase in the number of people suffering from hunger. Plants are the first in the chain of food production to be hit by salinity stress, which hampers their basic physiological and biochemical processes, such as water absorption and photosynthesis, thus resulting in overall reduced growth (Vaishnav et al., 2016). However, plants develop various morphological, physiological, biochemical, and molecular strategies in response to salinity in their environment (Meng et al., 2018).

In recent decades, significant overviews about mechanisms of salinity tolerance in plants have been shared by several authors (Munns and Tester, 2008; Deinlein et al., 2014; Gupta and Huang, 2014; Meng et al., 2018). In general, two types of plant adaptation to high salt concentration in the soil are distinguished: avoidance and tolerance strategy (Dajic, 2006). The first consisting of creating physical, physiological or metabolic barriers that counteract the penetration of stress factors in the plant, which causes morphological and physiological changes at the whole plant level. The tolerance strategy is based instead on plant ability to survive in stress conditions through cellular, molecular, and biochemical modifications aimed at minimising stress effects (Bahmani et al., 2015). Interestingly, those plants have a strong influence on shaping the rhizosphere and endorhiza microbiome (Abedinzadeh et al., 2019).

In particular, some plant growth-promoting rhizobacteria (PGPR) may exert a direct stimulation on plants' growth and development by providing them with fixed nitrogen, phytohormones (IAA, GB), iron (Egamberdieva et al., 2019), and soluble phosphate (Shrivastava and Kumar, 2015) that help to overcome the effects of salinity stress. Another mechanism that minimizes the impact of salinity consists in the production of substances (proline, trehalose, glycine, betaine) that work as osmoprotectant for plants' cells (Kushwaha et al., 2019). Moreover, under various environmental stresses, plants typically produce ethylene, from the precursor 1-aminocyclopropane-1-carboxylic acid (ACC). It is a hormone that limits plants' growth in conditions of high salinity, high temperatures, drought, or at the presence of toxic metals or organic pollutants. PGPRs produce a particular enzyme that degrades ethylene (ACC-deaminase), allowing the plants' roots to develop despite the environmental stress (Glick, 2012).

When salt meddles between soil, plant and microorganisms in and around the rhizosphere, a unique extreme environment is created, that provides a scene for mutualistic relationships. In this

system, plant-endophytes and plant-microbiome interactions play a crucial role in the activation or stimulation of different adaptation mechanisms for survival in saline soil (Acosta-Motos et al., 2017). In this review, we provide information about natural and anthropic causes of soil salinity and discuss the plant's strategies for stress management. An overview of the known interactions occurring between endophytic communities and host plants is provided along with a discussion on the potential of plant growth-promoting rhizobacteria (PGPR) for increasing plant's salt stress tolerance.

NATURAL AND ANTHROPIC CAUSES OF SOIL SALINITY AFFECTING AGRICULTURAL LAND USE

All soils naturally contain a mixture of salts soluble in water, and some of these are essential for plant development. Their origin is mainly from the meteoric alteration of the igneous rocks of the lithosphere. Following the hydrological and climatic events, in past ages, there has been the deposition of large quantities of salts in sedimentary rocks, in surface and underground waters, in seas and oceans (Daliakopoulos et al., 2016; Zaman et al., 2018). From these deposits, through various mechanisms, the salts reach the soil. In particular conditions, the formation of a water evaporation front in the soil moves water by evaporation rather than by percolation, so the salts remain in the soil and accumulate (Datta and de Jong, 2002).

Soil salinity can be distinguished in primary and secondary. The first depends on factors related mostly to the lithology of the substrate (in particular hydrological characteristics), morphological characteristics of the area, intrinsic chemistry of soils and climatic factors (Schofield and Kirkby, 2003). If soil parent rock contains carbonate minerals or feldspar, physical or chemical weathering operated by water bring salts in solution, increasing their concentration in groundwater and consequently on the wetted topsoil layer. Soil porosity, texture and mineral composition influence soil hydrological properties which also depend on the salts' accumulation on the soil surface. The amount of saline precipitates is in turn modulated by soil transpiration and the extent and characteristics of the capillary fringe. This kind of accumulation process is reported in different European areas (Schofield and Kirkby, 2003; Kováčová and Velísková, 2012; Gkioukakis et al., 2015; Daliakopoulos et al., 2016).

Another group of naturally saline soils originate from salty marine seawater in the coastal regions. This phenomenon can occur under short- or long-term periods. In short term periods, soil salinization can be caused by stochastic rapid events like floods or tsunamis that cause salinization on the surface of the geographical area beaten by the waves (Central Water Commission, 2017). In contrast, in a geological time scale, prolonged high tides can cause the formation of marine deposits. Soils of the coastal areas can be therefore easily affected by salinization, due to the intrusion of the marine seawater wedge inside the fresh groundwater (Daliakopoulos et al., 2016). Despite the saltwater in a natural environment usually

stays below the fresh, because of its higher density, in many cases the water extraction by wells for different human uses could interfere by breaking this balance, inducing the recall of the saline wedge with rising saltwater and the consequent formation of an area of brackish water near the ground surface. This anthropic cause is mainly due to inadequate agricultural water management practices and could induce “secondary soil salinization” along time by increasing the salt content in the irrigation waters (Napoli and Vanino, 2011; Greggio et al., 2012).

Agriculture plays an essential role as one of the leading causes of secondary salinization given by the use of poor-quality water, often worsened by the presence of soil components that limit the leaching of salts, such as the presence of impermeable horizons and an unfavorable physiographic position.

Secondary salinity could also depend by the overuse of fertilizers, insecticides and fungicides, unsustainable use of the land, excessive drainage of the water tables (Datta and de Jong, 2002).

Salinization caused by overuse of fertilizers is due to different phenomena. The excessive use of nitrogen fertilizers both chemicals or organic can cause a rise in nitrate content in the soil that can be detected as a rise in Electrical Conductivity (EC) or interpreted through signs of crop disease often associated with high soil salinity (Gkiouglis et al., 2015). The use of fertilizers with a high content of potassium and sodium can cause soil degradation by the accumulation of these salts in particular conditions (Chang et al., 2018). The application of incorrect irrigation practices added to an excess of fertilization, together with particular climatic conditions sometimes may favor the accumulation of salts (Daliakopoulos et al., 2016). Poorly permeable silty-clay soils in climatic conditions with limited rainfall and high temperatures favor high evapotranspiration and therefore the quick accumulation of salts in the first layers of the soil (Chang et al., 2018).

It should be considered, however, that even in areas irrigated using “good” quality water, moderate levels of salinity were detected as a consequence of the irrigation methods applied and local arid climatic conditions. Vice versa, the phenomenon of salinization may not occur on lands irrigated for several years with waters rich in salts. These examples indicate that each area is characterized by different and peculiar balances, which influence its possible evolution. In certain soils, drained and with particular thermo-pluviometric trends, the accumulation of salts in the soils could be only temporary. A balance could quickly be established between accumulation and leaching that would allow over time, and with due care, to maintain agricultural activity (Machado and Serralheiro, 2017).

Secondary soil salinization caused by incorrect irrigation strategies is also associated with the use of wastewaters. When the use of poor quality water exceeds the natural buffering effect of the soil, a whole series of substances, such as insecticides and fungicides remain on the ground which, as a result, cause an increase in salinity (Kováčová and Velisková, 2012; Rodríguez-Liebana et al., 2014). Another common cause of secondary salinization phenomenon is the consequence of the replacement of spontaneous vegetation (polyannual and primarily arboreal

species) with crops (annual and exclusively herbaceous), characterized by superficial, less deep root systems than the pristine vegetation. This artificial change of vegetation causes a drastic modification of the delicate hydrological balance. In essence, the reduced root systems of the new plant species, requiring less water than the original tall trees leads, over time, to a progressive rise of the water table and lower solubilization of the salts in the subsoil, causing them to rise together with the water until it affects the layer occupied by the roots of the crops (Bui, 2013). The water absorption from the roots and the evapotranspiration process inevitably causes a gradual accumulation of the salts on the surface, making the soil progressively inhospitable to agricultural plants and unsuitable for agriculture (Hanson et al., 1993; Machado and Serralheiro, 2017). The saline sources in secondary salinization could also come from the use of soil improvers that are themselves saline (i.e. gypsum or elemental sulphur), from manure and chemical fertilizers (Wallender and Tanji, 2012).

Classification and Extension of Saline Soils

As reported by Bui in 2013, the definition of saline soil is confusing. Are considered saline the soils in which salt concentration can interfere with the capability of plants to absorb water, affecting their growth or more specifically a soil with an electric conductivity (EC) on a saturated soil paste extract $>4 \text{ dS m}^{-1}$ (Bui, 2013; Shrivastava and Kumar, 2015; Zaman et al., 2018). Salts concentration and the osmotic pressure of a saline soil also depend on soil texture and relative water characteristics more than only on the salt content (Darwish et al., 2005; Daliakopoulos et al., 2016; Zaman et al., 2018).

In literature, there are two most accepted classifications for saline soil. One is the *US Salinity Laboratory Staff Classification* in which it is most commonly used the term “salt-affected soil” to indicate saline, saline-sodic and sodic soils. In this classification, a saline soil has an $\text{EC} \geq 4 \text{ dS m}^{-1}$, exchangeable sodium percentage (ESP) <15 and $\text{pH} < 8.5$ while saline-sodic differs only for $\text{ESP} \geq 15$ and $\text{pH} \geq 8.5$. Sodic soils have $\text{EC} < 4 \text{ dS m}^{-1}$, $\text{ESP} \geq 15$ and $\text{pH} > 8.5$. These soils are also characterized by the loss of permeability to water caused by the disruption of soil aggregate operated mostly by the Na^+ ions (Daliakopoulos et al., 2016; Machado and Serralheiro, 2017; Zaman et al., 2018) and the ensuing collapse of soil structure in the Natric Horizon.

According to the classification approach of *World Reference Base Classification* (IUSS Working Group WRB, 2014), salt-affected soils are divided into two classes: solonchacks (saline soils with $\text{ECe} > 15 \text{ dS m}^{-1}$ in the top 125 cm) and solonetz (are sodium-rich soils with an $\text{ESP} > 15$), both divided into subclasses (Zaman et al., 2018).

The Reference Group of Solonchacks is quite widespread in all the arid and semi-arid areas of North Africa, Near East, Central Asia, India, Iran and Iraq, Australia and the Americas. The Reference Group of Solonetz, mainly located in the steppe climatic regimes and flat landscapes with poor drainage, were mapped in Ukraine, Russian Federation, Eastern Europe, China, India, USA, Canada, Southern and Eastern Africa and Australia.

Last studies and maps at world level reported an estimated area of about 260 Mha and 135 Mha covered by Solonchacks and Solonetz, respectively (Cherlet et al., 2018).

Apart from mapping the soils classified as “saline”, the exact extension and localisation of salinity based on its causes were hardly addressed geographically. Primary (natural) salinization was estimated to be slightly under 1 billion ha worldwide, secondary (artificially induced by human activities) occurs on around 77 Mha, mostly in intensively cultivated and irrigated areas of India, Pakistan, China, Iraq, and Iran. Because of the human activities linked to wrong water management, large areas of the Mediterranean Basin, Australia, Central Asia, the Middle East and Northern Africa are interested in the risk of salinization (Cherlet et al., 2018).

In Europe, secondary salinization affects approximately 4 Mha of European soils (Daliakopoulos et al., 2016) and focusing on the Mediterranean region. Soil salinization affects 25% of irrigated agricultural land. Along the Mediterranean coasts, soil salinity is the primary cause of desertification due to human activities. Artificially induced salinization caused by irrigated agriculture is affecting significant parts of southern Italy (Napoli and Vanino, 2011), Spain (e.g. the Ebro Valley), Hungary (e.g. Great Alfold), Greece, Cyprus, Portugal, France (West coast), the Dalmatian coast, Slovakia and Romania. Besides, North Europe countries (e.g. Denmark, Poland, Latvia, and Estonia) are facing similar issues (Daliakopoulos et al., 2016).

Limitation to Agricultural Uses

Salinization represents a critical threat for agriculture because it can cause the alteration of the delicate balance of ecological processes occurring in soil.

The phenomenon of salinization represents only one of the multiple aspects that the accumulation of salts in soil causes (Shrivastava and Kumar, 2015; Nouri et al., 2017). The loss of soil occurs in terms of both surface subtraction (Peng et al., 2019), and the alteration of its chemical characteristics Nouri et al., 2017). The salinized soil readily undergoes to a further series of degradation processes, such as erosion (Ishida et al., 2009), a decrease of organic matter content (de Souza and Fay, 2014), local or widespread contamination (Hanson et al., 1993), sealing, compaction, and decline of biodiversity (Nouri et al., 2017). These processes contribute to compromising the quality of the soil and its ability to interact with the ecosystem to maintain biological productivity, environmental quality and promote the health of all living organisms (Salvati and Ferrara, 2015; Zaman et al., 2018).

Saline and sodic soils reduce the value and productivity of large areas around the world (Qadir et al., 2014; Acuña-Rodríguez et al., 2019). It is estimated that every day in the world 2000 hectares of arable land are lost due to salinization, and this problem can cause loss of yield for many crops of 10–25% and in exceptional conditions can lead to desertification (Zaman et al., 2018).

Geographical areas affected by salinity problems have an impact on both the environmental and socioeconomic sphere.

These areas face the loss of productivity (Datta and de Jong, 2002) with a different degree of magnitude depending on the crops (Qadir et al., 2014) seeing their farmers sometimes reduced to poverty or forced to migrate searching for a new source of income (Ammari et al., 2013; Salvati and Ferrara, 2015; Zaman et al., 2018). Soil can become saline at first, causing a decrease in agricultural yield and then, in the long run, it can progressively turn into completely sterile (desertification) (Gorji et al., 2017; Cuevas et al., 2019).

The pollution from chemicals causes the water that returns to the water cycle to be of poor quality with negative consequences on agriculture and health, giving rise to a vicious cycle (Zewdu et al., 2017). Soil salinization has a significant impact on the environment, causing landscape and consequently, ecosystem fragmentation (Cramer and Hobbs, 2002). A poor vegetative growth leads to the reduction of the protective role of the plants cover, enhancing soil degradation and erosion. The presence of chemical residues on the soil surface, together with salts, cause the elevation of toxic clouds of dust (Zewdu et al., 2017).

The enrichment of the soil with organic matter, humic substances, the application of biofertilizer containing microorganism and the application of fertilizers through irrigation water (fertigation) could help to alleviate the negative effect of salt accumulation on salt-sensitive crops (Darwish et al., 2005).

Given the importance of agricultural production, it is crucial to understand the impacts of salinization on different crops and to find effective strategies to reduce economic losses in salt-affected areas.

EFFECTS OF SALINITY STRESS ON PLANT GROWTH

Soil salinity affects (directly or indirectly) both growth and reproduction of plants as a consequence of complex interactions between physicochemical properties of soil (salt content, poor aeration, an increase of crusting, hard setting, reduced infiltration, reduction of water uptake, and difficult root penetration) and plants' morphological and physiological features (Rogers et al., 2005; Akbarimoghaddam et al., 2011).

Salinity causes low water potential in the soil, which negatively affects plants' water and nutrients uptake. Plants collect salts simultaneously with the water they use and often accumulate Na^+ and Cl^- ions, that result toxic to plants' cells due to ion imbalance mechanisms. What is more, enzymatic activity in cells may be disturbed. These factors trigger different responses in plants, manifested by a variety of symptoms both at cell and organ's level (**Figure 1**).

A reduction in respiration characterises stressed plants, which also show altered assimilates distribution, inhibited photosynthesis process and lower production of new leaves. Simultaneously, increased morphological changes of organs (leaf thickening and succulence, a decrease of internode lengths), wilting, drying and even necrosis of organs and entire plants are observed (Parida and Das, 2005; Kumar and Verma, 2018). Not to mention that

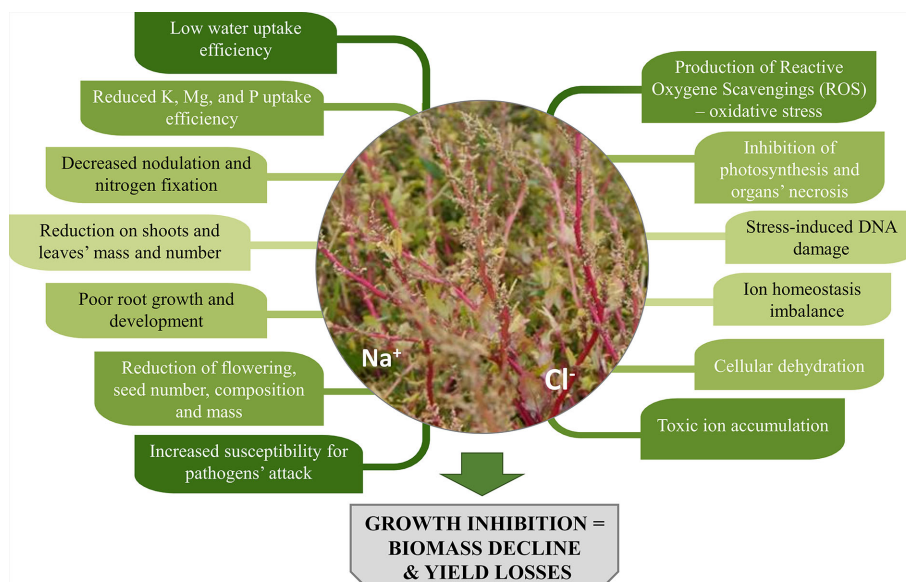


FIGURE 1 | The impact of soil salinity on morphology and physiology of plants.

cultivation of crops in saline areas can adversely affect their palatability. Some plants under salinity stress might accumulate higher amounts of compounds such as oxalate and tannins, which are bitter, whereas for others, higher sugar content, and an improved flavour was observed (Masters et al., 2005; Rahnesan et al., 2018).

Different models may describe plants' salt tolerance. One of the most popular is the Maas-Hoffman model and its modifications (van Straten et al., 2019). This mathematical tool reflects the relationship between crop yield in response to soil salinity, and for most crops, this follows a sigmoidal function. The breakpoint between the first part of the plot, being horizontal, and the second, which is sloping downward, is known as a threshold (EC_t) or salt tolerance. Based on threshold and slope values, a classification in sensitive, moderately sensitive, moderately tolerant and tolerant crops can be made. There is a large variation in salt tolerance between plants' species, from the extremely sensitive (like chickpea) to more tolerant (like cotton or beet). Among agricultural species, greens are particularly sensitive throughout the whole ontogeny of the plant, whereas the majority of cereals are highly resistant to salt impact (Munns and Rawson, 1999; Machado and Serralheiro, 2017).

In each crop, salt tolerance or sensitivity depend on the ability to uptake water and nutrients from saline soils avoiding at the same time an excessive accumulation of salt ions in the tissues. The absolute sensitivity and tolerance of plants to salinity, vary in a wide range, depending upon species, climate, soil type and its features, and agricultural practices including water management, e.g. irrigation or waterlogging methods, their frequency and intensity (Shrivastava and Kumar, 2015 and references therein).

The plants are not susceptible to salt for the whole life since their sensitivity changes during the various growth stages. In

general, it was found that plants are more sensitive to salt stress when they are in their early growth stages (seedling or establishment) compared to later development phases (Machado and Serralheiro, 2017).

As mentioned, it is believed that the most sensitive to salinity stage of the plant's growth is the germination and seedling (Läuchli and Grattan, 2007). Plants, which are better adapted to soil salinity must either have a high tolerance to salt during germination or can delay germination. Moreover, tolerance to salinity differs widely among crops without always correlating to salt-tolerances based on yield-response functions. Cotton, for instance, which is considered a salt-tolerant crop, based on lint yields, showed to be susceptible to poor stands when growing in fields irrigated with saline-sodic water (Grattan and Oster, 2003).

With maturation, plants acquire a higher tolerance to salinity. However, a prolonged exposure to salinity causes a reduction in biomass, due to lower stems numbers and smaller leaf area. A decreased leaf area commonly expresses salt-affected reduction in shoot growth, which is crucial for water uptake by the plant (Munns and Tester, 2008). In sugar beet leaves, cells' elongation was found to be more salt-sensitive in comparison to leaf initiation related to cells' division (Rozema et al., 2015). Hu and Schmidhalter (2007) reported that cells' division of grass leaves was reduced by salinity. Khan et al. (2017) found in chickpea (*Cicer arietinum* L.) a reproductive failure due to reduced supply of assimilates to reproductive tissues, decreased leaf area and reduced photosynthesis, water restriction and hormonal imbalances.

Roots are the organs directly exposed to the saline environment, and they control the uptake and internal translocation of water, nutrients and salts. The anoxic situation often present in saline soils can have a more significant impact on radical architecture than

salinity itself. In the case of root systems, oxygen deficiency contributes significantly to poor uptake of nutrient ions and decreased ability to toxic ions (such as Na^+) removal (Barrett-Lennard, 2003). It is believed that roots are generally affected by excess salinity but commonly still less than aboveground organs (Rahneshan et al., 2018).

BACTERIAL AND FUNGAL COMMUNITIES ASSOCIATED WITH ROOTS AND RHIZOSPHERE IN SALINE SOILS

According to Matilla et al. (2007), two selective forces of different nature are essential for microorganisms to colonise the rhizosphere: stress adaptation and the availability of particular nutrients. In saline soils, microbial communities associated with the rhizosphere, phyllosphere, and endosphere of halophytes comprehend members of Archaea and Bacteria domains and kingdom Fungi (Mukhtar et al., 2019). These communities are directly or indirectly involved in the osmoregulation of halophytes that allows them to survive under salinity stress conditions. Endophytic bacteria and fungi are those organisms whose life-cycle take place partly or entirely inside a plant. These organisms can live in the intercellular spaces of different tissues and plant's organs (Kandel et al., 2017) without causing visible external sign of infection or a negative effect of the host (Weyens et al., 2009). Endophytes' population in a specific environment or within a single plant may differ with tissue type, plant growth stage and dimension of the ecological niches. Several studies comparing rhizosphere and endophytic microbial communities showed how species assemblages are significantly different but also that endophytic bacteria and their communities have some peculiar, commune, traits (Kushwaha et al., 2020).

Endophytic bacterial species, for example, have larger genomes as compared to rhizosphere bacteria (Pini et al., 2011). Moreover, the diversity, richness and evenness of rhizosphere bacterial communities seem higher compared to endophytic ones (Huang, 2018). Bacterial phyla are different when considering rhizospheric soil and the plant's tissues, with a predominance of *Proteobacteria* and *Chloroflexi* in the former and *Acidobacteria*, *Bacteroidetes*, and *Planctomycetes* in the latter (Kandel et al., 2017; Huang, 2018). However, Compant et al. (2010) pointed out that the majority of plant-associated bacteria derives from the soil environment. Some of them can penetrate plants' roots thanks to specific mechanisms that are responsible for rhizosphere and endophytic competence. Chemotaxis, quorum sensing, flagella, antibiotic secretion, siderophore production are only some of the tools that rhizosphere bacteria need to be able to inhabit some specific plant-associated niches (Matilla et al., 2007; Compant et al., 2010). Liu et al. (2017) suggested that plant roots act as "gatekeepers" because they select soil bacteria from the rhizosphere and rhizoplane. This result in an endophytic root microbiome dominated by *Proteobacteria*, *Actinobacteria* and to a lower degree, by *Firmicutes* and *Bacteroidetes*, but with *Acidobacteria* and *Gemmatimonadetes*

resulting almost depleted (Liu et al., 2017). The traits that characterise the bacteria that can successfully colonise and establish in endophytic niches are motility, reactive oxygen species scavenging, plant cell-wall degradation abilities. The salinity is in itself a critical environmental filter that selects species with very particular characteristics, which are probably upstream of the further selection that the rhizosphere first, and the tissues of the plant then, exert in soil microbial communities. In some studies, it has also emerged a decisive role of salinity in defining the type of endophyte associations that a plant establishes. Qin et al. (2016) provided further evidence that plants gain a higher advantage from association with a diverse microbial community (microbiome) compared with the interaction with single members of a community.

In addition to bacterial communities, also mycorrhizal symbioses showed a fundamental role in the improvement of plant nutrition, especially at the presence of environmental stresses (Qin et al., 2017). Plant-associated mycobiota belong to arbuscular mycorrhizal fungi (AMF), ectomycorrhizal fungi (EMF), non-mycorrhizal basidiomycetous fungi (NMF), and a consistent number of ascomycetous species (Zuccaro et al., 2014). Different groups, mostly based on host colonisation pattern and type of transmission, have been delimited in plant-fungal endophytes. Some fungal endophytes exhibit a vertical transmission through the host seeds or a horizontal transfer with soil- or air-borne spores (Rodriguez et al., 2009). Moreover, habitat-adapted symbiosis has been observed in some groups of fungal endophytes, and they impart a host-specific tolerance to stress in limiting environments (Qin et al., 2017). Certain groups of soil fungi have only recently been associated with the rhizosphere and endophytic situations, particularly in extreme environments or where one or more forms of stress exist (Khidir et al., 2010). Like some bacterial species, that live both as saprophytes in the soil and as endophytes, many fungal species can be isolated from both the free soil, far from the roots of plants and in close association with them (Maciá-Vicente et al., 2012). However, it must be said that several studies, conducted at different functional scales, have shown that in saline soils, the gradient of salinity as well as the presence of a high spatial heterogeneity favor the presence of species with very different functional traits and an extremely uneven distribution of communities (Maciá-Vicente et al., 2012). A similar phenomenon has been observed in the distribution of communities of bacteria in Mediterranean saline soil. Canfora et al. (2014) showed apparent differences in bacterial community distribution, diversity and composition, according to an increasing degree of soil salinity, as a consequence of a multi-scale spatial variability. A patchy distribution of vegetation structure and soil chemical properties coincided with a heterogeneous distribution of many bacterial groups. Conversely, some bacterial phyla resulted spread in the whole study area, along with the occurrence of a significant number of "salinity unrelated" phyla (*Nitrospira*, *Spirochaetes*). Canfora et al. (2014) hypothesized that a patchy saline environment could be "compared to a set of islands that allow the formation of different communities, separated from each other by the discontinuity of the limiting and stress factors".

Therefore, a patchy saline environment would contain not a homogeneous microbial community developed to tolerate an extreme environment, but a whole set of different communities. By comparing this evidence to the rhizosphere, it is also possible to imagine that the roots and tissues of halophilic or halotolerant plants in a saline soil represent islands of biodiversity and constitute a complicated system in which processes, at the microscale level, are particularly relevant. The living conditions within the tissues of a plant in an extreme environment such as a saline soil can reasonably be less limiting than those an organism can experience when living free in the soil. Therefore, it can be hypothesized that in saline soils, it is possible to find species as endophytes that in other systems are more easily found free in the soil. Kearl et al. (2019) isolated bacteria from halophytes (*Salicornia* and *Allenrolfea*) and observed that there were different populations in samples collected at different times of the year, with a majority of the genera, however, present independent of when the samples were collected.

Thiem et al. (2018) analysed the community structure of plant-associated endophytes of *Alnus glutinosa*, that is a dual mycorrhizal tree that forms ectomycorrhizal (EM) and arbuscular (AM) root structures, and can typically associate also with nitrogen-fixing actinomycetes. The authors sampled the plant's root microbiome present at two forest test sites (saline and non-saline). They found that the dominant type of root microsymbionts of alder were ectomycorrhizal fungi, whose distribution depended on the site (salinity). In contrast, representatives of fungal saprotrophs or endophytes displayed the opposite tendency.

Same applies to fungi, as the Pleosporalean taxa (i.e. Pleosporales order, within the class Dothideomycetes, Ascomycota) and other generalist endophytes and epiphytes that seem particularly present in high salinity environments (Quin et al., 2017). Pleosporalean fungi like those belonging to the genera *Pleospora*, *Alternaria*, and *Phoma*, are very frequent colonisers in halophytes (Quin et al., 2017 and references therein), and in plants from arid environments (Khidir et al., 2010), and have common traits of endophytes from other adverse environments. More recent studies based on a comprehensive molecular analysis involving both fungal and bacterial communities have also highlighted a close relationship between the two. Furtado et al. (2019) examined the microbiome of the non-mycorrhizal halophyte *Salicornia europaea* and showed a significant influence of the *Salicornia* bacterial community on the fungal one, but not the other way around. They also found that the sampling season was not influencing the biodiversity. Seasonality did not appear to be an essential factor in shaping endophytic microbial communities in saline soils also from other studies (Thiem et al., 2018).

Recent studies illustrated some main mechanisms that emerged as capable of supporting directly or indirectly plant growth under saline stress (Kushwaha et al., 2020). Plants' strategy to survive under salinity conditions comprises the synthesis and accumulation of osmolytes, as free amino acids (i.e. proline) and sugars to sustain an adequate osmotic cellular pressure needed for the functioning of cellular metabolism

(Kushwaha et al., 2020). Endophytes in plants living in saline soils proved to help them accumulating osmolytes and antioxidant compounds (Vaishnav et al., 2019).

STRATEGIES FOR INCREASING SALT STRESS TOLERANCE

Halophytes are considered model plants, enabling the study of adaptive mechanisms like the induction of enzymes with antioxidant functions, the accumulation of toxic ions in their vacuoles, the storage of compatible soluble substances, occurring in the cell in response to cellular stress. Consequently, the salt-resistance genes involved in the above processes can be expressed in conventional crops, increasing their resistance to environmental salinity. So far, however, this strategy has proved to be inefficient and was implemented mainly under laboratory conditions. An equally costly and environmentally unfriendly approach in the production of salinity resistant plants is the pre-treatment of biological materials with specific, selective, chemicals, e.g. ascorbic acid, nitric oxide, H_2O_2 , Ca^{2+} , K^+ , paraquat and glutamate, silicon, phosphorus and humic acid, glycine betaine, jasmonates and salicylic acid, 5-aminolevulinic acid (El-ESawi et al., 2018) or with physical effectors like UV-B irradiation (Dhanya Thomas et al., 2020). However, these methods are not recommended for sustainable agriculture. Instead, the use of soil bacterial and fungal community colonizing plant's roots and stimulating plant's growth under stress conditions are promising for the increase of agricultural productivity in saline areas. Kloepper and Schroth (1978) introduced the term "rhizobacteria" to describe this microbial community collectively. Three years later, the same authors expanded the term to "plant growth-promoting rhizobacteria" (PGPR). PGPRs can be distinguished between "extracellular plant growth-promoting rhizobacteria (ePGPR)" and "intracellular plant growth-promoting rhizobacteria (iPGPR)" (Martinez-Viveros et al., 2010). The ePGPRs are bacteria that can have their niche in the rhizosphere, on the rhizoplane, or in the interstitial microenvironments of the root cortex. The iPGPRs live inside specialised nodular structures of root cells (Bhattacharyya and Jha, 2012). Some ePGPRs and iPGPRs show tolerance to a high concentration of salts and thus can also grow in saline soils and be associated to halophytes, or more in general, in niches characterized by low water potentials due to salt stress or to a dry climate (Khan et al., 2019) (**Supplementary Material Table 1**). Salt-tolerant PGPRs (ST-PGPR) include mainly bacteria of the genus *Bacillus*, *Pseudomonas*, *Enterobacter*, *Agrobacterium*, *Streptomyces*, *Klebsiella* and *Ochrobactrum* (Sharma et al., 2016). Whipps (2001) roughly distinguished between three strategies of interaction of the rhizobacteria with the plants: neutral, negative, or positive. Neutral interaction means that the rhizobacteria, although living in the plant's rhizosphere have no visible effect on the growth and metabolism of the host (Beattie, 2006). Positive interactions comprise those ST-PGPRs (salt-

tolerant plant growth promoting rhizobacteria) that have a promoting effect on plant physiology and tolerance to salinity (Figure 2). The ST-PGPRs, in turn, from a utilitarian point of view, can be subdivided into biofertilizer microorganisms, phytostimulators, and biopesticides. Negative interactions comprehend all those situations where the bacteria are phytopathogenic or produce substances toxic to plants (i.e. hydrogen cyanide or ethylene). However, sometimes, the secretion of the toxic substance, such as hydrogen cyanide by PGPRs, is also beneficial as it is one of the many ways to protect the plant from phytopathogens. ST-PGPR can improve plant's growth and tolerance to salinity stress through the accumulation of osmolytes (proline, trehalose, and glycine betaines) (Supplementary Material Table 2), the production of phytohormones (auxins, gibberellins, cytokinins), ion homeostasis, the improvement of nutrients uptake (N_2 fixation, solubilising of P, K, Zn, and Si), the activation of plant's antioxidative enzymes, and the synthesis of ACC deaminase, indole-3-acetic acid (IAA), exopolysaccharides, and siderophores (Egamberdieva et al., 2019).

Plant growth-promoting fungi (PGPFs) are soil-borne, non-pathogenic, saprophytic microorganisms. This group contains several fungal taxa, and among the most quoted genera,

especially in saline environments, there are *Penicillium* spp., *Fusarium* spp., *Alternaria* spp., *Aspergillus* spp., *Sclerotium* spp., and *Phoma* spp., which colonise plants roots and form symbiotic interactions with them (Gangwar et al., 2017; Naziya et al., 2020). Pereira et al. (2019), for example, surveyed the culturable endophytic mycobiota of *Festuca rubra*, a perennial grass diffused in coastal environments with low nutrient availability, wind, and salinity. Taxa belonging to *Fusarium*, *Diaporthe*, Helotiales, *Drechslera*, *Slopeiomyces*, and *Penicillium* were constant inhabitants of the plant's roots (occurred in more than 20% of the plants the authors analysed). Seventy-one point eight percent of the strains they could culture were halotolerant. When the authors used the isolates to inoculate the grass *Lolium perenne*, a *Diaporthe* strain increased leaf biomass production under both normal and saline watering regimes (200 mM NaCl). PGPFs enhance the plant growth directly and indirectly by the production of IAA, siderophore, cellulase, chitinase, gibberellins, and increasing phosphorus solubilisation and availability (Mishra et al., 2017; Naziya et al., 2020).

High salinity increases susceptibility to various phytopathogens and promotes some fungal soil-borne diseases in plants. Crops protection against pathogens is extremely difficult, because the use of chemicals agents e.g. fungicides, bactericides and nematicides

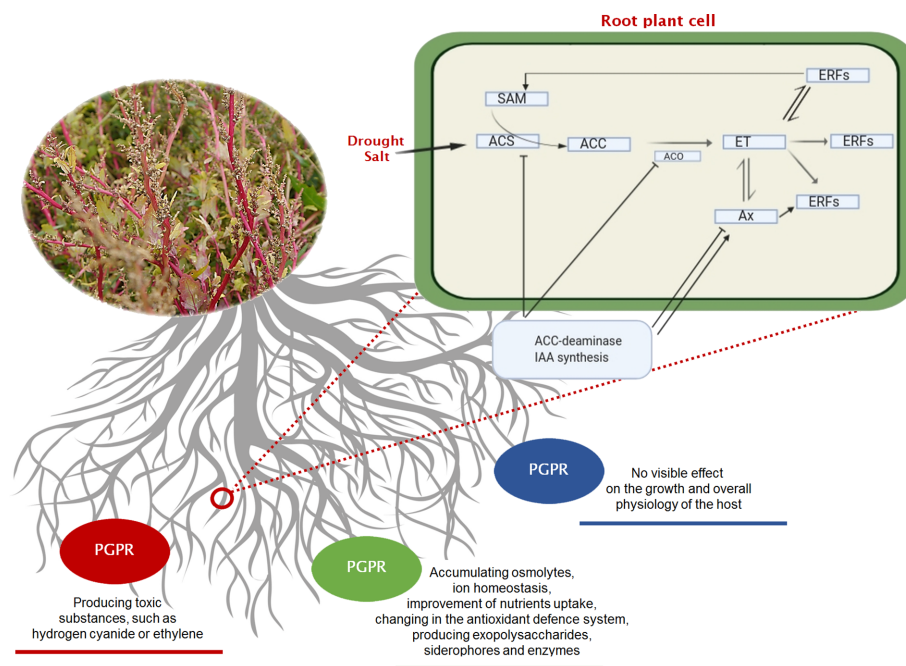


FIGURE 2 | Three basic strategies of interaction (neutral—blue line, negative—red line, positive—green line) existing between the rhizobacteria and growing plants. In the negative interaction, it is highlighted the pathway of ethylene (ET) that occurs under soil stress, and the effect of mitigation that can be played by PGPRs on a plant root's cell. Regulation of ethylene signalling and plant stress response. Ethylene pathway in plants. ACC (1-aminocyclopropane-1-carboxylic acid) the amino acid methionine is converted to SAM (S-adenosylmethionine) by the action of ACC synthase enzyme (ACS). ACC is then converted to ethylene by the enzyme ACC oxidase (ACO), triggering different ethylene response factors (ERFs). Plant growth-promoting bacteria can alter all steps of ethylene signalling. Some bacteria species can increase the ethylene levels by producing ACC oxidase (microbial ethylene-forming enzyme), by inducing ACC synthase in plant or by affecting other plant hormones indirectly. They can also modulate ethylene response by producing plant hormones that interact with ethylene signalling. Other microorganisms can also decrease ethylene production by cleaving its precursor ACC. ACC, 1-aminocyclopropane-1-carboxylate; ACS, ACC synthase; ACO, ACC oxidase; Ax, Auxine; ET, Ethylene; ERFs, Ethylene Response Factors; SAM, S-adenosyl methionine.

has a negative impact on the environment and organisms while increases the costs of cultivation (Egamberdieva et al., 2017; Sharma et al., 2017). An alternative to the use of chemicals is the use of PGPRs and PGPFs, which could synthesise the aforementioned lytic enzymes, siderophores, IAA, and antibiotics. These compounds could reduce and inhibit phytopathogens that cause plant infections (Labuschagne et al., 2010; Murali et al., 2012; Gangwar et al., 2017). *Trichoderma* isolates widely used in bio-fungicidal formulations can be ineffective at high salinity conditions, since most of these fungal species have a low osmotolerance (Mohamed and Haggag, 2006; Bheemaraya et al., 2013). However, some studies targeted halotolerant biological control fungi. This is the case of the experiments by Gal-Hemed et al. (2011) who isolated *T. atroviride* and *T. asperelloides* from the Mediterranean sponge *Psammocinia* sp. and used them to reduce *Rhizoctonia solani* disease in beans, and more recently the study by Sánchez-Montesinos et al. (2019). They isolated *Trichoderma* strains from the seagrass *Posidonia oceanica* and evaluated its capacity to control the disease caused by *Pythium ultimum* in melon seedlings under various levels of salt stress.

Osmoprotectants

In response to salinity stress, ST-PGPRs accumulate many metabolites called compatible (organic) solutes, among which amino acids and derivatives (e.g., glutamate, proline, peptides, and N-acetylated amino acids), quaternary amines (e.g., glycine betaine and carnitine), sugars (e.g., sucrose and trehalose), and tetrahydro pyrimidines (ectoines). These metabolites enhance the stability of protein conformation, the balance of cell redox condition, cytosolic pH, complex II electron transport, membrane integrity, and the activity of enzymes such as ribulose biphosphate carboxylase/oxygenase (RUBISCO) (Saghafi et al., 2019). Moreover, the storage of osmolytes represents a successful stress response mechanism that helps the bacteria to limit water loss, increasing their cytoplasmic concentration of K^+ . In some halophilic bacteria, the internal concentration of osmoprotectants due to salt stress may reach up to 1 M (Saum and Müller, 2007). Rodríguez-Salazar et al. (2009) observed that *Azospirillum* spp. accumulates proline, trehalose, and glycine betaine as a mechanism for protection against osmotic stress. Kushwaha et al. (2019) found in *Halomonas* sp. that accumulation of betaine suppresses the *de novo* synthesis of ectoine at low NaCl concentrations, however, at higher NaCl concentrations the amount of ectoine is significantly larger than betaine. It means that the salinity stress transcriptionally up-regulates ectoine accumulation. The expression of the genes *proH*, *proJ*, and *proA* involved in proline biosynthesis was induced in some of the halophilic bacteria at higher salt concentration, leading to the highest accumulation of proline (Saum and Müller, 2007). Also, several reports determined the link between proline accumulation and pyrroline-5-carboxylate synthase (P5CS) gene expression level after PGPR inoculation and hypothesized that bacterial treatment upregulates the P5CS gene expression in plant roots, causing intracellular storage of free proline (Kim et al., 2007; Kumari et al., 2015a; Kumari et al., 2015b). Many ST-PGPRs showed a high expression of genes

implicated in trehalose biosynthetic pathways (e.g. trehalose 6-phosphate gene) (Qin et al., 2018). Trehalose is an osmoprotectant, and its role in salt-stress tolerance has been well documented (Garg et al., 2019; Orozco-Mosqueda et al., 2019; Shim et al., 2019). Figueiredo et al. (2008) reported that *Rhizobium tropici* and *Paenibacillus polymyxa* were modified to overexpress trehalose 6-phosphate gene and were co-inoculated in *Phaseolus vulgaris* plants resulting beneficial for plants grown under saline stress, with higher nodulation and N content. A differential gene expression analysis of the tissues of the nodules compared to normal roots revealed upregulation of stress tolerance genes, which suggested that extracellular trehalose works as an osmoprotectant, including tolerance to salinity (Figueiredo et al., 2008).

Ion Homeostasis

One strategy used by bacteria to limit salt uptake, also by plants, is by trapping cations in their exopolysaccharide matrix. This mechanism results in an altered root structure with the formation of extensive rhizo-sheaths (agglutinated soil that adheres to roots when they are removed from the pot or field). Moreover, at the rhizosphere level, it has been found differential regulation of the expression of genes involved in ion affinity transporters. PGPR often impact the mineral nutrient exchange of both macro and micronutrients as a strategy to react to nutrient imbalance due to a higher uptake of Na^+ and Cl^- ions (Saghafi et al., 2019). Both fungi and bacteria can help plants to keep cellular ion homeostasis and sustainable K^+/Na^+ ratios in shoots. This has been documented as a mechanism that reduces Na^+ and Cl^- accumulation in leaves, by increasing Na^+ exclusion *via* roots and boosting the activity of high-affinity K^+ transporters (Ilangumaran and Smith, 2017). Zhang et al. (2008a) reported that the inoculation of *Arabidopsis thaliana* with *B. subtilis* moderated the adverse effects of salinity by regulating HKT1 potassium transporter. This bacterium also stimulated the overexpression of the AtHKT1 gene, expressing for a high-compatibility transporter for potassium ion, in *Arabidopsis* under conditions of salt stress. *Puccinellia tenuiflora*, a salt-excluding halophytic grass, when inoculated with *B. subtilis* showed lower levels of Na^+ accumulation. The plant at the same time upregulated plasma membrane Na^+/H^+ transporters SOS1, and HKT-type protein and tonoplast Na^+/H^+ antiporters genes. However, one of the HKT genes was downregulated in roots under high salt concentrations (Zhang et al., 2008a; Niu et al., 2016). This showed how the bacterium synergistically regulated Na^+ homeostasis by controlling Na^+ -transport systems at the whole-plant level under both lower and higher salt conditions, differentiating the mechanisms at play in case of high or mild salinity conditions. According to Rojas-Tapias et al. (2012), the inoculation of auxin-producing strains of *Azotobacter* in maize plants exposed to saline stress resulted in better K^+ uptake and Na^+ exclusion from plant's tissues.

Moreover, the authors showed that after PGPR inoculation chlorophyll, proline, and polyphenol contents in maize leaves increased along with a better general plant stress response (Rojas-Tapias et al., 2012). Ilangumaran and Smith (2017) reported that in many studies on the interaction between

plants and PGPR species, the genes involved in ion homeostasis showed differential expression under saline stress. For example, in an experiment where *Arabidopsis thaliana* was treated with *Burkholderia phytofirmans* the expression of both bacterial and plant's genes involved in ion homeostasis (KT1, HKT1, NHX2, and SOS1) was rapidly altered as a result of an imposed saline stress (Pinedo et al., 2015).

Also, fungi can play a decisive role in a plant's ability to improve nutrients' uptake and regulate the osmotic balance in soils affected by salinity. Arbuscular mycorrhizal fungi are plants symbionts that increase root phosphorus uptake and confer the mycorrhizal plants' tolerance to salinity. Romero-Munar et al. (2019) demonstrated that in *Arundo donax* a commercial inoculum containing the arbuscular mycorrhizal *Rhizophagus intraradices* and *Funneliformis mosseae* improved the nutritional status by enhancing nutrient use efficiency. The authors suggested that increased use efficiency of phosphorus could have improved ion (Na^+ and K^+) uptake and allocation. Arbuscular mycorrhizal fungi have also been reported to enhance the ability of wheat plants to modulate the reactive oxygen scavenging system when coping with salinity stress (Talaat and Shawky, 2011) (Table 1).

Symbiotic fungi can modulate gene expression of the host plant to modify its phenotype in order to improve the tolerance to abiotic stress factors caused by soil salinity (Khan et al., 2013). Molina-Montenegro et al. (2020) showed that inoculation of plants' roots with Antarctic fungal endophytes improves growth and survival by changing the expression of a gene responsible for Na^+/H^+ antiporters proteins integrated with vacuolar membranes. In particular, the NHX proteins are involved in the maintenance of cell turgor through ionic balance control and are associated with the capacity of accumulating Na^+ inside vacuoles.

The Improvement of Phosphorus, Potassium, and Zinc Solubilisation

Phosphorus (P) is one of the essential macronutrients for plants, although its availability is limited due to its low solubility. P is required as an essential nutritional element for photosynthesis, energy transfer, biosynthesis of macromolecules and respiration (Fernandez et al., 2007). The average content of phosphorus ions in the soil is 0.05% (w/w). Still, often only 0.1% of the total P is available to plants because of its precipitation in soil (Etesami and Beattie, 2018). Salinity leads to depletion and sedimentation of absorbable phosphorus. Phosphate-solubilising halotolerant PGPRs (*Bacillus*, *Pseudomonas*, *Achromobacter*, *Alcaligenes*, *Brevibacterium*, *Serratia*, *Xanthomonas*, and *Rhizobium*) provide an opportunity to enhance P availability to plants without deteriorating soil salinity levels. These microorganisms can hydrolyse inaccessible phosphorus forms into absorbable forms via various mechanisms like chelation, ion exchange, and acidification by secreting low molecular weight organic acids, such as gluconic acid, citric acid, succinic acid, propionic acid, and lactic acid (Choudhary, 2012; Etesami and Beattie, 2018; Saghaei et al., 2018). In salt-affected soils, the inoculation of wheat with *Bacillus aquimaris* increased plant P content under salinity stress (Upadhyay and Singh, 2015). Khan et al. (2019)

identified highly salt stress-tolerant strains of *Arthrobacter woluwensis*, *Microbacterium oxydans*, *Arthrobacter aurescens*, *Bacillus megaterium*, and *Bacillus aryabhattai*, which showed to increase phosphate uptake in several plants: *Artemisia princeps*, *Chenopodium ficifolium*, *Echinochloa crus-galli*, and *Oenothera biennis* (Khan et al., 2019). Salcedo et al. (2014) identified seven best phosphate-solubilising actinobacteria strains out of the 57 strains that they isolated from soil. El-Tarabily and Youssef (2010) screened the mangrove *A. marina* rhizosphere and identified 129 bacterial strains capable of solubilising rock phosphate. In particular, *Oceanobacillus picturae* showed to be able to mobilize 97% of the available mineral P. Many other genera of bacteria isolated from halophytes (i.e. *Arthrobacter*, *Bacillus*, *Azospirillum*, *Vibrio*, *Phyllobacterium*) were found capable to implement P absorption in halophytes under salinity stress (Banerjee et al., 2010; Yasmin and Bano, 2011). Bashan et al. (2000) showed that in the leaves of halophytes inoculated with halotolerant PGPRs, as species of the genera *Azospirillum*, *Vibrio*, *Bacillus*, and *Phyllobacterium*, the P content increased. Vaishnav et al. (2016) used a hydroponic system to demonstrate how insoluble phosphate-solubilising bacteria that solubilise sedimentary phosphorous actively increased the availability of assimilable P to plants in salinity stress conditions. Yadav et al. (2011) reported the contribution of *Aspergillus niger*, *Penicillium citrinum*, and *Trichoderma harzianum* in phosphate solubilisation and their beneficial effects on chickpea growth. Amongst studied fungal genera, the highest P-solubilizing ability was attributed to *Aspergillus* and *Trichoderma* species. Ceci et al. (2018) showed that many saprotrophic fungi could mobilize P from insoluble forms according to a variety of mechanisms, with strains of *Rhizopus stolonifer* var. *stolonifer*, *Aspergillus niger* and *Alternaria alternata* among the best performing strains in terms of amounts of insoluble phosphate solubilisation.

Apart from phosphorus, another essential nutrient ingredient for plants is potassium (K). This element plays a vital role in plant metabolism and improves the quality of the crop production due to its role in grain filling, and in promoting disease resistance, leading to a higher resistance of plants to stress. The concentration of potassium in the soil solution is usually 1%–2% (Sindhu et al., 2010). The potential of soil application of potassium solubilising microorganisms (KSBs) is widely studied, especially in saline soils where this element is even less available to plants. These bacteria solubilise potassium-containing minerals (mica and orthoclase) by producing tartaric, succinic, citric, oxalic, and alpha-ketogluconic acids (Saghafi et al., 2019). Singh et al. (2010) reported that *Bacillus mucilaginosus*, *Azotobacter chroococcum*, and *Rhizobium* sp. were able to increase potassium absorption by wheat and corn.

Several authors showed that also fungi, especially ectomycorrhizal species, can weather silicate minerals to extract nutrients like P actively, K, Ca, Mg, and Fe, in particular under conditions of nutrient limitation. Mycorrhizal (ecto- and endo-) contribution to K^+ acquisition by plants has also been demonstrated (Benito and Gonzalez-Guerrero, 2014). Fungi are already used at industrial level to mobilize or precipitate also other metals, like Cu, Mn, Zn, even though they are poorly applied for

TABLE 1 | The effect of halophilic PGPRs and PGPFS on alleviating salt stress in halophytes.

Host halophyte	Microorganisms	Bacterial/Fungal activity	Plant response	References
<i>Arthrocnemum macrostachyum</i> L. (glaucous glasswort)	<i>Bacillus alcalophilus</i> , <i>Bacillus thuringiensis</i> , <i>Gracibacillus saliphilus</i>	IAA production, siderophore, and phosphate solubilization	Mitigating the effects of high salinity on plant growth and physiological performance	Navarro-Torre et al., 2017
<i>Aster tripolium</i> L. (sea aster)	<i>Bacillus cereus</i> , <i>Serratia marcescens</i>	IAA production, siderophore production, N ₂ fixation, and ACC deaminase activity	–	Szymańska et al., 2016
<i>Atriplex leucoclada</i> L. (orache)	<i>Arthrobacter pascens</i>	Phosphate solubilization and siderophore production	Increase in root and shoot length, fresh and dry weight, accumulation of osmolytes (e.g., sugar, proline), increase in activity of antioxidant enzymes	Ullah and Bano, 2015
<i>Bassia indica</i> L. (<i>Kochia indica</i>)	<i>Bacillus subtilis</i>	IAA and ACC deaminase production	Improving root and shoot growth, total lipid content, the phospholipid fraction, photosynthetic pigments (chlorophyll a and b and carotenoid contents); 200 mM NaCl	Abeer et al., 2015
<i>Beta vulgaris</i> L. (beet)	<i>Micrococcus yunnanensis</i> , <i>Planococcus rifietoensis</i> , <i>Variovorax paradoxus</i>	ACC deaminase production	Improving germination and plant biomass, higher photosynthetic capacity and lower stress-induced ethylene production; 50–125 mM NaCl	Zhou et al., 2017
<i>Brassica napus</i> L. (canola)	<i>Rhizobium leguminosarum</i> , <i>Sinorhizobium mellilote</i> , <i>Bacillus aryabhattai</i> , <i>Brevibacterium epidermidis</i> , <i>Micrococcus yunnanensis</i>	IAA, ACC deaminase production and phosphate solubilizing IAA, ACC deaminase, ammonia production, nitrogen fixation, phosphorus and zinc solubilization, thiosulfate oxidation, production of extracellular hydrolytic enzymes	Increasing in all of the growth indices (plant height, root and shoot dry weight), nutrient uptake and restricted availability for plants 40% increase in root elongation and plant dry weight; 150 mM NaCl	Saghafi et al., 2018 Siddiquee et al., 2010
<i>Catharanthus roseus</i> L. (Madagascar periwinkle)	<i>Enterobacter cloacae</i> , <i>Paenibacillus xylanexedens</i>	IAA, ACC deaminase production	Enhance plant root elongation	Yaish et al., 2015
<i>Cicer arietinum</i> (chickpea)	<i>Achromobacter xylosoxidans</i>	ACC deaminase production, nitrogen fixation, increasing the level of antioxidative enzyme	Decreasing stress ethylene level; influence on germination, plant height and root weight	Karthikeyan et al., 2012
<i>Coriandrum sativum</i> (coriander)	<i>Halomonas variabilis</i> , <i>Planococcus rifietoensis</i>	EPS production	Increasing the plant growth and soil aggregation	Qurashi and Sabr, 2012
<i>Glycine max</i> L. (soybean)	<i>Pseudomonas pseudoalcaligenes</i> , <i>Pseudomonas putida</i>	P-solubilization, photosynthetic pigments, IAA, ACC deaminase production and increasing the level of POD	Improving plant growth and root system	Al-Garni et al., 2019
<i>Helianthus annuus</i> L. (sunflowers plant)	<i>Pseudomonas sp.</i>	EPS production	Effects on the elongation of shoots and roots, number of lateral roots, shoot and root fresh weight, and decreased Na ⁺ /K ⁺ ratio under salinity stress.	Kasotia et al., 2016
<i>Prosopis strombulifera</i> (creeping screwbean)	Bacterial strains	ACC deaminase production	Increasing plant height, shoot dry weight and root dry weight, phosphorus, potassium contents, and K ⁺ /Na ⁺ ratio in the shoot	Kiani et al., 2015
<i>Salicornia brachiata</i> (glasswort)	<i>Achromobacter xylosoxidans</i> , <i>Bacillus licheniformis</i> , <i>Bacillus pumilus</i> , <i>Brevibacterium halotolerans</i> , <i>Lysinibacillus fusiformis</i> , <i>Pseudomonas putida</i>	IAA production, siderophore production, N ₂ fixation, ACC deaminase activity, gibberelin production, protease and antifungal activity	–	Sgroy et al., 2009
	<i>Brachyбактерium saurashtrense</i>	IAA production, siderophore production, N ₂ fixation, ACC deaminase activity	–	Gontia et al., 2011
	<i>Agrobacterium tumefaciens</i> , <i>Brachyбактерium saurashtrense</i> , <i>Brevibacterium casei</i> , <i>Haererohalobacter sp.</i> , <i>Zhinguelluella sp.</i>	IAA production, phosphate solubilization, siderophore production, N ₂ fixation, ACC deaminase activity	Increase in amino acids, IAA, content of Ca ²⁺ , P, N of the inoculated plants; in the percentage of water content in roots and shoots in inoculated plants; increase total biomass; increased in plant length and dry weight compared to un-inoculated plants	Shukla et al., 2012

(Continued)

TABLE 1 | Continued

Host halophyte	Microorganisms	Bacterial/Fungal activity	Plant response	References
Solanum lycopersicum L. (tomato)	<i>Leclercia adecarboxylata</i>	IAA, ACC deaminase and osmoprotectants production	Improving plant growth	Kang et al., 2019
	<i>Achromobacter piechaudii</i>	ACC deaminase production	Increasing fresh and dry weights of tomato seedlings grown in the presence of up to 172 mM NaCl salt	Mayak et al., 2004
<i>Suaeda salsa</i> L. (seepweed)	<i>Pantoea agglomerans</i> , <i>Pseudomonas oryzae</i> habitant, <i>Pseudomonas putida</i>	Gibberellic acid production, IAA production, ACC deaminase activity, siderophore production, abscisic acid production, and antifungal activity	–	Teng et al., 2010
<i>Triticum aestivum</i> L. (wheat)	<i>Bacillus methylotrophicus</i> , <i>Bacillus siamensis</i> , <i>Bacillus</i> sp.	IAA, ACC deaminase, and EPS production	Influence on the germination rate of wheat seedlings, root and shoot length, and photosynthetic pigments.	Amna et al., 2019
	<i>Klebsiella</i> sp.	Auxin and siderophores production	Increasing the plant biomass; 0.25 M and 0.45 M NaCl	Acuña et al., 2019
	<i>Serratia</i> sp.	IAA, ACC deaminase and ammonia production, nitrogen fixation and phosphate solubilization	Increasing root and shoot length and total fresh plant weight	Orhan, 2016
	<i>Bacillus gibsonii</i> , <i>Bacillus</i> sp., <i>Halomonas</i> sp., <i>Oceanobacillus oncorhynchi</i> , <i>Zhihengliuella</i> spp.			
<i>Triticum turgidum</i> subsp. durum (durum wheat)	rhizospheric and endophytic bacteria	Nitrogen fixation, ACC deaminase and auxin production, inorganic phosphate solubilization and siderophore production	Improving survival in inoculated plants under high salinity stress conditions, faster germination rates, and seedling growth	Albdaiwi et al., 2019
<i>Zea mays</i> L. (maize)	<i>Serratia liquefaciens</i>	antioxidant enzymes (APX, CAT, SOD, POD), non-enzymatic redox antioxidants (ascorbic acid and glutathione) induction and osmoprotectants production	Reduction of oxidative stress markers and increase the maize growth and biomass production along with better leaf gas exchange, osmoregulation, antioxidant defence systems, and nutrient uptake under salt stress (80 and 160 mM NaCl)	El-Esawi et al., 2018
	<i>Pantoea alli</i> , <i>Pseudomonas reactans</i> , <i>Rhizoglyphus irregularis</i>	Osmotic adjustment	Consortium tended to mitigate ion imbalances in plants across the gradient of NaCl (0–5 g/kg of soil), promoting maize growth and nutritional status.	Moreira et al., 2020
<i>Arundo donax</i> L. (giant reed)	<i>Rhizophagus intraradices</i> <i>Funnelliformis mosseae</i>	Improving the nutritional status of plants by enhancing nutrient use efficiency	Improving plant growth (1, 75, and 150 mM NaCl)	Romero-Munar et al., 2019
<i>Brassica napus</i> L. (canola)	<i>Trichoderma parareesei</i>	Increasing the expression of genes related to the pathways of ethylene	Increasing rape seed yield	Poveda, 2020
<i>Lolium perenne</i> (ryegrass)	<i>Diaporthe</i> strain S69	Ion homeostasis	Promoting leaf biomass production (200 mM NaCl)	Pereira et al., 20192019
	<i>Arthrinium gamsii</i> sp., <i>Stereum gausapatum</i> sp., isolated from <i>Salicornia europaea</i>	Siderophores, polyamines, IAA and cellulolytic, proteolytic, lipolytic and chitinolytic enzymes production	Increasing the length, fresh and dry weights of the shoots and roots	Furtado et al., 2019b
<i>Sesamum indicum</i> L. (sesame)	<i>Penicillium</i> sp.	Chlorophylls, proteins, amino acids, and lignans production	Increasing the length of shoot and root, and fresh and dry seedling weight (150 mM NaCl)	Radhakrishnan et al., 2014
Wheat (cv. Yongliang 4)	<i>Trichoderma longibrachiatum</i>	Improvement of the antioxidative defense system and gene expression in the stressed plants	Increasing root and shoot length and total fresh plant weight	Zhang et al., 2016
Wheat	<i>Trichoderma reesei</i>	Flavonoid, phenolic compounds, phytohormones, including IAA and gibberellic acid production	Improving plant growth. Increased amount of chlorophyll a and b, carotenoids	Ikram et al., 2019

plant nutrition. There are studies, however, for the application of fungi and bacteria as solubilizers of specific nutrients, and some bacteria are already applied in fertilization treatments to this aim.

Zinc deficiency, for example, is a significant problem for the plant, especially in saline arid and semi-arid soils. Plants absorb Zn mainly in the form of Zn^{2+} , zinc hydrate, and organic zeolite and use it in biochemical reactions, for the stability of biological membranes, the activity of oxidative and carbonic anhydrase enzymes and the synthesis of the enzyme auxin (Broadley et al., 2007; Alaghemand et al., 2018). The most important method to provide plants with Zn is the application of rhizobacteria together with Zn-containing fertilizers. These bacteria can increase the solubility of poorly soluble Zn compounds by employing different mechanisms, such as chelation by siderophore (Tariq et al., 2007), reduction of soil pH by the production of organic acids (2-ketogluconic acid, gluconic acid) and proton secretion (Subramanian et al., 2009). Abaid-Ullah et al. (2015) reported that *Serratia* sp. could increase wheat yield through solubilisation of ZnO under different climates.

Biological Nitrogen Fixation by PGPRs

Halophytic crop species used in agriculture can be limited by the lack of available nitrogen often affecting saline soils. PGPRs can fix nitrogen through symbiotic and non-symbiotic mechanisms (Saghafi et al., 2019). The first method involves the formation of nodes in the host roots by bacteria which results in nitrogen content of approximately 65% of the total nitrogen assimilation by plants (Rajwar et al., 2013). The other group of nitrogen-fixing bacteria, including *Azospirillum*, *Azotobacter*, *Burkholderia*, *Herbaspirillum*, *Bacillus*, and *Paenibacillus* is not plant-specific (Goswami et al., 2015). Salt-tolerant N_2 -fixing PGPRs are an essential source of available N in saline soils, and the amount of nitrogen fixed by these bacteria has been estimated as $20\text{--}30 \text{ kg h}^{-1} \text{ year}^{-1}$ (Oberson et al., 2013). The potential benefits of nitrogen-fixing strains to halophytes and salt-sensitive crops underline the need of implementing the studies on N_2 -fixing halotolerant PGPRs to be used as boosters in saline soil-based agriculture (Goswami et al., 2015; Ilangumaran and Smith, 2017).

Siderophore Production

Some strains of bacteria produce siderophores, especially in the rhizosphere, this increases plant growth and prevent phytopathogens from proliferation by inhibiting them from accumulating iron (Scavino and Pedraza, 2013). Siderophores are Fe(III)-chelating compounds, usually small and with high-affinity so that plants, in need for iron nutrient, quickly access the iron-siderophore complexes. Iron is an integral part and cofactor of enzymes involved in plants' respiration, photosynthesis, N_2 fixation and many other biochemical processes (Abbas et al., 2015; Etesami and Beattie, 2018). Bacterial siderophores have a higher affinity for iron than fungal pathogens, which require iron for their metabolism and plants' infecting mechanisms (Miethke and Marahiel, 2007). Many halotolerant PGPRs and PGPFs, particularly those isolated from halophytes (Table 1) produce iron siderophores. Among biocontrol agents, the strains belonging to *Pseudomonas* sp. secreting non-fluorescent and fluorescent siderophores such as pyochelins and pseudobactins are most effective competitors of Fe^{3+} . The potential

uses of siderophore producing bacterial strains have been reported in the suppression of fungal pathogens of rice and wheat (Labuschagne et al., 2010). Moreover, pyoverdine synthesised by *P. aeruginosa* under low-iron stress condition could inhibit the growth of *Aspergillus flavus*, *A. oryzae*, *F. oxysporum*, and *Sclerotium rolfsii* (Manwar et al., 2004).

Also, fungi, however, produce siderophores that can act as protectants against plants' pathogens. Fungal strains belonging to *Aureobasidium* and *Emerizellopsis* genera synthesise siderophores, which are part of the biocontrol strategies occurring in *Salicornia* plants (Furtado et al., 2019).

IAA Production

Indole-3-acetic acid (IAA) is the most common plant hormone of the auxin class, and it regulates various aspects of plant growth and development. IAA acts as an effector molecule between bacteria and IAA producing plants, and in bacterial-bacterial interactions (Spaepen and Vanderleyden, 2011). It is involved in many processes such as seed germination, root system development, or increasing plant tolerance to stress conditions (Aeron et al., 2011). IAA-producing microorganisms increase the root growth and root length of plants, which contributes to a greater root surface area enabling the plant to acquire more nutrients from the soil (Boiero et al., 2007). Tryptophan is the IAA precursor in most biosynthetic pathways. However, several reports indicate the possibility of IAA synthesis in tryptophan-independent reactions (Sitbon et al., 2000; Saghafi et al., 2018).

The positive impact of IAA produced by PGPRs on the growth of various plants in conditions of salinity stress has been determined. For example, IAA-producing, halotolerant and halophilic bacteria significantly affected root and shoot elongation and freshly available mass of *Triticum aestivum* plants under salt stress conditions (Orhan, 2016). Also, *Brassica napus* L. seedlings inoculated with IAA producing *Rhizobium* bacteria showed an improved growth rate under salt stress, regarding especially plant height, as well as root and shoot dry weight (Saghafi et al., 2018). It has been confirmed that the salt-tolerant *B. subtilis* promotes the growth and fitness of Indian bassia plants (*Bassia indica*) under salt stress by providing an additional supply of IAA, and induces salt stress resistance by reducing ethylene levels. Inoculation of unstressed and salt-stressed Indian bassia with *B. subtilis* has significantly improved root and shoot growth, total lipid content, the phospholipid fraction, the content of photosynthetic pigments and also increased oleic, linoleic, and linolenic acids in plant leaves, as compared to uninoculated plants (Abeer et al., 2015). IAA-producing bacteria are also involved in suppression of plant disease-causing pathogenic fungi. *Pseudomonas extremorientalis* and *P. aureantiaca* were successfully used for control cucumber root infection caused by *F. solani* (Egamberdieva et al., 2014). Other examples are given in Table 1. Sodium chloride (NaCl) induces a decline in the IAA level in rice seedlings (Sakhabutdinova et al., 2003). Auxin activates the transcription of various genes known as primary auxin response genes in *Arabidopsis*, rice and soybeans (Hagen and Guilfoyle, 2002). Auxin negatively regulates the expression of the rice gene adenosine phosphate isopentenyltransferase (OsIPT) that

encodes a key enzyme in CTK biosynthesis in nodes, thus inhibiting the growth of tiller buds in rice (Liu et al., 2011). Therefore, the identification of new genes that respond to high saline conditions offers investigators the opportunity to develop new approaches to select varieties with different mechanisms of tolerance to salinity stress (Zhu, 2002).

ACC Deaminase Production

The production of ACC deaminase enzyme is an essential mechanism for the direct promotion of plant growth by PGPRs. Bacterial IAA affects the level of ethylene in plants by increasing the activity of ACC deaminase, catalysing the hydrolysis of 1-amino-cyclopropane-1-carboxylic acid (ACC), an ethylene precursor, to ammonia and α -ketobutyric acid (Glick, 2005; Etesami et al., 2015). In fact, under stress conditions, including salinity, ethylene level increases in the plant and 1-aminocyclopropane-1-carboxylate (ACC) is more consistently enzymatically converted into ethylene (**Figures 2, 3**). Ethylene is a plant growth regulator and stress hormone which plays a key role in causing physiological changes in plants at the molecular level (Pierik et al., 2009). Ethylene interferes with plant growth under salinity stress by inhibiting roots elongation (Glick, 2005), causing defoliation, premature senescence (Shaharoona et al., 2006; Bari and Jones, 2009). To increase resistance against

the harmful effects of ethylene, plants are commonly treated with ACC deaminase-producing bacteria (Glick, 2005; Etesami et al., 2015).

So far, many researchers focused on the performance of PGPR with ACC deaminase activity to mitigate the adverse effects of elevated ethylene levels caused by salinity stress (**Table 1**). Siddikee et al. (2010) reported that 25 out of 140 halotolerant bacterial strains isolated from coastal soils of the South Korean Yellow Sea showed ACC deaminase activity. In particular, three of them *Brevibacterium epidermidis*, *Micrococcus yunnanensis*, and *Bacillus aryabhatai* generated more than 40% increase in root elongation and plants' dry weight when compared to uninoculated salt-stressed canola seedlings (Siddikee et al., 2010). In the study by Kiani et al. (2015), sunflower plants were inoculated with ACC deaminase-producing bacteria, which resulted in better growth in terms of plants' height and dry weight of shoots and roots. It has also been confirmed that *Achromobacter piechaudii*, an ACC deaminase-containing PGPR, can significantly increase the fresh and dry masses of tomato seedlings (Mayak et al., 2004). The study by Amna et al. (2019) investigated the role of halotolerant ACC deaminase-producing *Bacillus* spp. strains to help wheat seeds germination and seedling growth at different NaCl levels. There are pieces of evidence that ACC deaminase producing PGPRs enhance uptake of essential nutrients like N, P, and K, which

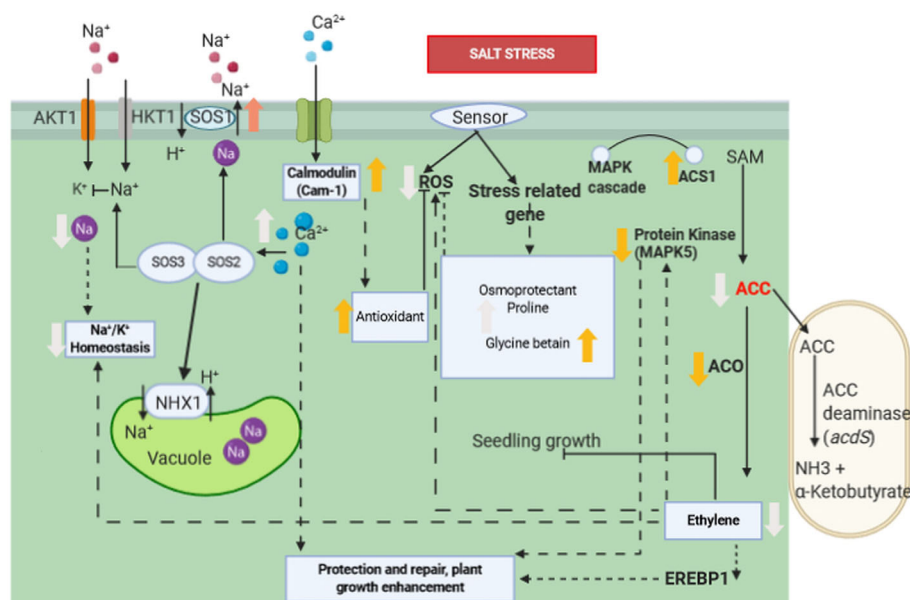


FIGURE 3 | Molecular interaction of ACCD-producing endophytic bacteria associated with plant roots under saline stress. Salt stress induces the ethylene biosynthesis pathway by upregulation of ACS1. However, ACC is consumed as a result of the activation of *acdS* gene encoding ACCD of PGPR, whereas ACO1 and EREBP1 are down-regulated and ethylene production is reduced as a consequence. The reduction of ethylene induces a lower expression of MAPK5 and reduces the accumulation of ROS. Increase in proline, betaine, and glycine improves salt tolerance in plant's roots. Ca^{2+} content is increased, and $\text{Na}^{+}/\text{K}^{+}$ ratio is decreased, which are correlated with up-regulation of *Cam1*, *SOS1*, and *NHX1* genes. Ca^{2+} signal activates the *SOS3/SOS2* protein kinase complex, which negatively regulates the activity of Na^{+} ion channel. Association of Ca^{2+} and calmodulin activates antioxidant enzymes which subsequently inhibits ROS. Bold orange arrow indicates gene regulation, bold white arrow indicates plant physiological regulation, black arrow indicates positive regulation, dashed arrow indicates indirect positive regulation, black line with bar-end indicates inhibition and dashed line with bar-end indicates indirect inhibition.

consequently increase K^+/Na^+ ratios in the stressed plants (Nadeem et al., 2009).

Some fungi use similar mechanisms to alleviate plants stress. Some *Trichoderma* strains produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase that regulates plants' endogenous 1-aminocyclopropane-1-carboxylic acid (ACC), which is the direct precursor of the plant hormone ethylene. The ACC level regulates the plant's tolerance to abiotic stress (Zhang et al., 2019). Poveda (2020) showed an impact of the fungi on plant's hormones as well. The author determined the role that the enzyme chorismate mutase plays in *Trichoderma parareesei* ability to promote tolerance to salinity and drought in plants. This enzyme is at the base of a mechanism that increases the expression of genes related to the hormonal pathways of abscisic acid (ABA) under drought stress, and ethylene (ET) under salt stress.

Exopolysaccharides Production

The biofilm formation and exopolysaccharide (EPS) production by soil bacteria constitute important strategies to assist metabolism during stress imposed by salinity. EPS produced by PGPRs have a significant impact on plant growth and stress tolerance, such as drought or high salt concentration. They are hydrating compounds that are available for use before the decay of roots or germinating seeds. Bacteria produce polymeric biofilms on a variety of surfaces such as roots and soil, cementing particles, and forming aggregates. This can improve crop performance and soil physicochemical properties (Qurashi and Sabr, 2012; Amna et al., 2019). Salt-tolerant *Halomonas variable* and *Planococcus rifietoensis* strains can improve plant growth and aggregation of soil. These strains showed the formation of a biofilm and accumulated exopolysaccharides as a result of increasing salt stress (Qurashi and Sabr, 2012). Bacterial EPS can help to alleviate salinity stress by reducing the Na^+ content available for plant uptake (Upadhyay et al., 2011). The kind of EPS produced by *Pseudomonas* spp. (Kasotia et al., 2016), and *Bacillus* spp. (Amna et al., 2019) helped in the binding of free Na^+ from the soil, thus making Na^+ unavailable to the soybean and wheat plants, respectively.

Increased Antioxidant Activity

High soil salinity is also responsible for increased production of reactive oxygen species (ROS) by plants, such as superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH\cdot$), and alkaline radicals which have a negative impact on proteins, DNA, lipids, and other biomolecules and cause oxidative effects including plant cell damage and premature senescence or necrosis (Møller et al., 2007; Miller et al., 2010; Habib et al., 2016; Zhang et al., 2018). ROS are produced at a low level in organelles (chloroplasts, mitochondria, peroxisomes) under optimal plant growth conditions. However, under stress conditions, their concentration increases significantly (Miller et al., 2010). A critical system responsible for the production of ROS is the plasma membrane-bound NADPH oxidase (RBOH), which controls cellular redox homeostasis under salinity stress (Hossain and Dietz, 2016; Hossain et al., 2017). Many other plant

cell components also play a role in regulating intracellular ROS levels. Among them, the most important are antioxidant enzymes such as peroxidases (POD), catalase (CAT), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), dehydroascorbate reductase (DHAR), monodehydroascorbate reductase (MDAR), glutathione peroxidases (GPX), or glutathione s-transferase (GST) (Yan et al., 2013; Hossain and Dietz, 2016; Sukweenadhi et al., 2018). Non-enzymatic components include glutathione, ascorbic acid, tocopherol, phenolic or polyphenolic compounds (Yan et al., 2013; El-Sayed et al., 2014; Fatma et al., 2014; El-Esawi et al., 2018a). To some species of endophytic fungi or ectomycorrhizal fungi are attributed antioxidant properties that could improve plant's resistance to plant's endogenous reactive oxygen species. *Trichoderma* is a genus of soilborne filamentous fungi that comprehend species capable of triggering plants' defensive mechanisms and inducing tolerance to abiotic stress. Plants' roots colonised by *T. harzianum* increased the production of antioxidant enzymes (Zehra et al., 2017). Also, Mastouri (2010) showed that plants colonised by *T. harzianum* showed a lower accumulation of lipid peroxides and a higher production of antioxidant compounds such as glutathione, resulting in a mechanism based on the control of the accumulation of reactive oxygen species that occur in stressed plants. *T. harzianum* can accelerate seeds germination while reducing the adverse effects caused to seeds and seedlings by thermal, osmotic, saline, and water stress. Examples of PGPRs and PGPFs, increasing the level of antioxidant enzymes and non-enzymatic redox antioxidants are listed in **Table 1**.

Biosynthesis of Hydrolytic Enzymes

One of the main indirect mechanisms of plant's pathogens biocontrol by PGPRs is the production of cell wall degrading enzymes, like chitinases, glucanases, proteases and cellulases that cause lysis of the fungal cell walls (Siddique et al., 2010; Berrada et al., 2012; Goswami et al., 2014). Teng et al. (2010) reported that halotolerant *Pseudomonas* sp. strain isolated from *Suaeda salsa* is a source of proteinases active against phytopathogenic fungi like *Fusarium oxysporum* (Teng et al., 2010). Two bacterial strains (*B. halotolerans* and *B. pumilus*) isolated from the halophyte *Prosopis strombulifera* were able to produce proteinases inhibiting the growth of *Alternaria* (Sgroy et al., 2009). *B. cereus* and *B. thuringiensis* isolated from salty Tunisian soils were able to produce N-acetyl- β -D-glucosaminidases, chitobiosidases, endochitinases, and they were active against *F. roseum* (Sadfi et al., 2001). The extracellular chitinases of *Serratia marcescens* and *Enterobacter agglomerans* have been indicated as biocontrol agents against *Sclerotium rolfsii*. The ability to suppress *Fusarium oxysporum* and *Rhizoctonia solani* was found in *Peaenibacillus* spp., *Bacillus* spp., and *Streptomyces* spp., which synthesise β -1,3-glucanase that are lytic enzymes able to destroy the cell walls of some fungi (Compant et al., 2005; Labuschagne et al., 2010).

Other Extracellular Molecules

Bacteria secrete many extracellular molecules such as lipochitooligosaccharides, bacteriocins, polyamines and volatile

organic compounds (**Supplementary Material Table 2**). It has been demonstrated that these molecules often control metabolic pathways and have a role in regulatory functions that increase the plant's defence and stimulate its growth, stress tolerance, and disease resistance.

Lipo-chitooligosaccharides (LCOs), in particular, are produced by rhizobia and have been found to initiate nodule formation in response to root exudates and flavonoids (Ilangumaran and Smith, 2017). The LCOs molecules have a conserved core and a variable N-Acetyl chain length, with different substitutions (sulfation or glycosylation) and degree of saturation, which account for host specificity (Oldroyd, 2013). Miransari and Smith (2009) reported how *Bradyrhizobium japonicum*, when inoculated in soybean under different salinity levels (from 36 to 61 mM NaCl), enhanced nodulation and growth of plants with the effects becoming more consistent with time.

Rhizobacteria secrete bacteriocins, that are small proteinaceous or peptidic toxins. These act as bactericidal or bacteriostatic agents against competing bacteria and indirectly promote microbial diversity under salinity stress. Some bacteriocins showed a role in plant's resistance to stress. *Bacillus thuringiensis* is a soybean endosymbiont that, *in vitro*, produces "thuricin 17", a bacteriocin that is capable of manipulating plant proteome profile, enhancing its tolerance to salinity (Subramanian et al., 2016).

Polyamines (Pas) such as spermidine, spermine, and putrescine, consist of low molecular weight aliphatic amines that can have antioxidant activity. These compounds are present practically in all living organisms and impact reactive oxygen species by scavenging free radicals and inducing the expression of genes related to cellular antioxidant mechanisms. Among all, spermidine which is secreted by *Bacillus megaterium* showed to increase the cellular accumulation of polyamines in plants. The mechanism in *Arabidopsis* involved osmotic stress tolerance via the activation of polyamines-mediated cellular signalling, which resulted in greater biomass, higher antioxidant enzyme activity and high photosynthetic capacity in the inoculated plant, compared to the untreated control (Zhou et al., 2016; Ilangumaran and Smith, 2017). Some authors used the definition "systemic induced resistance (SIR)" to cover several bio-protecting mechanisms induced by the PGPRs in plants and that act on multiple functions, once activated at the presence of a pathogenic infection (Numan et al., 2018).

Volatile organic compounds (VOC) are low molecular weight compounds, such as aldehydes, alcohols, ketones and hydrocarbons, which can enter the atmosphere as vapours due to significantly high vapour pressure. They are released from by PGPRs and stimulate plant growth, resulting in increased shoot biomass, and modulated stress responses (Ilangumaran and Smith, 2017). The role of VOCs in the biocontrol of plant's pathogens and antibiosis is not fully understood, but some of the mechanisms at play gained attention in the last decades and will require further research (Bailly and Weisskopf, 2012). *Paraburkholderia phytofirmans* produced VOCs such as 2-undecanone, 7-hexanol, 3-methylbutanol that stimulate plant

growth and induce salinity stress tolerance as demonstrated both *in vitro* and in soil. Growth parameters of *Arabidopsis* plants treated with these VOCs and measured as rosette area, fresh weight, and primary root length were higher than in the control plants (Ledger et al., 2016; Ilangumaran and Smith, 2017). VOCs emitted by *Bacillus subtilis* can stimulate many different hormonal signals in *Arabidopsis thaliana*, which includes cytokinins, salicylic acid, gibberellin, auxin and brassinosteroids (Zhang et al., 2007; Zhang et al., 2008b). Almost 600 genes related to metabolism, auxin homeostasis, cell wall modification and stress response were identified, and these studies showed that VOCs could play an essential role in plant growth and development. PGPRs VOCs can stimulate many chemical and physical changes, some of which could be addressed to improve plants' tolerance towards abiotic stress (Mantelin and Touraine, 2004; Zhang et al., 2008a).

Several antimicrobial metabolites are the basis of biocontrol mechanisms activated by PGPRs against other microorganisms, and also phytopathogenic species. Strains of *Arthrobacter* spp., *Pseudomonas* spp., *Bacillus* spp., *Streptomyces* spp., can synthesise one or several types of bioactive compounds including amphisin, bacillomycin, 2,4-diacetylphloroglucinol (DAPG), fengycin, hydrogen cyanide, iturin, macrolactin, phenazine-1-carboxylic acid (PCA), pyoluteorin, pyrrolnitrin, surfactin, tensin, tropolone, and viscosinamide (Compant et al., 2005; Hinarejos et al., 2016). However, among bacterial biocontrol agents, the most cited are *Bacillus* spp. (*B. amyloliquefaciens*, *B. cereus*, *B. licheniformis*, *B. pumilus*, *B. subtilis*) and *Pseudomonas* spp. (*P. chlororaphis*, *P. fluorescens*, *P. putida*). Their antagonistic properties against bacterial (*Ralstonia solanacearum*, *Xanthomonas axonopodis*) and fungal (*F. oxysporum*, *F. culmorum*, *P. ultimum*, *Rhizoctonia solani*) phytopathogens of barley, chickpea, maize, peanut, rice, and wheat were widely proven (Raaijmakers et al., 2002; Yuttavanichakul et al., 2012). Moreover, due to multifaceted mechanisms of action in preventing pathogens' infections, some strains of *Bacillus* sp. have been commercialized and used for improving crop production (Radhakrishnan et al., 2017; Nakkeeran et al., 2020). The formation of a biofilm around plant's roots by some *Bacillus* species and the secretion of antagonistic metabolites inhibit pathogenic communities and reduce the occurrence and frequency of the diseases in plants (Radhakrishnan et al., 2017).

One of the antibiosis mechanisms adopted by *Pseudomonas* spp. is the production of hydrogen cyanide, which inhibits the terminal cytochrome c oxidase in the respiratory chain and binds to metalloenzyme (Ramette et al., 2003). However, hydrogen cyanide antagonistic potential against phytopathogens, mainly fungi, is still a matter of discussion (Rijavec and Lapanje, 2016). Ramette et al. (2003) showed a broad spectrum of antifungal activity, whereas Rudrappa et al. (2008) and Blom et al. (2011) reported that hydrogen cyanide is unlikely a biocontrol agent. The authors indicated that pigments and other antibiotic substances are more effective against fungi. Interestingly, Rijavec and Lapanje (2016) proved that HCN regulates phosphate availability of PGPRs and host plants.

THE INTERPLAY BETWEEN HALOPHYTES AND THEIR MICROBIOME: A GLIMPSE INTO THE FUTURE

For many years, a wide range of PGPRs and PGPFs have been studied, and some bacterial and fungal isolates, including species of the genera *Pseudomonas*, *Bacillus*, *Enterobacter*, *Klebsiella*, *Azobacter*, *Variovorax*, *Azospirillum*, *Serratia* and *Trichoderma*, *Aspergillus*, *Penicillium*, *Phoma* have been used in commercial products (Glick, 2012; Jahagirdar et al., 2019). Nevertheless, the application of PGPRs and PGPFs in the agricultural industry is only a small part of agricultural practice worldwide (Bashan et al., 2014). Disadvantages regarding the utilization of bacteria are connected with properties of the inoculated PGPRs and chiefly depend on their survival in soil, their interaction within indigenous soil microflora, and other complex environmental factors (Martinez-Viveros et al., 2010). Moreover, the modes of action of PGPRs are incredibly varied, and not all rhizobacteria have the same effects with identical mechanisms (Choudhary, 2012; Arora et al., 2020). Little is known about PGPFs compared to bacteria regarding their effectiveness in the plant growth-promoting processes. However, many researchers reported beneficial effects of PGPFs application to plants growth by activation of induced systemic resistance (ISR) (Murali et al., 2012; Naziya et al., 2020). Another frontier is the exploitation of interactions between several microorganisms, as it is often from the interaction of several species that the production of bioactive compounds is obtained. Complex interactions between different mycorrhizal species were documented, for instance. Poveda et al. (2019) showed, for example, that plant roots' colonization by *Trichoderma harzianum* biocontrol strain increases the colonization of the same host by arbuscular mycorrhizal fungal species. The authors, analysing the expression profile of defence-related marker genes, suggested that the phytohormone salicylic acid could play a key role in the modulation of the roots' colonization process when both fungi are jointly applied.

According to Gangwar et al. (2017) and Egamberdieva et al. (2019), an ideal plant growth-promoting microorganisms

(PGPMs) should possess a high rhizosphere competence, enhance plant growth capabilities, have a broad spectrum of action, be safe to the environment, be compatible with other rhizobacteria, and be tolerant to heat, UV radiation, and oxidizing agents. So far, organisms with interesting properties have been isolated, and some possess more than one of the qualities required for a perfect PGPR, however, imagining such an ideal organism capable of accomplishing all the necessary actions, is a kind of extreme. Nevertheless, the direction is the right one because by continuing to search and experimenting, it is possible to find different organisms that together can work with complementary mechanisms. The research on PGPMs as biofertilizers is the most natural and realistic aspiration to face a global agricultural productivity requirement, capable of feeding the world's population, which is going to escalate to 9 billion people by 2050.

AUTHOR CONTRIBUTIONS

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/fpls.2020.553087/full#supplementary-material>

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Bioaugmentation of Entomopathogenic Fungi for Sustainable *Agriotes* Larvae (Wireworms) Management in Maize

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Soil microorganisms influence biotic and abiotic stress tolerance of crops. Most interactions between plant symbiotic and non-symbiotic soil microorganisms and plants occur in the rhizosphere and are sustained through plant exudation/rhizodeposition. Bioaugmentation, *i.e.*, the introduction or amplification of certain plant beneficial microbes (*e.g.*, entomopathogenic fungi) into the rhizosphere, could contribute to controlling insect crop pests and replacing chemical, environmentally unfriendly insecticides. Wireworms, the soil-burrowing larval stages of click beetles (Coleoptera: Elateridae), are major pests of crops including maize, wheat and potatoes, worldwide. Alternative strategies for controlling wireworms are needed because several chemical pesticides used successfully in the past are being phased out because of their ecotoxicity. Therefore, virulence to *Agriotes lineatus* L. wireworms and plant beneficial traits of entomopathogenic fungi were investigated in a series of laboratory experiments. Tested taxa included environmentally retrieved *Metarhizium brunneum* Petch. (two strains), *M. robertsii* Bisch., Rehner & Humber (Hypocreales: Clavicipitaceae), and *Beauveria brongniartii* (Sacc.) Petch. and commercially formulated *B. bassiana* (Bals.-Criv.) Vuill. (Cordycipitaceae) and *Bacillus thuringiensis* Berliner 1915 var. *kurstaki*. In-house reared larvae were dipped in conidial suspension, and maize and wheat seeds were coated with fungal conidia. *Metarhizium brunneum* strains 1154 and 1868 significantly increased wireworm mortality. Fungi were significantly more often re-isolated from maize than wheat rhizoplanes in laboratory assays. The strains tested were rarely isolated as endophytes. *Metarhizium brunneum* strain 1154 stimulated wheat growth, while *M. robertsii* 1880 stimulated maize growth, whereas *M. brunneum* 1868 and others did not affect root or shoot length or plant biomass significantly in laboratory settings. *Metarhizium brunneum* strain 1868, re-isolated most often from maize rhizoplane, caused the highest wireworm mortality. It was further evaluated whether *M. brunneum* 1868 can protect maize varieties FeroXXY, LG 34.90 and Chapalu from wireworm damage and promote plant growth at field conditions. Plants of all three varieties stemming from seeds treated with conidia of *M. brunneum* 1868 showed significantly less wireworm damage 3 to 4 weeks after sowing (5-

to 6-leaf stage) resulting in a significantly higher initial maize stand. However, only in the variety LG 34.90 a significant increase of the maize stand was observed at harvest time.

Keywords: biological control, biopesticide, plant-microbe interaction, plant-microbe-insect interaction, rhizosphere, sustainable agriculture, plant-microbe-pest interaction, biocontrol

INTRODUCTION

Wireworms (Coleoptera: Elateridae) damage potato and other crops including wheat and maize. They start feeding on seed potatoes shortly after planting without causing plant losses initially. However they reduce the market quality of the produce as they penetrate into near harvest potatoes (Benjamin et al., 2018). Once potatoes are damaged, also secondary microbial infections occur and the yield of entire potato crops can become unmarketable in high pest pressure areas or organic production settings (Brandl et al., 2017). Due to their hidden life cycle belowground, wireworms can hardly be controlled, especially in organic farming, where persistent, non-specific soil insecticides cannot be used (Schepl and Paffrath, 2007; Brandl et al., 2017; Benjamin et al., 2018). In maize and wheat, wireworms target germinating seeds and young sprouts, what results in typical herbivory symptoms such as leaf drilling holes and dead central leaves. However, in case of severe infestations, plant stand and yield can be significantly decreased (Reddy et al., 2014; Furlan et al., 2017). It has been emphasized that the abandonment of ecotoxicologically problematic soil insecticides may increase wireworm-related problems (Parker and Howard, 2001; van Herk and Vernon, 2013).

Organophosphates, organochlorines, and carbamates effectively controlled wireworms in the second half of the 20th century (Merrill, 1952). However, due to their ecotoxicity (Costa, 2015), biomagnification in non-target organisms (Mitra et al., 2011), and yearlong availability in soils (Wilkinson et al., 1976), these pesticides are no-longer used in agriculture. Newer types of chemical insecticides used in the past two decades included pyrethroids, phenyl pyrazoles, and neonicotinoids (Jeschke et al., 2011), which in some cases function *via* pest repellency or morbidity, rather than mortality (van Herk et al., 2008; Vernon et al., 2009). However some of the neonicotinoids and phenyl pyrazoles are already prohibited due to negative effect on bees and other pollinators (Zhang and Nieh, 2015), aquatic invertebrates and fish (Werner and Moran, 2008) or beneficial spiders and mites (Douglas and Tooker, 2016).

Accordingly, several nonchemical methods for wireworm control were proposed including crop rotation (Willis et al., 2011), crop residue removal, biofumigation (Furlan et al., 2010), weeding (Schepl and Paffrath, 2007), trap and cover crop use (Rogge et al., 2017), mechanical soil disturbance and biological control (Reddy et al., 2014; la Forgia and Verheggen, 2019). Also entomopathogenic fungi (EPF) can significantly reduce insect pest pressures. Typically, they penetrate the insect cuticle, paralyse and destructively colonize insect bodies (Yousef et al., 2018). Several commonly occurring species of *Metarhizium* and *Beauveria* are known to be effective against wireworms (Kabaluk and Ericsson, 2007a; Kabaluk and Ericsson, 2007b; Kabaluk et al.,

2013; Razinger et al., 2013; Reddy et al., 2014; Brandl et al., 2017; Rogge et al., 2017; Benjamin et al., 2018; Razinger et al., 2018b). They also infest wireworms as naturally occurring soil fungi (Kabaluk et al., 2005). In addition to causing pathogenicity in pest insects, EPF also have other beneficial functions as they enhance plant growth and mineral nutrition and exclude phytopathogens from rhizosphere niches (Herbst et al., 2017; Rivas-Franco et al., 2019; Ahmad et al., 2020).

Various studies have stressed that plants may actively shape their root microbial communities through rhizosphere depositions (Dennis et al., 2010; Canarini et al., 2019) and some have speculated that rhizosphere colonizing entomopathogenic fungi could protect plants from plant insect pests in tritrophic interactions (Vega et al., 2009; Bruck, 2010; Steinwender et al., 2015). Therefore, we hypothesized that utilizing rhizosphere competent EPF could contribute to potentially long-lasting pest management solutions, and decrease the amount of required biopesticide product. Accordingly, we are trying to identify microbial agents with plant beneficial metabolic or ecological traits that can be bioaugmented in the rhizosphere (Compant et al., 2010). In previous studies we screened the virulence of several EPF species (Razinger et al., 2013; Razinger et al., 2018b). The aim of this study was to apply EPF onto maize kernels as a one-step prophylactic strategy to protect maize plantlets during germination and sprouting against wireworm herbivory. In addition, the plant × microbe interactions were investigated in a series of laboratory experiments.

MATERIALS AND METHODS

Wireworm Rearing

Wireworms of the species *Agriotes lineatus* L. were reared in a glasshouse following methods described by Kölliker et al. (2009). For collecting adult click beetles, a 5–10 cm thick layer of freshly mown grass was placed on top of a 2 m² plastic (PVC) sheet placed from late April to late June on either meadows or grass areas juxtaposing agricultural fields. The grass on the sheet was inspected twice a week, and adults were collected. Beetles identified as *A. lineatus* were then placed in plastic containers with added food (dry baker's yeast and honey mixture 1:9 w:w). After 24 h, 5–15 beetles were transferred to individual 10 L plastic pots filled with commercial planting soil (Potgrond H, Klasmann-Deilmann GmbH, Geeste, Germany). Wheat, barley, or maize was sown into the pots; small plastic cups filled with honey and yeast mixture were placed on top of the substratum to provide an additional food source for the adults. To prevent escape of insects, an insect rearing bag (BugDorm, Taiwan) was

erected aboveground. Plants in pots were watered and wheat or corn seeds re-sown when needed. After approximately 10 months wireworms of average length 15 ± 1 mm were collected from the rearing pots. Rearing was performed in a glasshouse under natural illumination at 15–30°C and 40–65% RH.

Fungi Collection and Growing

Entomopathogenic fungi such as *Metarhizium brunneum* (strains 1154, isolated from soil, and 1868, from dead *Agriotes* sp. adult), *M. robertsii* (1880, unknown host), and *Beauveria brongniartii* (from *Melolontha melolontha* L.), isolated from agriculturally used areas in Slovenia, were used for *in-vitro* mortality bioassays. The isolates are kept in the mycological collection of the Agricultural Institute of Slovenia. Fungal cultures were incubated at $24 \pm 1^\circ\text{C}$ in darkness for 14 d on full or 1/3rd strength potato dextrose agar (PDA, Biolife Italiana S.r.l., Milan, Italy). Tween 80 (0.05%, Sigma-Aldrich, Germany) was used for preparing conidial suspensions (1×10^8 spores ml^{-1}) and conidial viability was assessed as in Razinger et al. (2014).

Laboratory Experiments

Virulence and Pathogenicity Assessment

Wireworms were dipped for 10 s in 3.5 ml of spore suspensions with continuous gentle agitation. Additionally, Naturalis (Andermatt biocontrol AG, Grossdietwil, Switzerland, based on *Beauveria bassiana* ATCC 74040) (recommended concentration of 0.1 v/v) and Delfin (Andermatt biocontrol AG, Grossdietwil, Switzerland, based on *Bacillus thuringiensis* var. *kurstaki*) (0.05%, v/v) were used as reference biopesticides. Tween 80 (0.05%) was used as the negative control. Commercial potting substrate (Special substrate, Floragard, Germany) was steam treated for 1 h in loosely closed plastic bags, in which the top of the soil reached 92°C. Sterile 50 ml centrifuge tubes were filled with 25 ml of steamed substrate and moistened with 2.5 ml sterile demineralized water. One infected wireworm was placed on top of the substrate of each centrifuge tube and next to a 2 mm thick slice of organically produced carrot. The tubes were gently capped so that air could freely circulate. Ten wireworms were used per treatment and the experiment was repeated twice independently ($n = 20$). Experiments were observed on a weekly basis for 8 weeks. At each observation wireworms were classified as living, dead and dead and mycotic. Both experiments were carried out in an environmental chamber at $20 \pm 1^\circ\text{C}$, 80 \pm 5% relative humidity, in darkness.

Plant \times Microbe Interactions

The laboratory experiments investigating plant \times microbe interactions were performed in the absence of wireworms. Seeds were prepared according to Razinger et al. (2018a). Approximately 200 ml of wheat seeds of variety Renan and 1,200 ml of maize seeds of variety DKC4190 were surface disinfected by immersing seeds for 3 min in 70% ethanol with hand shaking, rinsing twice with sterile demineralized water and drying in a laminar flow chamber. For seed coating exposure, conidial suspensions of a concentration of 2×10^7

viable conidia ml^{-1} were prepared in 1% carboxymethyl cellulose (CMC; Sigma-Aldrich Chemie GmbH, Steinheim, Germany). The amount of conidia attached to the seeds was estimated by washing the conidia off five seeds per fungal treatment with 0.05% Tween 80. The number of conidia was assessed by plating serial dilutions on 1.5% malt extract agar (Sigma-Aldrich Chemie GmbH, Steinheim, Germany). Washing and plating were performed in triplicate. The coating success was checked twice in triplicate ($n = 6$) and measured in means \pm SE. Retrieved number of conidia were $7.0 \times 10^4 \pm 6.1 \times 10^3$ per maize seed and $9.4 \times 10^4 \pm 3.9 \times 10^4$ per wheat seed (*M. brunneum* 1154); $8.3 \times 10^4 \pm 3.9 \times 10^3$ (maize) and $6.6 \times 10^4 \pm 2.5 \times 10^4$ (wheat) (*M. brunneum* 1868); $1.2 \times 10^5 \pm 1.3 \times 10^4$ (maize) and $1.1 \times 10^5 \pm 1.9 \times 10^4$ (wheat) (*B. brongniartii* 1877); $1.0 \times 10^5 \pm 1.7 \times 10^4$ (maize) and $8.1 \times 10^4 \pm 2.7 \times 10^4$ (wheat) (*M. robertsii* 1880). No conidia were retrieved from the negative control, i.e., untreated maize or wheat seeds, surface disinfected seeds, and surface disinfected seeds coated with 1% CMC without conidia. The fungal treatments were compared to the surface disinfected and CMC-treated controls. The CMC control was in turn compared to the untreated and surface disinfected control to determine if the CMC coating or surface disinfection had an effect on germination rate or plant growth.

The effect of fungal coatings or control treatments on the germination success was evaluated according to the International Seed Testing Association protocols. In brief, maize was sown into moist sand and incubated for 7 d at 20°C with 8:16 d:n regime, whereas wheat was placed on moist filter paper, chilled for 5 d at 7°C and then incubated for 7 d at 20°C with 16:8 d:n regime. In a second experiment, the effect of fungal coatings or control seed treatments on seedling emergence (evaluated on days 2, 3, 4, 7, and 15), plant biomass (fresh above- and belowground plant tissue weight and length), and rhizoplane and endophytic plant tissue colonization was assessed in quartz sand in a growth chamber 15 d post sowing (20°C, 16:8 d:n). Fungus-coated or non-coated seeds were sown 2 cm deep into moist sand. Both experiments were performed independently twice with four biological replicates containing 25 seeds each. The fresh above- and belowground plant tissue weight and length were measured on 16 plants per treatment per experiment repetition and rhizoplane and endophytic plant tissue colonization was assessed on three plants per treatment per experiment repetition.

The ability of strains to colonize rhizoplane and roots or leaves as endophytes was evaluated on plants from the second experiment 15 d after sowing according to Herbst et al. (2017) with slight modifications. Five 2-cm-long pieces of roots were sampled per plant to evaluate rhizoplane colonization. The root pieces were washed once with tap water and five times with sterile demineralized water. The washed root pieces were transferred to CTC agar medium (Fernandes et al., 2010), which promotes the growth of EPF semi-selectively. The plates were incubated for 14 d at $22 \pm 1^\circ\text{C}$. Another collection of five washed root pieces and five washed leaf pieces (root pieces 2 cm in length; leaf pieces 1–2 cm^2) per plant were surface disinfected for evaluating endophytic colonization by the fungi. Surface disinfection was performed in 50-ml centrifuge tubes by submersing pieces in 5 ml 70% ethanol for 3 min. During the

3 min of submersion in ethanol the tubes were vigorously vortexed three times for 10 s. Plant tissue pieces were then washed with sterile demineralized water. To evaluate the efficiency of the surface disinfection, 100 µl of the final wash-water was plated onto CTC plates. In all cases, no fungal colonies grew on the CTC plates from the final wash-water. Fungi emerging from washed or surface disinfected plant tissue pieces were isolated by transferring single hyphal tips to clean agar plates. After an incubation of 10–14 days at 25°C, retrieved fungal isolates were identified through morphological comparisons with strains used for inoculations.

Field Experiments

Experimental Sites, Design and Crop Management

Four field experiments (three in 2017 and one in 2018) were conducted to assess the biopesticidal efficacy of *M. brunneum* strain 1868 against wireworms by comparing plants that emerged from 1868-coated and uncoated seeds. Three commonly used maize varieties (FeroXXY, LG 34.90 and Chapalu) were tested at two different locations in Eastern Slovenia. Location 1 was at Bučecovci (46°35′07″N, 16°06′37″E; 0.112 ha) on dystric planosol with FeroXXY sown on April 27, 2017 and Chapalu on May 9, 2018; location 2, Laporje (46°35′14″N, 15°61′04″E; 0.112 ha) on dystric gleysol with FeroXXY and LG 34.90 sown on May 8, 2017. Both sites are characterized by mild continental climate, low-medium rainfall during maize growing season, no available irrigation, and a medium grain yield potential (<12 t ha⁻¹). Two plots of the same size were designed for each maize variety. Each plot consisted of four maize rows spaced 0.7 m apart and of 100 m length giving 0.028 ha per plot. Approximately 2,400 seeds were sown per plot giving a theoretical plant stand of 85,000 ha⁻¹. The same crop and weed management was applied for both plots in each experiment; thus the two plots differed only in the EPF seed treatment. All field experiments were managed with standard, farmer-owned equipment suited for field scale applications. The field experiments were evaluated in springtime to assess seedling emergence and wireworm damage to the seedlings. Pre-harvest-related parameters were assessed in autumn.

Wireworm Damage and Emergence Evaluation

Wireworm damage was evaluated 3 to 4 weeks after sowing at the 5- to 6-leaf stage. Four to six 20 m segments within the 100 m plots were randomly selected and marked with wooden poles. In these segments, all emerged plantlets were categorized into three groups: wireworm-damaged plantlets (e.g., leaves exhibiting drilling holes, dead central leaf, yellow stripes on leaves), undamaged plantlets, and a sum of the previous two categories—total plantlet stand. The number of total spring observations per treatment (i.e. biological replicates, each consisting of >100 plants) was 10 for variety FeroXXY, four for LG 34.90 and six for Chapalu.

Pre Harvest Evaluation

In mid-September (2017) or the second half of August (2018) the previously marked segments were evaluated to assess the

following parameters: final stand—reduced growth, no ears (i.e., plants reduced in growth or damaged and having no ears), final stand—plants carrying corn ears, and final stand—total plant stand. Additionally, from each segment, 10 fresh plants (with ears) and ears with husks alone were weighed and the number of ears counted. The number of total autumn observations per treatment (i.e. biological replicates, each consisting of >100 plants) was 12 for variety FeroXXY, six for LG 34.90, and six for Chapalu.

Statistical Evaluation of Data

Plant × microbe interaction data was first analyzed for normality of distribution by D'Agostino–Pearson omnibus K2 test. In case of normal distribution it was analyzed by one- or two-way analysis of variance, and in case significance was observed, individual treatments were subjected to Bonferroni's multiple comparison *post-test*. When data was not normally distributed it was analyzed using the Kruskal–Wallis test followed by Dunn's multiple comparison test (Motulsky, 1995). The time-based wireworm mortality was analyzed using Kaplan–Meier survival analysis. When multiple survival curves were compared, the significance threshold was corrected according to the Bonferroni method (Panevska et al., 2019). The field experiment data were analyzed by general linear model (GLM), where the effect of factor *treatment* (*M. brunneum* and negative control), *maize variety* (FeroXXY, LG 34.90 and Chapalu) and their interaction were analyzed on the dependent variables described in the section *Pre Harvest Evaluation*. Further, Fisher's least significance difference (LSD) procedure at 95% confidence level was used to discriminate between the treatments within the field experiment dataset. The difference was considered significant at $P < 0.05$. If not stated otherwise, data presented are mean values ± standard error (SE). The number of biological replicates (n) is indicated in the figure or table captions. The analyses were performed with the statistical software Statgraphics Centurion XVI (StatPoint Technologies, Inc., The Plains, VA, USA) and GraphPad Prism 5.00 (GraphPad Software, Inc., La Jolla, CA, USA).

RESULTS

Laboratory Experiments

Virulence and Pathogenicity Assessment

On average 5.6% of wireworms died in the negative control group for unknown reasons until day 56 of the laboratory experiments. Kaplan–Meier survival analysis showed a significant mortality increase of wireworms treated with *M. brunneum* 1154 (50.0 ± 10.0% mortality) and 1868 (52.8 ± 2.78%). The other fungal strains and the Naturalis and Delfin biopesticide formulations did not cause a significant mortality increase during the 8-week experiment (Figure 1).

All fungal isolates and Naturalis formed mycelium and sometimes conidiogenous structures on wireworm surfaces. The highest incidence of sporulation was observed on wireworms treated with *M. robertsii* and Naturalis, where mycoses were

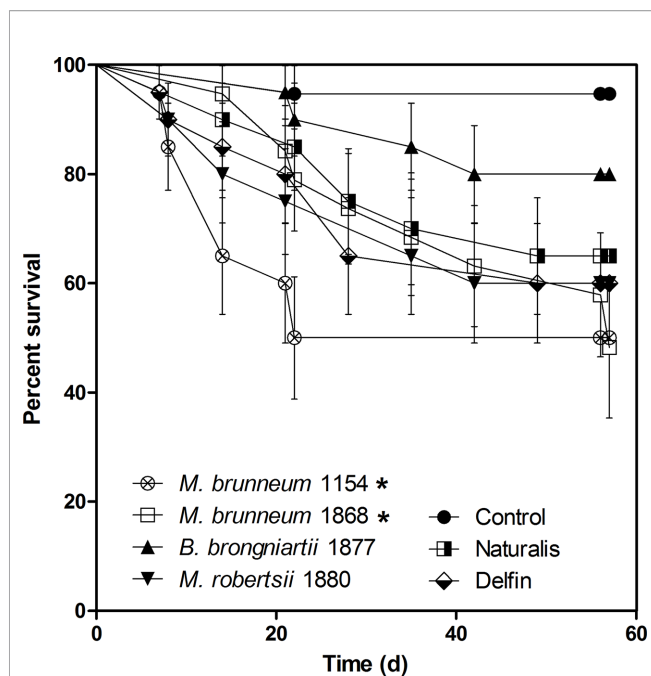


FIGURE 1 | Mortality of wireworms treated with entomopathogenic fungi and two reference biopesticides in laboratory assays. The experiments were evaluated on a weekly basis for 8 weeks post infection. Asterisks denote significant difference from the control mortality ($P < 0.05$). Data from two independent experiments were pooled and analyzed with Kaplan–Meyer survival analyses ($n = 20$). Naturalis—commercial product based on *Beauveria bassiana*; Delfin—commercial product based on *Bacillus thuringiensis* var. *kurstaki*.

observed on all dead insects. *Metarhizium brunneum* 1154 caused mycoses in 75% and strain 1868 on 80% of dead insects. *Beauveria brongniartii* sporulated on 67% of insect cadavers.

Plant × Microbe Interactions

Germination on Filter Paper

Fungal treatment did not have any effect on maize seedling emergence when compared to the CMC control, while wheat seedling emergence was inhibited by *M. robertsii* (Kruskal–Wallis statistic and Dunn’s post-test, **Figure 2**). Seed disinfection or seed disinfection with CMC coating did not result in a significantly different seedling emergence when compared to untreated control seeds in both plant species evaluated (average germination of maize/wheat was $25 \pm 0.3/24 \pm 0.3$, $24 \pm 0.3/23 \pm 0.7$ or $24 \pm 0.3/25 \pm 0.3$ for control CMC, disinfected seed or untreated seeds, respectively).

Speed of Seedling Emergence

A significant effect of factor treatment ($F_{6, 245} = 14.0$; $P < 0.0001$), time ($F_{4, 245} = 1148$; $P < 0.0001$) and their interaction ($F_{24, 245} = 3.1$; $P < 0.0001$) was observed on wheat germination timing. However, no differences were observed between CMC-treated surface disinfected control and fungal treatments. The only significant differences were observed between (i) CMC-treated, surface disinfected seeds and surface disinfected seeds at day 3 ($P < 0.0001$), and (ii) CMC-treated surface disinfected seeds and

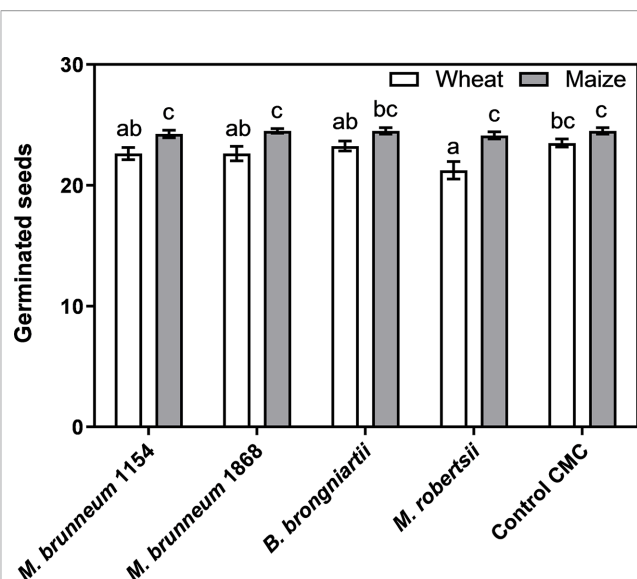


FIGURE 2 | Maize and wheat seed germination after 7 d incubation on moist filter paper. Data from two independent experiments, each containing four biological replicates of 25 seeds each, were pooled and given as means \pm standard error ($n = 8$). Bars not sharing the same lower-case letters are significantly different. Control CMC—seeds were surface disinfected and coated with 1% carboxymethyl cellulose.

untreated seeds at days 2 ($P < 0.01$) and 3 ($P < 0.0001$). Consistently, seed germination was slower in CMC-treated and surface disinfected controls. No significant effects of seed treatment procedures were observed on the timing of maize seeds (not shown).

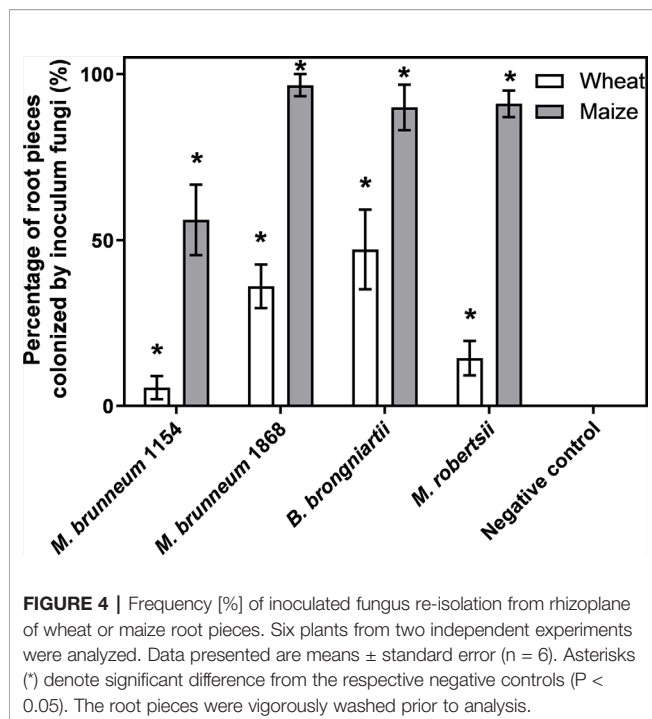
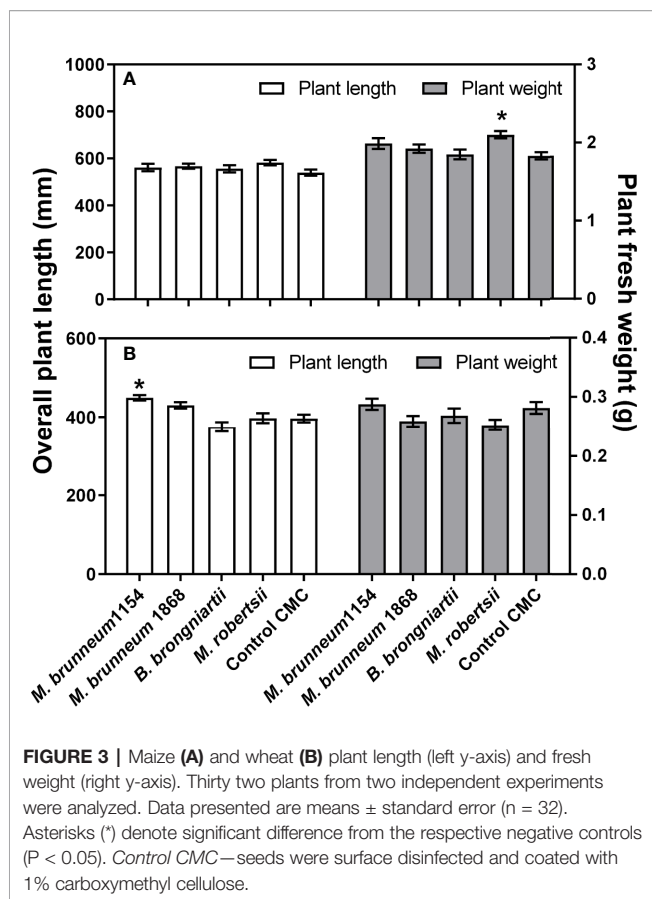
Effect on Plant Growth

Fungal treatments had a significant effect on plant fresh weight ($P = 0.0054$), but not on plant length ($P = 0.2856$) in maize, e.g. *Metarhizium robertsii* significantly increased the weight of maize plantlets compared to CMC-treated surface disinfected controls. In contrast, length of wheat plants ($P < 0.0001$) but not plant fresh weight (0.0857) was significantly affected by the fungal treatments in wheat, e.g. *M. brunneum* 1154 significantly increased wheat plantlets’ length compared to CMC-treated surface disinfected controls (**Figure 3**). Seed disinfection or seed disinfection with CMC coating did not result in a significantly different plantlet length or fresh weight compared to untreated control seeds in both crops (not shown).

Rhizoplane and Endophytic Plant Tissue Colonization

Two-way ANOVA showed a significant effect of factors’ treatment ($F_{4, 49} = 37.1$; $P < 0.0001$) and plant species ($F_{1, 49} = 124$; $P < 0.0001$) and their interaction ($F_{4, 49} = 9.22$; $P < 0.0001$) on rhizoplane colonization by the inoculated fungus. In all cases the inoculated fungi were significantly more often re-isolated from maize root pieces (**Figure 4**).

Endophytic tissue colonization was rarely observed. No endophytic colonization was observed in root or leaf tissue of wheat. Endophytic colonization of maize roots was observed for



M. brunneum 1154 ($16.7 \pm 11.4\%$), *M. brunneum* 1868 ($18.8 \pm 9.3\%$), and *M. robertsii* ($12.8 \pm 6.6\%$). Endophytic colonization of maize leaves was observed only for *M. brunneum* 1868 ($8.3 \pm 5.7\%$).

Field Experiments

The field experiments were evaluated in the springtime to assess seedling emergence and aboveground wireworm damage and in autumn, to assess harvest-related parameters. A significant effect of factor treatment and maize variety, but not their interaction, was observed on certain parameters assessed in the field experiments (Table 1). Treatment with *M. brunneum* resulted in a significant decrease of wireworm damaged plants and a significant increase of emerged undamaged plants during the spring evaluation in all three maize varieties tested. At harvest time a significant influence attributed to *M. brunneum* treatment was observed in the number of plants exhibiting reduced growth without ears, plants carrying corn ears and the number of total plants. However, in these three parameters also maize variety exhibited a significant effect. For example, only in the variety LG 34.90 a significant increase of the number of maize plants carrying corn ears was observed.

Among other parameters observed at springtime, wireworm damage was significantly decreased and consequently the number of undamaged plantlets was significantly increased in all three maize varieties treated with *M. brunneum* 1868. However these beneficial effects were observed at harvest time only for variety LG 34.90, where the number of plants carrying corn ears was significantly increased (Figure 5).

The *M. brunneum* treatment resulted in a significant increase (+17.9%) in the number of plants carrying corn ears at harvest time in variety LG 34.90 (Figure 5C). This allowed us to calculate a theoretical fresh grain yield increase. Based on the measured weights of ears with husks and differences in numbers of plants carrying ears at harvest time, an increase of 5.23 t ha^{-1} fresh ear yield was calculated attributed to *M. brunneum* treatment for variety LG 34.90. Similarly, the *M. brunneum* treatment may reduce the wireworm related collapse of entire plants and could lead to an increase of 11.03 t ha^{-1} fresh aboveground biomass for variety LG 34.90. These calculations average fresh weight of 10 plants and fresh weight of ears from the same 10 plants and pooled data from treated and untreated maize variety LG 34.90 as the fungal treatment significantly affected only final stand of LG 34.90 plants carrying ears but not their fresh weight or the weight of fresh corn ears.

DISCUSSION

Of the several EPF strains tested in the laboratory, *M. brunneum* 1868 showed the highest virulence to *Agriotes lineatus* wireworms and was most often re-isolated from washed pieces of maize roots that emerged from seeds coated with conidia of that strain. Frequency of retrieved re-isolation events suggests that *M. brunneum* 1868 is rhizosphere competent. Based on these findings we evaluated its potential to reduce damages of

TABLE 1 | Results of statistical analyses of the observed variables assessed in field experiments.

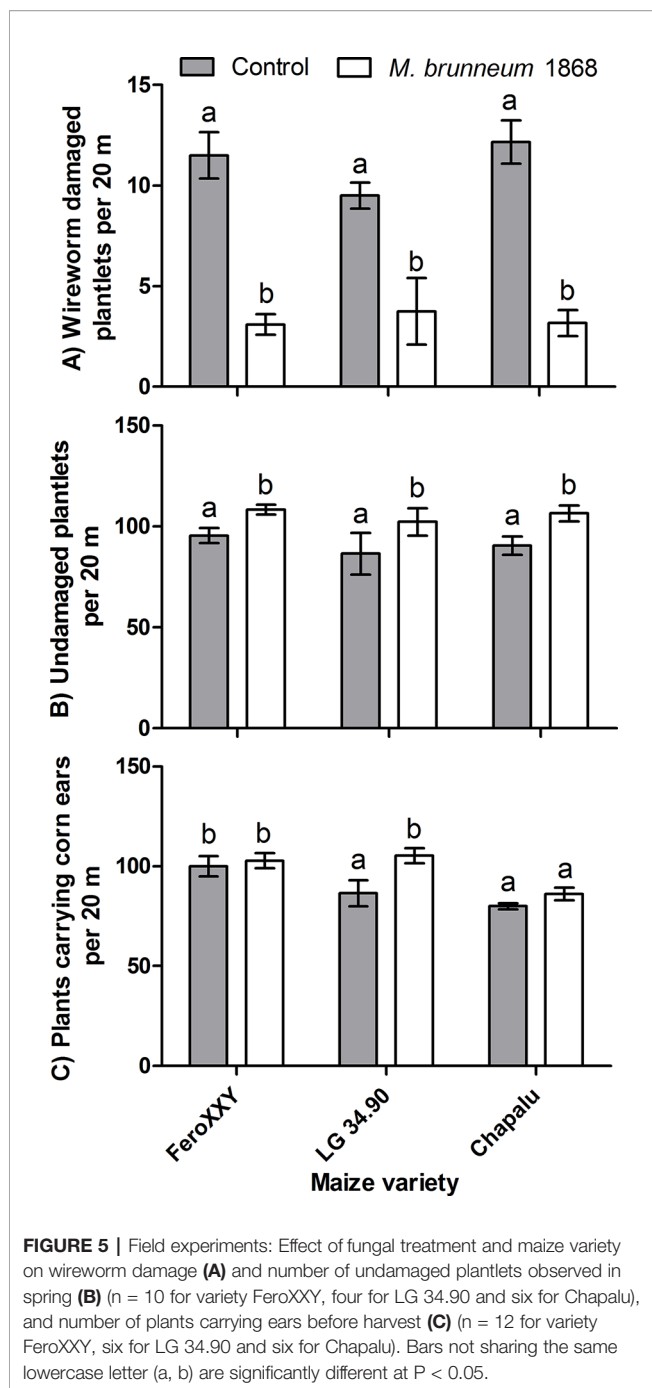
Maize variety	Chapalu		FeroXXY		LG 34.90		Significance	
	Treatment	Control	M. brunneum	Control	M. brunneum	Control	Maize Variety	MV × T
Spring (emergence and wireworm damage) evaluation								
Wireworm damaged plants	12.17 ± 1.05 ^a	3.17 ± 1.05 ^b	11.50 ± 0.81 ^a	3.10 ± 0.81 ^b	9.50 ± 1.29 ^a	3.75 ± 1.29 ^b	ns	F _{1, 39} = 78.4; P = 0.0000
Emergence—undamaged plants	90.50 ± 4.81 ^a	106.50 ± 4.81 ^b	95.40 ± 3.73 ^a	108.30 ± 3.73 ^b	86.50 ± 5.89 ^a	102.25 ± 5.89 ^b	ns	F _{1, 39} = 13.9; P = 0.0007
Emergence—total plants	102.67 ± 4.64	109.67 ± 4.64	106.90 ± 3.60	111.40 ± 3.60	96.00 ± 5.69	106.00 ± 5.69	ns	ns
Autumn (final stand) evaluation								
Final stand—reduced growth, no ears	9.33 ± 0.87 ^a	8.67 ± 0.87 ^a	3.25 ± 0.62 ^a	2.83 ± 0.62 ^a	4.33 ± 0.87 ^a	0.83 ± 0.87 ^b	F _{2, 47} = 37.9; P = 0.0000	F _{1, 47} = 5.55; P = 0.0232
Final stand—plants carrying corn ears	80.00 ± 5.43 ^a	86.17 ± 5.43 ^a	100.03 ± 3.84 ^a	102.82 ± 3.84 ^a	86.50 ± 5.43 ^a	105.33 ± 5.43 ^b	F _{2, 47} = 7.63; P = 0.0015	F _{1, 47} = 5.25; P = 0.0271
Final stand—total plants	89.33 ± 5.10 ^a	94.83 ± 5.10 ^a	103.25 ± 3.61 ^a	105.67 ± 3.61 ^a	90.83 ± 5.10 ^a	106.17 ± 5.10 ^b	F _{2, 47} = 4.02; P = 0.0253	F _{1, 47} = 4.15; P = 0.0479
Fresh weight of 10 plants [kg]	5.47 ± 0.55	5.35 ± 0.55	6.64 ± 0.39	6.73 ± 0.39	9.84 ± 0.55	10.29 ± 0.55	ns	ns
Weight of ears from 10 plants [kg]	2.67 ± 0.23	2.71 ± 0.23	2.71 ± 0.17	2.64 ± 0.17	3.77 ± 0.23	4.01 ± 0.23	F _{2, 47} = 39.9; P = 0.0000	ns
Number of ears on 10 plants	10.83 ± 0.69	10.67 ± 0.69	11.08 ± 0.49	10.92 ± 0.49	10.00 ± 0.69	10.33 ± 0.69	ns	ns

Different lowercase letters indicate significant effect of fungal treatment within varieties. ns, not significant; MV, maize variety; T, treatment.

wireworms in field settings. Maize plants stemming from kernels inoculated with *M. brunneum* 1868 conidia showed significantly less wireworm damage at emergence in the field experiment resulting in significantly higher undamaged maize stand in springtime. While the early plant belowground herbivory avoiding effect could be measured in all three varieties, increased fresh ear yield and fresh aboveground biomass was observed only in variety LG 34.90 at harvest time.

While chemical insecticides have an immediate effect on wireworm fitness, no such immediate effect can be expected through inoculating seeds or roots with entomopathogenic fungi (Razinger et al., 2013; Razinger et al., 2018b). Even after inoculating insect larvae directly with dense conidial suspensions, long incubation is required for observing an effect on insect larvae. In the laboratory experiments, wireworm mortality reached less than 60% after 8 weeks (Figure 1), whereas wireworms damage corn seedlings already within three to four weeks after sowing. It is therefore possible that the reduction of *Agriotes* herbivory by *M. brunneum* is due to larval repellence or other mechanisms. Also Kabaluk and Ericsson (2007a) reported that wireworms were not killed but repelled by *Metarhizium anisopliae* (Metchnikoff) Sorokin contaminated soil and that repellence increased with the conidial concentration in soil in laboratory experiments. Based on these results they postulated that the plant stand density in field settings increased possibly due to larval repulsion (Kaballuk and Ericsson, 2007b). Similar maize stand density increase was also observed when an encapsulated *M. brunneum* formulation, registered for wireworm management in potatoes (Brandl et al., 2017), was tested against wireworms in maize (prof. S. Vidal, personal communication). Metabolite production by endophytes of other species of the Clavicipitaceae or endophyte mediated production of volatile organic compounds has frequently been discussed as a mechanism resulting in herbivory repellence (reviewed in Johnson et al., 2016). Similarly, predatory bugs such as *Anthocoris nemorum* L. sense the presence of *Beauveria bassiana* after leaf inoculation with conidia of that species (Meyling and Pell, 2006). Meyling and Pell (2006) also observed that *A. nemorum* avoided contact with the thus inoculated leaves. It is thus possible that the high conidial inoculum mediated presence of *M. brunneum* can protect crop plants from wireworms non-parasitically.

Metarhizium brunneum did not stimulate early maize growth in the here described laboratory settings, which was also reported by Kabaluk and Ericsson (2007b) for *M. anisopliae*. Specifically, no effect of fungal treatment was observed on the speed of seed germination in maize and wheat. The only significant effect on the speed of seed germination was attributed to CMC-treatment used in the seed coating procedure, revealing a need for additional experiments and especially improved formulation for testing possible effects of *Metarhizium* on plant growth. By using a simple inoculation technique of immersing seeds in conidial suspension for 2 h, Ahmad et al. (2020) reported a *M. robertsii* mediated increase of maize height and aboveground biomass compared to control plants in laboratory experiments. Ahmad et al. (2020) also observed a much higher proportion of endophytically colonized maize leaves as we did, and they



calculated a positive correlation between plant height and aboveground biomass and the proportion of endophytic root and leaf colonization by *M. robertsii*. *Metarhizium brunneum* strain 1868 well developed on maize root surfaces (Figure 4) and clearly better than on cauliflower (Razinger et al., 2014) or broccoli (Herbst et al., 2017) roots. This tight association with maize roots is in sync with the report by Hu and Bidochka (2020), who suggested that *Metarhizium* has a preference for monocots such as barley and corn. As a rhizosphere colonizer, *Metarhizium* spp. might also protect corn from other detrimental factors like soil pathogens (Kabaluk and Ericsson,

2007b; Vega et al., 2009). Furthermore, a higher frequency of endophytic colonization was detected in maize roots (18.8%) compared to leaves (8.3%). This could be the result of fungal preference towards different tissues within the plant host, i.e. plant root preference by *Metarhizium* species as postulated by Behie et al. (2015).

Laboratory results are often inconsistent with field trials (Kabaluk et al., 2005; Kölliker et al., 2011; Sufyan et al., 2017). To achieve the highest EPF mediated control effectiveness, many factors must be considered, such as landscape properties, soil characteristics, crop type, etc. In addition, different *Agriotes* species can be differently susceptible to a certain entomopathogenic taxon or individual strain or formulation product (Kölliker et al., 2011). The challenge is thus to find a strain or a mixture of different strains that performs equally well in different environmental conditions and against different pests. One might consider using multiple EPF strains of the same species to cover a wider range of ecological conditions, but such a strategy would be very difficult to put into practice due to registration constraints (Gadhav et al., 2016; Humber, 2016).

CONCLUSIONS

The investigated entomopathogenic fungi exhibited multifaceted functions (Vega et al., 2009), i.e. pathogenicity to wireworms, rhizosphere competence and some growth promoting effects of maize and wheat plants in lab settings. However, observed effects were either depending on the EPF strain, plant species or variety. *Metarhizium brunneum* strain 1868 reduced *Agriotes* herbivory and increased initial plant stands of all three maize varieties tested in field settings. Interestingly, pre-harvest maize stand density and yield were increased only in one of the three varieties. Similar differential interactions between EPF and different plant varieties were reported by Canassa et al. (2020) for two root-inoculated strawberry varieties. This highlights the problems of generalizations and warrants further studies on the mechanisms of plant × fungus interactions. Insect repellence (Meyling and Pell, 2006; Kabaluk and Ericsson, 2007a) mediated through fungal rhizosphere competence may be the underlying mechanism for the measured increase of plant biomass. The ability of tested fungi to improve stand and robustness of young maize plants could contribute to wireworm stress resilience as this pest limits maize growth especially after crop emergence and less towards harvest time (Taupin, 2007).

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JR provided the initial concept and design of the study and designed and led the execution of the field trial; JR and H-JS

performed laboratory trials; JR and EP wrote the manuscript. H-JS contributed to study design and manuscript drafting. 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/fpls.2020.535005/full#supplementary-material>

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Fungal Endophyte-Mediated Crop Improvement: The Way Ahead

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Endophytes are non-disease causing microbes (bacteria and fungi) surviving in living tissues of plants. Their intimate association and possible coevolution with their plant partners have resulted in them contributing to an array of plant growth benefits ranging from enhanced growth and biomass accumulation, tolerance to abiotic and biotic stresses and in nutrient acquisition. The last couple of decades have witnessed a burgeoning literature on the role of endophytes (Class 3 type) in regulating plant growth and development and their adaptation to abiotic and biotic stresses. Though the underlying mechanisms of plant-endophyte interactions are far from clear, several studies have raised the hope of their potential application in agriculture, especially in mitigating abiotic and biotic stresses. The use of endophytes is envisaged as a route to reduce the production cost and burden on the environment by lessening the dependence on breeding for crop improvement and agrochemicals. Unfortunately, save a few well documented examples of their use, a little of these insights has been translated into actual agricultural applications. Here, we reflect on this paucity and elaborate on some of the important bottlenecks that might stand in way of fully realizing the potential that endophytes hold for crop improvement. We stress the need to study various facets of the endophyte-plant association for their gainful application in agriculture.

Keywords: agriculture, abiotic stress, biotic stress, agrochemicals, crop breeding

INTRODUCTION

Endophytes are microbes residing within plants without causing any harm to their growth and development. Unlike disease-causing microorganisms, endophytes are non-pathogenic and many of them are known to enhance their plant host's fitness (Mendes et al., 2013; Philippot et al., 2013). Fungal endophytes (FE) are classified in to four Classes based on their symbiotic criteria (Rodriguez et al., 2009). Class 1 endophytes are Clavicipitaceous fungi which survive in some cool season grasses and are transmitted vertically with their seeds. Class 2 endophytes colonize extensively the shoot, root, and rhizome of many plants and are transmitted both vertically and horizontally. Class 3 endophytes have a broad host range exhibiting restricted colonization of the shoot; they are transmitted horizontally. Class 4 endophytes which are also horizontally transmitted are restricted to the roots. The Class 3 endophytes which we address

here, are effective in combating several abiotic stresses faced by their host plants, such as drought, salinity, nutrient deficiency, and metal toxicity, etc., and biotic stresses caused by pathogens and insect pests (Waller et al., 2005; Hardoim et al., 2008; Rho et al., 2018a; Manasa et al., 2020; Sampangi-Ramaiah et al., 2020). They are also known to produce pharmaceutically important secondary metabolites and enzymes (Shweta et al., 2010; Kusari et al., 2013; Kaushik et al., 2014; Kumara et al., 2014; Nagarajan et al., 2014; Uzma et al., 2019) and phytohormones (Bilal et al., 2017, 2018). In the past few decades, it became obvious that endophytes could be isolated from every plant studied (Strobel and Daisy, 2003; Hardoim et al., 2015; Suryanarayanan et al., 2018a; Giauque et al., 2019). These analyses showed that many attributes of endophytes, in particular their universal occurrence, sustained presence in plants, non-pathogenic nature, ability to enhance the biotic and abiotic stress tolerance of their plant hosts (Rodriguez et al., 2009), increase access to soil nutrients and increase the plant yield (White et al., 2019; Xia et al., 2019) project them as candidates holding high promise for use in crop improvement. Despite this, very few of the benefits associated with endophytes have been translated into real-world agricultural applications. Here, we reflect upon this gap and identify potential bottlenecks that might hinder the exploitation of endophytes in agricultural applications. We also discuss the possible approaches that might help pave the way ahead in allowing for a gainful application of endophytes in agriculture.

PROOF OF THE PRINCIPLE OF APPLICATION OF ENDOPHYTES IN AGRICULTURE

Although fungal endophyte presence in plants is well known, the mechanism of plant colonization by these fungi is hardly known. For instance, the ability of species of *Colletotrichum*, *Guignardia*, *Pestalotiopsis*, *Diaporthe*, and *Xylaria* to infect a wide range of plant species as foliar endophyte (Suryanarayanan et al., 2018a) has not been explained. One study shows that an endophytic *Pestalotiopsis* produces a chitin deacetylase enzyme, which modifies its chitin cell wall to escape detection by its plant host immune system (Cord-Landwehr et al., 2016). Yuan et al. (2019), based on transcriptomics and proteomics analysis conclude that the endophyte *Gilmaniella* sp. infects its host plant *Atractylodes lancea* by reducing its immune response. A leaf is usually colonized by many Class 3 endophyte species (Rodriguez et al., 2009) exhibiting restricted growth in the tissue. Of these, invariably one or two species dominate the endophyte assemblage of the leaf, while the rest occur as satellite species with low colonization frequencies (Suryanarayanan et al., 2018a; Vaz et al., 2018). The interactions of a foliar endophyte with co-occurring endophytes (fungal and bacterial) in the leaf are little understood. It is possible that such interactions among them as well as their cross talk with the host would ultimately define the composition and ecological functions of the endophytes. According to Schulz et al. (2015), a balanced antagonism operates among the various endophytes in a plant tissue to maintain

the endophyte community. For instance, an endophytic *Alternaria tenuissima* produced more antifungal polyketide stemphyrylenol in the presence of another endophyte, *Nigrospora sphaerica* (Chagas et al., 2013). Colonization by alien endophytes of a plant tissue is generally inhibited by the existing native endophytes (Mohandoss and Suryanarayanan, 2009; Suryanarayanan et al., 2018b).

Additionally, since the plant and its associated microbiome (which includes the endophytes) function as a mini ecosystem (the holobiome), to use endophytes gainfully it is essential to discern the different interactions operating here. Currently, there is very little information available on the functional aspect plant and its microbiome (Vandenkoornhuyse et al., 2015). We hardly know the roles of the core (dominant) and satellite (showing low degree of tissue colonization) endophyte species or of the ecological functions of key stone species in an endophyte assemblage. Plants generally resist infection by pathogens through pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) or effector-triggered immunity (ETI). The basic question of how the endophytes overcome such resistance responses while infecting the host has not been answered satisfactorily (Vandenkoornhuyse et al., 2015). This is intriguing since some symptomless pathogens infect plant tissues and survive as endophytes.

Despite such lacunae, work conducted over the last 2 decades across a range of agricultural crop plants has provided a strong proof of principle for the application of endophytes to agriculture (Suryanarayanan et al., 2017). The goal of this review is to highlight some of the salient studies to reiterate the potential application of endophytes in agriculture but not to review the literature on plant/endophyte interactions.

Laboratory experiments and glasshouse trials strongly indicate that endophytes could mitigate stresses in agriculturally important crops and increase productivity. A recent meta-analysis by Rho et al. (2018a) highlights the potential applications of endophytes in mitigating drought, salinity, and nutrient shortfalls in agricultural systems. For instance, inoculation of FE from wild barley in to a barley cultivar significantly increased its grain yield (Murphy et al., 2018). Treatment of wheat plants with the endophyte *Alternaria alternata* enhanced growth and imparted drought tolerance. Plants colonized by the endophyte effectively quenched stress-induced free radicals and also accumulated higher levels of osmolytes (Qiang et al., 2019). Growth promotion induced by endophyte could often be brought about indirectly as evident in peanut plants where the endophyte, *Phomopsis liquidambri* enhanced nodulation and nitrogen fixation by H₂O₂ and NO signaling (Xie et al., 2017). FE protect crops against abiotic stresses under laboratory conditions, as shown for salt (Baltruschat et al., 2008; Manasa et al., 2020; Sampangi-Ramaiah et al., 2020), heat and drought (Redman et al., 2002; Bailey et al., 2006; Hubbard et al., 2014; Ali et al., 2018; Sangamesh et al., 2018) stresses. A number of studies confirm that the root endophyte *Piriformospora indica* (*Serendipita indica*) ameliorates a broad range of abiotic stresses in many crop plants. In *Zea mays*, it solubilizes the insoluble phosphate in the soil, which is unavailable to the plant and transports it to the plant (Yadav et al., 2010; Aslam et al., 2019), increases the drought stress tolerance of barley (Ghaffari et al., 2019),

and, apart from improving stress tolerance, increases growth and nutrient acquisition in soybean plants (Bajaj et al., 2018). Endophytes also enhance tolerance of host plants to biotic stressors including pathogenic fungi (*Botrytis cinerea* in grapevine – Barka et al., 2002; *Phytophthora* sp. in cocoa – Arnold et al., 2003; *Cronartium ribicola* in *Pinus monticola* – Ganley et al., 2008; *Verticillium dahliae* in tomato – Fakhro et al., 2010, and *Phytophthora capsici* in hot pepper – Bae et al., 2011). The protection of plants against insect pests is well documented for Class 1 FE (Rodriguez et al., 2009), which are vertically transmitted within their grass host communities (Rodriguez et al., 2009; Kauppinen et al., 2016; Raman and Suryanarayanan, 2017), although, according to Faeth and Fagan (2002), such endophytes may not function as defensive mutualists in native plants. A few horizontally transmitted Class 3 FE are also reported to reduce insect attack of plants (Vega, 2008; Vidal and Jaber, 2015). The action of endophytes in plants may often be due to the synergy with other co-existing endophytes, fungi, or bacteria as was demonstrated by Bilal et al. (2020). They showed that the two EF, *Paecilomyces formosus* and *Penicillium funiculosum* acted synergistically to impart tolerance to drought, high temperature, and heavy metals (Bilal et al., 2020).

The underlying mechanisms of the benefits of endophytes on their host plants are currently being unraveled. It appears that a combination of alterations in the gene expression and physiology of the host induced by the endophyte is reflected as the plant's response to stresses. But much of the studies investigating the mechanisms are largely restricted to a few fungi, notably the root endophyte, *Piriforma indica*. For example, in rice, *P. indica* confers drought tolerance by regulating miR159/miR396 that target MYB and GRF transcription factors, involved in regulation of growth and hyposensitivity response (Mohseni Fard et al., 2017). In yet another study in rice, *P. indica* colonization led to the differential miRNA synthesis that targeted transcription factors involved in nutrient uptake, Na⁺ transport, and growth regulation including auxin responsive proteins (Kord et al., 2019). In soybean, *P. indica* colonization leads to the upregulation of genes within the phenylpropanoid and derivative pathway and in iron scavenging siderophores (Bajaj et al., 2018). More recently, a comparative transcriptome analysis of rice colonized by a salt adapted EF was shown to upregulate a number of genes involved in both abiotic and biotic stress tolerance, when the plants were subjected to salinity stress (Sampangi-Ramaiah et al., 2020). There is now increasing evidence that the endophyte effects on plants are mediated through specific signaling cascades which, upon perception by the host cell, alter host gene expression (Sampangi-Ramaiah et al., 2020).

Infection of a plant by FE rapidly upregulates defense related genes and the lignin and cellulose content of its cell walls (Soliman et al., 2013; Mejía et al., 2014); such responses of the plant as well as the chemicals of the resident endophyte enhance its ability to counter abiotic and biotic stressors (Estrada et al., 2013). Thus, it is apparent that the use of endophytes is a promising route for improving crop productivity by reducing the dependence on breeding and agrochemicals. However, the cost suffered by the plant for harboring endophytes is a facet

that has not been understood adequately. For the plant host, the presence of FE results in reduced photosynthesis, altered host nitrogen metabolism, and loss of photosynthates (Mejía et al., 2014). Alternatively, the hypothesis that respiratory CO₂ of endophytes could result in islands of low photorespiration thus enhancing photosynthesis in the leaf tissue (Suryanarayanan, 2013) appears to be true at least with reference to bacterial endophytes (Rho et al., 2018b). To fully appreciate the benefit accrued by endophyte association, the cost-benefit ratio for a plant should ideally be worked out by taking into account the entire community of endophyte it harbors.

In summary there is ample evidence to suggest that endophytes can mediate growth and other benefits such as adaptation to abiotic and biotic stresses in plants that could in principle lead to their gainful application in agriculture.

FROM THE LAB TO THE FIELD: STILL A CHASM

Long term studies confirm that plant association with mycorrhizal fungi is not accidental and results in increased stress tolerance of the associated plant (Gehring et al., 2017). It is conceivable that the adaptive capabilities of the host plant increase substantially due to the extensive metabolic potential of the associated mycorrhizal fungi (Lau et al., 2017). Such focused studies on endophyte association are lacking though endophyte technology has distinct advantage over inorganic agriculture practices. Since most of the endophytes used in agriculture colonize the underground and above ground tissues and develop together with their host plants, their metabolisms are adapted to each other. The balanced interactions during the entire symbiotic phase allows for better adaptation to environmental changes since the responses are the result of a synergistic interaction between the two partners, which is believed to be more than the sum of the responses of the two partners alone (Rosenberg and Zilber-Rosenberg, 2018). However, the successful use of these microbes depends largely on their performance under field conditions which now requires extensive research addressing the barriers for effective product development.

There are many publications endorsing the positive role of FE on plant growth and performance in adverse environments (Gundel et al., 2013). *Epichloë* (Class 1) endophyte strains selected for low toxicity to livestock and which increase the productivity of forage grasses and the robustness of turf grasses have been used in the United States, Australia, and New Zealand (Young et al., 2014; Kauppinen et al., 2016). The performance of *Epichloë* endophytes infected grasses is superior such that Kauppinen et al. (2016) assert their use while designing sustainable management strategies for agriculture. According to Johnson et al. (2013) endophyte mediated plant trait improvement contributed around \$200 million per annum to the economy of New Zealand. Root endophytic *Trichoderma* species increase yields of stressed crops by inducing biochemical pathways, which render the toxic reactive oxygen species generated during stress into less toxic compounds

(Harman et al., 2020). Despite such studies, with reference to non-*Epichloë* endophytes, there is very little by way of commercial products which are evident in the global market. To evaluate this, we searched the United States and Indian patent database to analyze patents filed with respect to endophyte treatment for plant health benefits. The comprehensive search was made using the keywords “endophyte” and “plant” and the exclusive patents typically describing the effective utilization of endophytes for plant benefits are compiled in **Table 1**.¹

We observed that a considerable number of fungal endophytes were acclaimed to improve the overall agronomic attributes; sometimes, an endophyte confers more than one beneficial effect on plant growth and yield. For example, in one case endophytes not only improved drought tolerance, but also reduced pest attack, and improved the yield attributes in cotton (United States patent # 9,277,751; **Table 1**). Many FE control pests and diseases in plants (**Table 1**). Only four

patents are listed in the Indian database,² of which only that of Arora et al. (2015) has been experimentally examined and approved (**Table 2**).

We contacted 66 authors referred to in the meta-analysis publication of Rho et al. (2018a) and obtained responses from eight scientists, of which one claimed translation of the research into a commercial product (Cheng et al., 2012). This demonstrates that product development is disappointingly low compared to the scientific investments. As mentioned by one respondent, the complex and protracted regulatory guidelines are mainly responsible for the low success rate in commercialization of the product developed by the scientific community.

Finally, we also compiled information on commercial endophytic products, which are already available in the market. An example is endophyte infected grasses commercialized by a New Zealand based company “Grasslanz”.³ The inoculation of an endophyte in the grass grown in airports and recreational

¹<http://patft.uspto.gov/netahtml/PTO/search-bool.html>; accessed on March 19, 2019

²<http://ipindiaservices.gov.in/publicsearch>; accessed on March 20, 2019

³<http://www.grasslanz.com/>

TABLE 1 | United States patents claiming endophyte's benefit in plants (2000–2018).

No	Organism	Patent claim	Patent #	Patent author
A. Endophytic fungi				
1	<i>Neotyphodium</i>	Resistance to invertebrate pests	61,11,170	Latch et al., 2000
2	Fungi	Insect resistance, disease resistance, drought resistance	61,80,855	Hiruma, 2001
3	<i>Neotyphodium</i> spp. or <i>Gliocladium</i> spp.	Insect resistance, disease resistance to Italian rye grass	65,48,745	Hiruma et al., 2003
4	<i>Neotyphodium</i> spp.	Pest control, no toxicity to grazing animal	68,05,859	Imada et al., 2004
5	<i>Muscodor albus</i> and <i>Muscodor roseus</i>	Pest control by volatiles	69,11,338	Strobel et al., 2005
6	<i>Muscodor albus</i>	Bio control of fungus	77,54,203	Strobel et al., 2010
7	<i>Neotyphodium lolii</i>	No ryegrass toxicosis, enhance growth under drought	79,76,857	Tapper et al., 2011
8	<i>Colletotrichum dematium</i>	Antifungal peptide	80,80,256	Strobel et al., 2011
9	<i>Muscodor albus</i> and <i>Muscodor roseus</i>	Disease and nematode resistance by volatile	80,93,024	Strobel et al., 2012
10	<i>Colletotrichum dematium</i>	Biological activity against either <i>Botrytis cinerea</i> , <i>Sclerotinia sclerotiorum</i> , or <i>Rhizoctonia solani</i>	87,65,147	Strobel et al., 2014
11	<i>Acremonium</i> spp.	Protection of grass plants from biotic or abiotic stress	89,75,489	Carven, 2015
12	<i>Clonostachys rosea</i>	Stimulation of nodules in legumes, enhanced plant growth under stress	81,01,551	Stewart et al., 2012
13	<i>Dothideomycetes</i> spp.	Increased boll retention, growth, and yield. Resistance to drought, cold, metal, salt, fungi, bacteria, virus and pests in cotton	92,77,751	Sword, 2016
14	<i>Neotyphodium lolii</i>	No ryegrass toxicosis, protection from pests and/or abiotic stress	93,74,973	Tapper et al., 2016
15	<i>Lophodermium</i> spp.	Antifungal activity in pine	94,69,836	Miller et al., 2016
16	<i>Phialocephala</i> spp.	Pest tolerance in conifers	95,49,528	Miller et al., 2017
17	<i>Sarocladium</i> spp.	Promotion of germination, resistance to nitrogen stress	96,87,001	Vujanovic et al., 2017
18	<i>Neotyphodium coenophialum</i>	No ergopeptide, black beetle resistance, enhanced biomass and yield in grasses	97,06,779	Roulund et al., 2017
19	Incertaesedis, Nectriaceae, or Plectosphaerellaceae	Improved tolerance to drought, pests, better yield in cotton	97,56,865	Sword, 2017
20	<i>Acremonium</i> spp.	Improved resistance to diseases and/or pests in <i>Brachiariaurochloa</i> grass	98,72,502	Spangenberg et al., 2018
21	<i>Trichoderma harzianum</i>	Enhanced growth or seed germination under abiotic stress	99,61,904	Rodriguez et al., 2018

TABLE 2 | Indian patents claiming endophyte's benefit in plants (2000–2018).

No	Organism	Patent claim	Patent #	Patent author
A. Endophytic fungi				
1	<i>Piriformospora indica</i> and <i>Azotobacter chroococcum</i>	Plant growth promotion	2017/DEL/2013	Arora et al., 2015
B. Endophytic fungi + Bacteria				
2	Fungi and bacteria	Patent awaiting improvement of germination rate, emergence rate, shoot biomass, root biomass, seedling root length, seedling shoot length, and yield.	201717043115	Karen et al., 2017
3	Fungi and bacteria	Patent awaiting improvement of agronomically important traits.	201717043114	Karen et al., 2018

areas deter insects, grazing animals, and birds. The “Grasslanz” homepage claims over 69 and 88% reduction in above and below ground insect pests, respectively. This application might be a long-term solution for bird strike issues in the airports.⁴ Another example comes from “BioEnsure” developed by Adaptive Symbiotic Technologies (Seattle, United States). “BioEnsure” is endophytic fungal based preparation which had shown between 3 and 30% increase in crop (corn, rice, wheat, soybean, and cotton) yield under drought, heat, and cold stresses.⁵

BOTTLENECKS AND WAY FORWARD

Taking FE as a standard, we discuss the constraints faced in using endophytes for crop improvement (**Figure 1**). The two basic steps for successful use of FE in crop improvement are: (1) the identification of a FE with a needed trait and (2) introduction of that FE into the crop which involves colonization, establishment, and confirmation of a successful symbiosis and sustained expression of the desired trait in FE-colonized crops (Hyde et al., 2019). The first step is straight forward and involves screening of FE isolated from plants for choice traits. The second step is fraught with uncertainties since it entails the interaction of the newly introduced FE with the crop host and its already existing endobiome. Here, the vertically transmitted grass endophyte *Epichloë* appears to be more tractable owing to its tight host specificity and systemic and sustained infection of the host (Saikkonen et al., 2016) though certain questions like the role of genetics in dictating the fungus-grass symbiosis and its effect on natural and agricultural grass ecosystems remain unanswered (Saikkonen et al., 2016). Relatively less information is available on the interactions of horizontally transmitted endophytes with their hosts. Although the broad host range of some of these like *Colletotrichum*, *Guignardia* (*Phyllosticta*), *Pestalotiopsis*, *Diaporthe* (*Phomopsis*), and *Xylaria* could be a salient feature in ensuring successful colonization of different crops, their influence on the native microbes of the host crop and higher trophic levels are not known. (Rabiey et al., 2019). Furthermore, many species of these FE genera are latent pathogens and it is possible that climate change

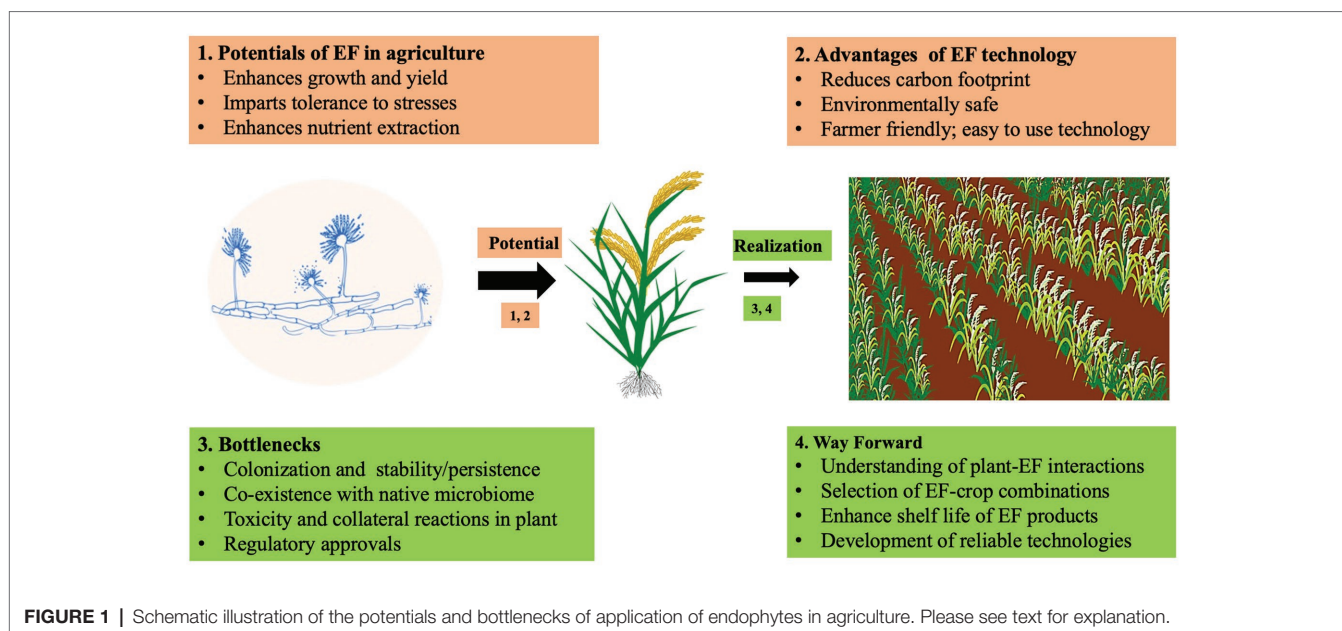
could tilt their lifestyle toward pathogenicity (Moricca and Ragazzi, 2008; Paolinelli-Alfonso et al., 2016). These facets of FE interactions should be unraveled for their efficient use in agriculture. Suffice it to say that little information with agricultural relevance is available for this step. Cogitation on the plant's defense reactions toward biotrophic and necrotrophic fungal pathogens at the structural, biochemical, and genome levels could be of heuristic value here.

First, as many fungi including endophytes produce toxic secondary metabolites including mycotoxins (Thirumalai et al., 2013, 2020), it is necessary to evaluate endophytes for the production of such metabolites when they are introduced in to crop plants; this becomes even more important if the endophytes reach the edible parts of the plant, such as the seeds or tuber (for human consumption) or forage (for animal consumption; **Figure 1**). Second, there is little information on the interplay that operates between the newly introduced microbes and the native plant endobiome. Hardly any information is available on the infection of a plant host by an endophyte, which is the very first step of FE establishment. One explanation for the colonization of a wide range of plants by non-host specific FE is that they escape detection by the plant immune response by altering the chitin in their cell wall, while infecting the plant (Cord-Landwehr et al., 2016). Endophyte enrichment technology should ensure that the introduced endophyte establishes itself in the plant host endobiome and its introduction does not perturb the native microbiome, which could result in negative impacts on plant performance or the environment/ecosystem or agrosystem (**Figure 1**). For instance, the presence of non-native endophytes which have not coevolved with the host could eliminate the native beneficial microbes resulting in a net loss for the plant (Whipps, 2001). Galls in trees induced by wasps are abscised by necrosis induced by the fungal endophyte *Apiognomonia errabunda*; however, this is more harmful to the host than the galls (Sieber, 2007). Thus, it is essential to consider the plant as a holobiont, i.e., the plant together with the diversity of microbes interacting with the plant and each other to achieve FE mediated enhance plant performance (Rosenberg and Zilber-Rosenberg, 2018; Rabiey et al., 2019).

The plant and its microbiome have to be considered as a mini-ecosystem in which the microbiome can be the essential determinant for plant performance (Aglar et al., 2016). For example, Thynne et al. (2019) showed that fungal pathogens

⁴<http://www.grasslanz.com/understanding-the-science>

⁵cf. <http://www.adaptivesymbioticttechnologies.com/field-results.html>



harboring horizontally acquired modular polyketide genes from bacteria exhibit a broader host range than those that are not housing these trans-genes. This highlights the importance of short-term and long-term interactions between plant and microbes and might have an important influence on the survival of a newly introduced endophyte. Furthermore, in the halophyte *Salicornia europaea*, the fungal endophyte community is influenced by the bacterial community but not vice versa (Furtado et al., 2019). Endophytes which do not produce antibiotics in pure cultures do so when co-cultured with another endophyte species (Schulz et al., 2015) suggesting that microbial interactions can profoundly influence the endobiome. It is conceivable that the complex network of such interactions (Schulz et al., 2015) can result in warding off the introduced non-native endophyte in the crop, or that the newly introduced endophyte alters the endobiome community such that it is no longer beneficial for the plant. The report that mango leaves could be colonized by alien FE species only after the native EF species are eliminated by systemic fungicide treatment is an example for the importance for this scenario (Mohandoss and Suryanarayanan, 2009). Similarly, an FE from a brown seaweed which effectively controls the insect pest *Helicoverpa armigera* in crops does not survive in the new host, even after infection with high spore doses, probably due to its poor competitive ability with the native microbiome (Suryanarayanan et al., 2018b). Alternatively, it is reasonable to assume that a new endophyte can dominate the holobiome and restrict growth of the endogenous microbes, which can result in loss of benefits for the plants. In this context, it is important to mention that barely anything is known about the role of newly introduced endophytes on existing mycorrhiza in crop plants, although many examples demonstrate that both symbionts can co-exist in the same root. Yet another factor to be considered is the possible negative effect of an introduced endophyte which could nullify the beneficial effect provided by it (Rabiey et al., 2019).

These include lack of substantial information on (1) field performance of endophytes, (2) influence of weather conditions on endophyte performance, (3) highly restricted colonization of plants leading to only localized results, and (4) alteration in the native community of associated microbes by the endophyte leading to reduced performance of the host (Rabiey et al., 2019).

Third, it is necessary to lay caution on the possibility that an introduced endophyte may elicit disease reactions in a non-host plant. Fungi such as *Leptosphaerulina crassiasca* (Suryanarayanan and Murali, 2006) and *Fusarium verticillioides* (Oren et al., 2003) could live in a plant as endophytes or pathogens. Endophytes alter their lifestyle depending on host genotype, web of interactions, the experience with co-occurring microbes in the endobiome, and the environmental conditions (Hardoim et al., 2015). Symptomless FE could become pathogenic due to heat stress such as that produced by climate change (Paolinelli-Alfonso et al., 2016). Factors which determine the lifestyle of a microbe in its host environment and influence its expression profile need to be understood to effectively use FE in crop management (Figure 1). Such investigations can be done in three directions – by analyzing the influences of the host milieu, of the microbes with and without the host, and of environmental conditions in which the crop is growing. Furthermore, for FE to be important for agricultural applications, it should be ensured that endophyte formulations maintain substantial viability and activity (Woodward et al., 2012). They also have to be consistent with cultural practices since agrochemicals can have a strong influence on EF communities associated with crops (Stuart et al., 2018).

Finally, crop breeding programs have not considered beneficial microbiota yet (Bakker et al., 2012; Raaijmakers and Mazzola, 2016), and genetic markers favoring endophyte association with crops are not known. Plant genetic loci controlling endophyte colonization in crop plants might be a helpful tool to promote agriculture with less agrochemical usage. Since information on

the influence of agricultural practices on FE of crops is meager, the performance of FE in real field situations cannot be predicted. A recent study reports that pest and pathogen management alters the leaf microbiome diversity of tea (Cernava et al., 2019). Agricultural practices including fertilizer application and mowing frequency influence the FE communities in agriculturally important grasses (Wemheuer et al., 2019). Application of agrochemicals such as triflurumuron and fenoxaprop-P-ethyl decreases the diversity, richness, and evenness of FE in soybean (da Costa Stuart et al., 2018). Gange et al. (2019), conclude that although endophytes could be crucial players in plant protection, experimental methodology, and inoculation methods to reintroduce FE into plant can skew the results. Since environment and agricultural practices influence interactions within the endobiome, these factors have to be taken into consideration for using FE in crop improvement (Figure 1). Since plant genotype influences the diversity and composition of its microbiome (Li et al., 2018), an FE effective for one crop may not be suitable for other crops. Hence, developing crop varieties which would readily accommodate an introduced FE will improve crop production and productivity (Schlatter et al., 2017).

Another formidable challenge for many biofertilizers, biopesticides, or bio-stimulants used in agriculture is obtaining registration for the product. The licensing laws differ with countries and registration may involve several regulatory bodies which is time-consuming (Timmusk et al., 2017). Therefore, this non-biological bottleneck requires more efficient administration such that research on endophyte-mediated crop improvement could be galvanized (Harman et al., 2010).

CONCLUSION

From the disparate studies so far, it seems that a deep understanding of the holobiont ecology and breeding of crops for better association with beneficial microbes (particularly endophytes) is the ideal way forward. Commercialization of

endophytes and/or their products can promote plant fitness and agricultural yields. In spite of an increasing demand for these biological products in today's market, we still need to address various gridlocks related to basic mechanisms of newly introduced FE into plants and eco-agrosystems. Field applications will help to understand whether results from the laboratory reflect those from the real world, in particular under adverse climate conditions.

AUTHOR CONTRIBUTIONS

RS along with VC planned the review. VC collated the data and prepared the draft. TS contributed to redrafting the manuscript and adding substantially to the section on the bottlenecks and way-forward and straightening the bibliography. KN, SP, and RO edited and contributed to several rounds of revision of the manuscript. All authors contributed to the article and 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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Changes in the Microbiome in the Soil of an American Ginseng Continuous Plantation

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American ginseng is an important herbal medicinal crop in China. In recent years, there has been an increasing market demand for ginseng, but the production area has been shrinking due to problems associated with continuous monocropping. We analyzed the microbiome in bulk soils to assess whether and, if so, what changes in the bulk soil microbiome are associated with continuous American ginseng cropping. The alpha diversity of fungi and bacteria was significantly lower in the soils planted with American ginseng than the virgin (non-planted) land. The relative abundance of *Fusarium* spp. and *Ilyonectria* spp., known plant root pathogens, was much higher in the soils cropped with American ginseng than the non-planted. On the other hand, a number of bacteria with biodegradation function, such as *Methylibium* spp., *Sphingomonas* spp., *Variovorax* spp., and *Rubrivivax* spp., had lower abundance in the soils cropped with American ginseng than the non-cropped. In addition, soil pH was lower in the field planted with American ginseng than the non-planted. Accumulation of fungal root pathogens and reduction of soil pH may, therefore, have contributed to the problems associated with continuous monocropping of American ginseng.

Keywords: American ginseng, microbial diversities, high-throughput sequencing, herbal crop, continuous cropping obstacles

INTRODUCTION

The American ginseng (*Panax quinquefolium*), which belongs to the Araliaceae family, is a perennial herb and native to the United States and Canada. It was introduced into China in 1975 from the United States, has become one of the most used herbal medicines in Asia, and now has been grown in more than 10 provinces in China with the total planting area close to 3,700 hectares. China is the third largest production country for American ginseng following the United States and Canada, producing 3,600 tons in 2016, and it is the largest country consuming American ginseng (Wang and Wu, 2003). However, problems associated with continuous monocropping have become a major limiting factor for growing American ginseng in China (Jin et al., 2006). Soil grown with American ginseng for one season (usually 4 years) could lead to very reduced productivity on the same land for 10 years or more

(Chen et al., 2012). As a result, land suitable for growing American ginseng is decreasing rapidly in China.

Problems associated with continuous monocropping occur in many crop species, such as apple, cherry, alfalfa, rice, corn, and strawberry; such problems are usually related to one or more of the following factors: deterioration of soil physicochemical properties, allelopathy/autotoxicity, soil-borne diseases, and changes in the soil microbial communities (Singh et al., 1999; Wu et al., 2008; Ying et al., 2012). Auto-intoxication is very common in continuous monocropping of rice, resulting in considerable yield loss (Chou, 1995). *Thielaviopsis basicola* is partially responsible for the cherry continuous cropping problem (Hoestra, 1968). *Rhizoctonia solani*, *Pythium intermedium*, and *Fusarium solani* are reported to play an important role in causing apple replant disease (Braun, 1995; Mazzola, 1998; Manici et al., 2003). Extracellular compounds released by *Dactylonectria torresensis* might have contributed to the severe growth reduction associated with apple replant disease (Manici et al., 2018).

Sanqi ginseng (*Panax notoginseng*) is also an important herbal plant in China. Autotoxic ginsenosides in the rhizosphere contributed to yield to a decline of Sanqi ginseng associated with continuous monocropping (Yang et al., 2015). Accumulation of soil-borne pathogen inocula and ginsenosides in Sanqi ginseng cultivated soils was considered to be one of the main reasons for replant failure in Sanqi ginseng (Li et al., 2019). Only one study was published on the continuous cropping of Asian ginseng (*Panax ginseng*), suggesting that changes in the rhizosphere microbiome due to inorganic fertilizers are one key factor resulting in the replant problem (Dong et al., 2017).

Many studies have been carried out to understand factors responsible for the replant problem in American ginseng (He et al., 2008; Bi et al., 2010), most of which have focused on toxic substances in soil. Nine phenolic compounds in the soil of American ginseng cultivation were detected and proved to inhibit radicle and shoot growth of American ginseng (Bi et al., 2010). Five groups of autotoxic compounds were found from aqueous extracts of fibrous roots of American ginseng (He et al., 2008). Adding root residue (0.02–0.5 mg/mL) to the hydroponics reduced seedling development of American ginseng and prolonged the leaf expansion period (Jiao et al., 2012).

In the present study, the main objective was to investigate the changes in soil microbial communities associated with the American ginseng production history to identify specific microbial groups related to the replant problem of American ginseng.

MATERIALS AND METHODS

Soil Sample Collection

Soil was sampled in the spring of 2017 from three fields (Zakoushi, Yingpan, and Miaotaizi) of Liuba county, Shaanxi province, China. The area of the fields varied from ca. 600 to 1,200 m². At Zakoushi, the fields were grown with American ginseng for 0–4 years, coded as LZCK for control (year 0) and LZ1, LZ2, LZ3, and LZ4 for years 1–4 years, respectively. At the Yingpan and Miaotaizi fields, American ginseng has been grown for 8 and 12 years; samples were coded as LZA2 for Yingpan and

TABLE 1 | Details of soil sample taken from three fields (Zakoushi, Yingpan, and Miaotaizi) with different cropping history of American ginseng in Liuba county, Shaanxi province, China.

Sample location	Longitude/ Latitude	Code of samples	Cultivation years	Number of samples
Zakoushi field	106°43′13″E/ 33°38′41″N	LZ1	1	3
		LZ2	2	3
		LZ3	3	3
		LZ4	4	5
		LZCK	0	3
Yingpan field	106°43′16″E/ 33°37′41″N	LZA2	8	5
		LZA2CK	0	3
Miaotaizi field	106°84′E/ 33°68′N	LZA3	12	3
		LZA3CK	0	3
Total				31

LZA3 for Miaotaizi with LZA2CK and LZA3CK as the respective controls (non-ginseng planted soil) (Table 1). In all the fields, American ginseng was grown with standard cultural practices. Briefly, the soil was fertilized with the humus of a primitive forest and plowed to the depth of ca. 30 cm prior to planting. American ginseng is planted in March and harvested in October of the fourth year. A total of 31 soil samples were collected from the fields with varying planting years (Table 1). For each sample, soil was collected from the top layer (0–20 cm) with a soil core sampler (2.5 cm in diameter) at five locations. Then, the soils were blended thoroughly and put into in sterile polythene bags. After the samples were transported to the laboratory, soils were immediately divided into two parts: one part was stored at –80°C until further use, and the other part was air-dried at room temperature for analysis of physiochemical properties.

Soil DNA Isolation and Sequencing

Total soil DNA was extracted with the NucleoSpin Soil Kit (Macherey-Nagel, Germany) following the manufacturer's instructions. The V4 region of bacterial 16S rDNA and the ITS1 region of fungal rDNA were amplified with the universal primer pairs 515F/806R (515F: GTGCCAGCMGCCGCGGTAA and 806R: GGACTACHVGGGTWTCTAAT) (Caporaso et al., 2011) and ITS1/ITS2 (ITS1: CTTGGTCATTTAGAGGAAGTAA and ITS2: GCTGCGTTCTTCATCGATGC) (Walters et al., 2016), respectively. Both forward and reverse primers were tagged with the Illumina adaptor and index sequences. PCR reactions were performed with the Phusion High-Fidelity PCR Master Mix (NEB, USA) in a 50-μL reaction, including 300 mM of each primer, 30 ng template DNA, and 25 μL PCR Master Mix. The PCR condition was as follows: pre-denaturation at 94°C for 3 min, 30 cycles of 94°C for 30 s, 56°C (V4)/55°C (ITS1) for 45 s, 72°C for 45 s, and final extension at 72°C for 10 min. PCR products from different samples were pooled together (equal volume) and purified with AGENCOURT AmpureXP beads (Beckman

Coulter, China) as a library. The library was quantified with the Agilent 2100 bioanalyzer and ABI StepOnePlus Real-time PCR system. Finally, the validated library was sequenced on the Illumina MiSeq platform by BGI (Shenzhen, China) to generate 250 bp paired-end reads.

Sequence Processing

Raw sequences were preprocessed to obtain clean sequence reads with the in-house pipeline of BGI Co., Ltd. Paired-end reads were then merged using fast length adjustment of short (FLASH) reads (v1.2.11). Minimal overlapping length was 15 bp with the maximum mismatching ratio in the overlapped region set to 0.1. Unique sequences with only one read were discarded. Then, all unique sequences were clustered into operational taxonomic units (OTUs) with USEARCH v7.0.1090 at 97% similarity with a representative sequence generated for each OTU. The SINTAX algorithm (https://www.drive5.com/usearch/manual/sintax_algo.html) then assigned each OTU sequence to taxonomic ranks by aligning the representative sequence against the Ribosomal Database Project (RDP) Classifier v2.2 trained on the bacterial database Greengene v201305 (Cole et al., 2014) and the UNITE_v7.2 fungal database (Kõljalg et al., 2013). A confidence value of 0.6 was used as the cutoff when assigning an OTU to a specific taxonomy group. Then, an OTU table was generated by aligning all sequences filtered with far less stringent criteria with the OTU representative sequences as described previously (Deakin et al., 2018). All OTU processing was carried out with the UPARSE pipeline (v10.0) (Edgar, 2013).

Data Analysis

The original OTU tables were normalized (rarefaction) with Qiime2 for all subsequent statistical analysis; the sampling depths were 57,000 and 42,000 for fungi and bacteria, respectively.

We conducted two types of analysis. First, we analyzed the effect of cropping years (0–4 years) at Zakoushi through a one-way ANOVA. Second, we compared microbial communities between the virgin land and the soils grown with American ginseng for multiple years across the three fields. In this analysis, samples of the fourth year (LZ4) were chosen at Zakoushi field to compare with other two fields, and the three fields (sites) were treated as a blocking factor; namely each field (site) represents a random sample of planting vs. non-planting comparisons. Thus, the statistical model is of the form: “Fields + Planting vs. Non-planting.”

Alpha diversity indices (Shannon, Chao1, and Robbins indices and observed feature) were calculated. The non-parametric methods of the Kruskal–Wallis test and trimmed means method were applied to assess the year effect at Zakoushi and planting effect across the three sites, respectively. To assess the differences in the overall microbial communities among samples, Bray–Curtis indices between samples were calculated and were subjected to non-metric multidimensional scaling (NMDS); the effects of treatments (planting years or planting vs. non-planting) were analyzed with the Adonis method through PERMANOVA (as implemented in R package vegan 2.3-1). The differences in fungal or bacterial relative abundance aggregated at each taxonomic level (with RDP Classifier v2.2 at the confidence of

0.6) between soils planted with and without American ginseng were analyzed with the compare group function in R package metacoder v0.3.3 (Foster et al., 2017) with *p*-values adjusted with the Benjamini–Hochberg method. The metacoder package was also used to display tree view graphs of this differential abundance analysis.

Measuring Soil pH and Nutrient Elements

Soil pH was measured for all individual samples with 1 mol (M) KCl as suspending media to avoid seasonal variability (Collins et al., 1970). Each soil sample was air-dried at room temperature for 3 days, ground thoroughly, and passed through 1-mm sieve. A subsample of 8.0 g soil was dissolved into 20.0 ml 1M KCl in a 50-ml beaker, mixed with a glass rod, and then kept at room temperature for 30 min for pH measurement with a pH meter (Mettler FE20 FiveEasy Plus™ pH, Germany). The standard pH buffer solutions of 4.01, 6.87, and 9.18 were used to adjust the pH meter. Available potassium was estimated with the flame photometric method (M410, Sherwood Scientific Ltd., Cambridge, UK). Available phosphorus was estimated with the spectrophotometric method (Shimadzu UV2450, Shimadzu Scientific Instrument, Kyoto, Japan).

RESULTS

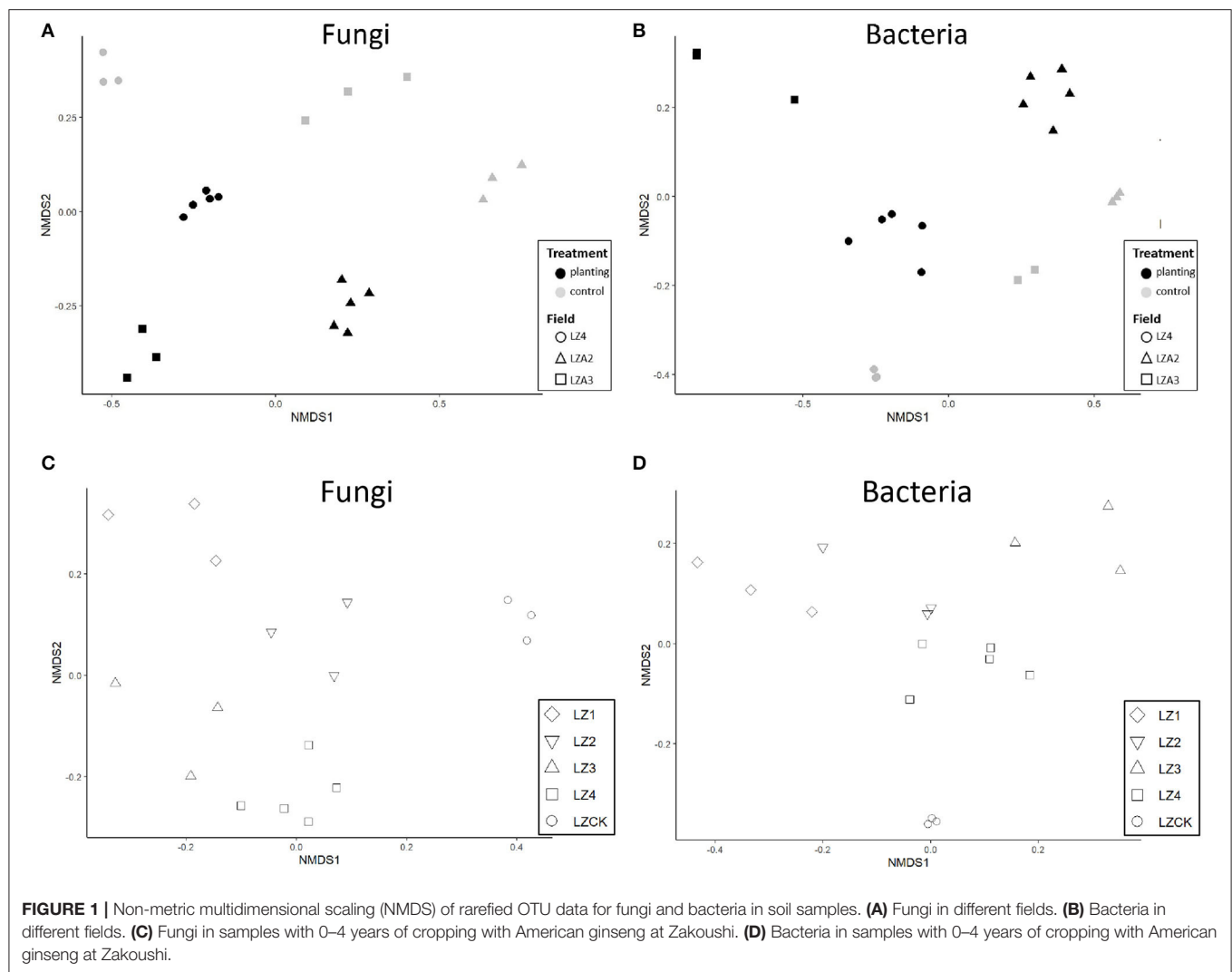
General Characteristics of Sequence Data

A total of 2,372,515 ITS sequences and 1,843,202 16S sequences were obtained from the 31 samples. The number of raw sequence reads per sample ranged from 78,729 to 83,757 for ITS and from 60,740 to 63,348 for 16S. The number of sequences per sample after quality filtering ranged from 72,492 to 78,934 (ITS) and from 58,430 to 60,313 (16S) per sample. The number of OTUs in each sample ranged from 383 to 877 (fungi) and from 1,976 to 4,088 (bacteria). Sequencing depth is sufficient for all samples (**Supplementary Figure 1**). Raw sequences were uploaded to the NCBI BioProject database (Accession: PRJNA640251).

Fungal Diversity

The alpha diversity indices (Shannon, observed feature, and Chao1) showed that fungal alpha diversity decreased ($P < 0.01$) in the soils planted with American ginseng compared to the non-planted, but there were no significant differences in the Robbins indices. Adonis analysis of the Bray–Curtis indices indicated significant ($P < 0.001$) differences between soils grown with American ginseng for multiple years and the virgin land. NMDS showed that samples from soils grown with American ginseng for multiple years were clearly separated from those virgin lands (**Figure 1A**); as expected, there were large differences between the three fields as well. There were clear separations between samples from the fields grown with American ginseng for 0–4 years at Zakoushi (**Figure 1C**). Multiple comparison based on PERMANOVA also showed that the overall fungal community from the field grown with the ginseng for 4 years (LZ4) differed ($P < 0.05$) from LZCK (control), LZ1, LZ2, and LZ3.

The OTUs with taxon for fungi were finally counted as 80 (**Supplementary Table 1**) not including those whose OTUs were identified as a taxon named unidentified in the database,



those whose OTUs were confirmed but could not be found in the database, and those whose relative abundance was $<0.05\%$ in all 31 samples. The tree view in **Figure 2A** was constructed using these data. At the class rank, the abundance of Dothideomycetes fungi decreased in the soils planted with American ginseng, and Eurotiomycetes increased compared to non-planted soils. At the order rank, two orders (Eurotiales and Pleosporales) increased significantly in the soils grown with American ginseng compared to the non-planted. At the family rank, Trichocomaceae had an increased relative abundance, whereas Phaeosphaeriaceae and Didymellaceae had decreased relative abundance. At the genus rank, *Sagenomella* had an increased relative abundance, and *Boeremia* and *Gibberella* had decreased relative abundance. At the species rank, only *Boeremia exigua* and *Paraphoma chrysanthemicola* had decreased relative abundance, whereas eight species, including known plant root pathogens (*Ilyonectria robusta*, *Vishniacozyma heimaeyensis*, and *Fusarium hostae*) of American ginseng, had increased relative abundance in the ginseng-cropping compared to non-planted soils.

Mortierella spp. was the dominant fungal species in all soil samples, and its relative abundance varied from 22.2 to 52.6% (**Supplementary Tables 2, 3**). *Fusarium* spp. was not detected in any control soil samples but was found in all soil samples from the ginseng-grown soils at Zakoushi. The relative abundance of *Fusarium* spp. increased with the cropping years, accounting for 1.4, 2.0, 2.2, and 3.2% in the first, second, third, and fourth years, respectively (**Supplementary Table 2**).

Bacterial Diversity

The bacterial alpha diversity decreased greatly in the fields grown with American ginseng for multiple years compared to the virgin lands. All the alpha diversity indices calculated showed significant differences: Shannon ($P < 0.01$), Chao1 ($P < 0.05$), Robbins ($P < 0.05$), and observed feature ($P < 0.001$). Similarly, the beta-diversity indices (Bray–Curtis indices) also differed ($P < 0.001$) between the soils grown with and without American ginseng across the three fields as shown by the Adonis analysis. NMDS analysis showed a clear separation of samples between the soils grown with and without ginseng at each field (**Figure 1B**).

Similarly, samples from the soils grown with ginseng for 0–4 years at Zakoushi were also separated (Figure 1D). The overall bacterial community in the soils grown with ginseng for 4 years differed ($P < 0.05$) from all other samples at Zakoushi.

The OTUs with taxon for bacteria were finally counted as 43 (Supplementary Table 4), not including those whose OTUs were identified as a taxon named unidentified in the database, those whose OTUs were confirmed but could

not be found in the database, and those whose relative abundance was $<0.05\%$ in all 31 samples. The tree view in Figure 2B was constructed using these data. As shown in Figure 2B, at the phylum rank, three phyla (Betaproteobacteria, Nitrospirae, and Bacteroidetes) decreased in the soil planted with American ginseng, and four phyla (Verrucomicrobia, Acidobacteria, Firmicutes, and Alphaproteobacteria) increased compared to non-planted soils. At the class rank, the

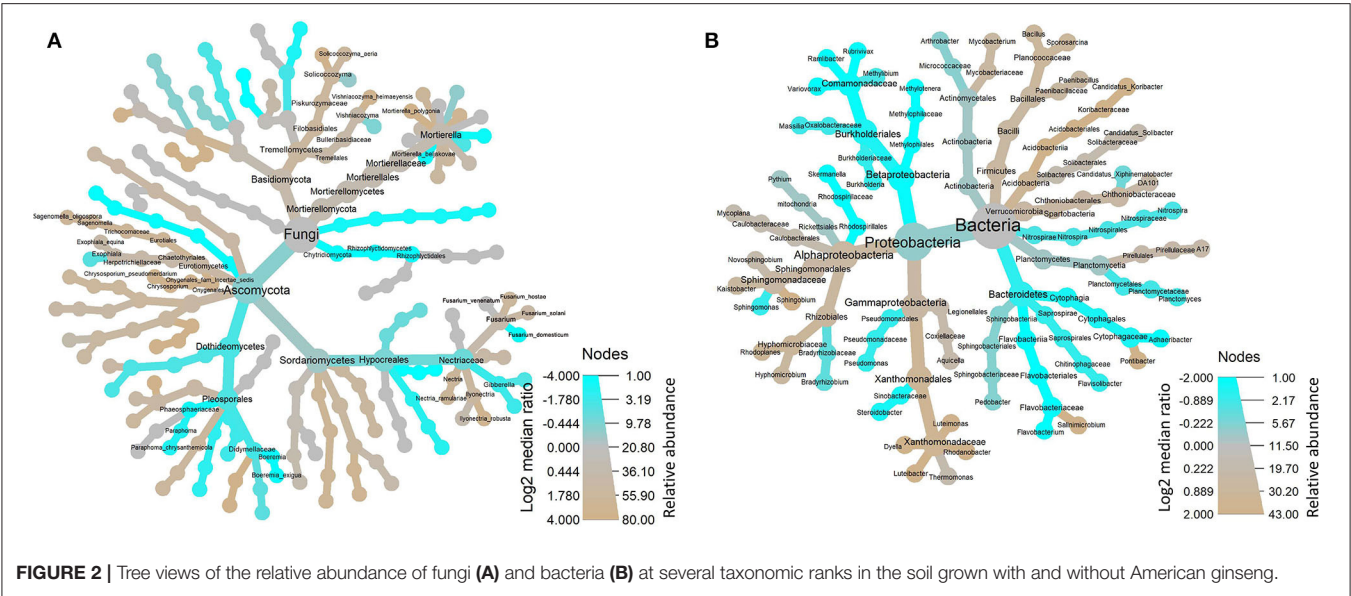


FIGURE 2 | Tree views of the relative abundance of fungi (A) and bacteria (B) at several taxonomic ranks in the soil grown with and without American ginseng.

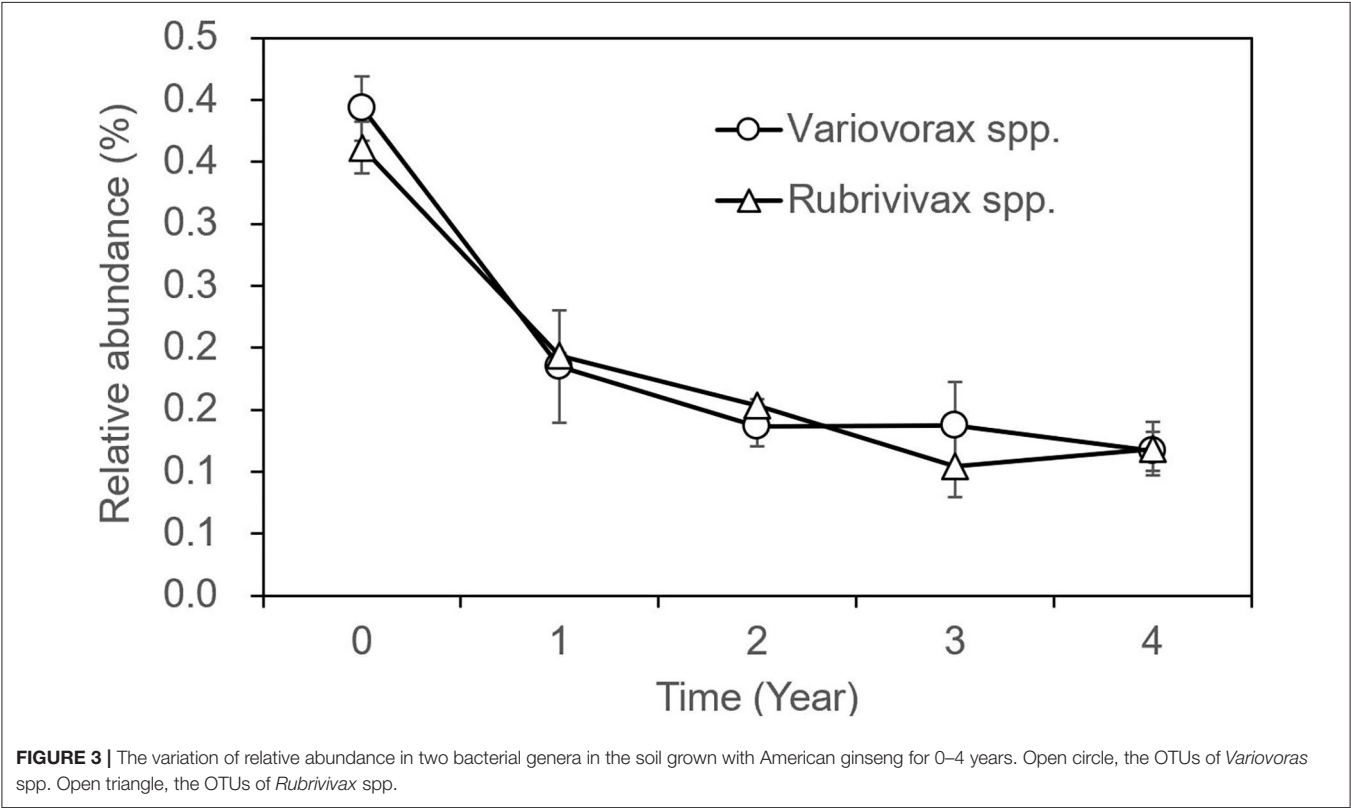


FIGURE 3 | The variation of relative abundance in two bacterial genera in the soil grown with American ginseng for 0–4 years. Open circle, the OTUs of *Variovorax* spp. Open triangle, the OTUs of *Rubrivivax* spp.

relative abundance of Solibacteres, Acidobacteriia, and Bacilli was higher in the soils planted with American ginseng than non-planted soils. At the order rank, five orders (Methylophilales, Burkholderiales, Saprospirales, Nitrospirales, and Pseudomonadales) had decreased relative abundance in the soils grown with American ginseng compared to non-planted soils. At the genus level, 13 genera decreased, whereas six genera grew in relative abundances. Most of the decreased genera, i.e., *Methylibium*, *Rubrivivax*, *Variovorax*, and *Sphingomonas*, were involved in the degradation of certain compound substances,

such as diphenyl ether, isoproturon, and atrazine. Interestingly, an alkali-tolerant bacterial genus, *Ramlibacter*, was found decreased, and one phylum, Acidobacteria, which are suitable for growing in acidic conditions, were found to be higher in the soils grown with American ginseng than non-planted soils. This coincides with the decreasing soil pH in the soil grown with American ginseng. Among the increasing genera, Xanthomonadaceae was proved to be a plant pathogen, and *Sphingbium* was involved in the degradation of phenol, which was one of the compounds inhibiting radicle and shoot growth of American ginseng. The relative

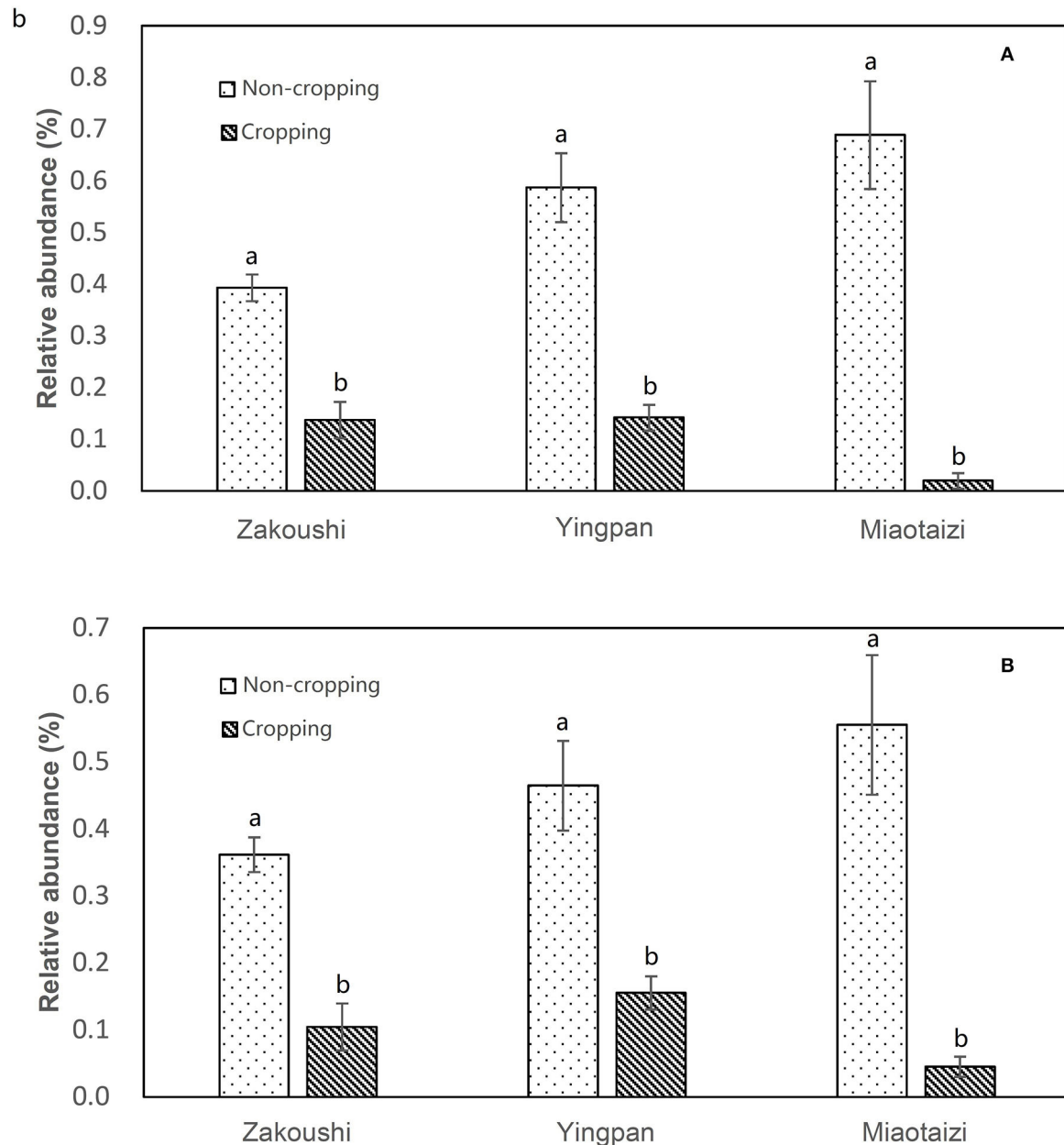
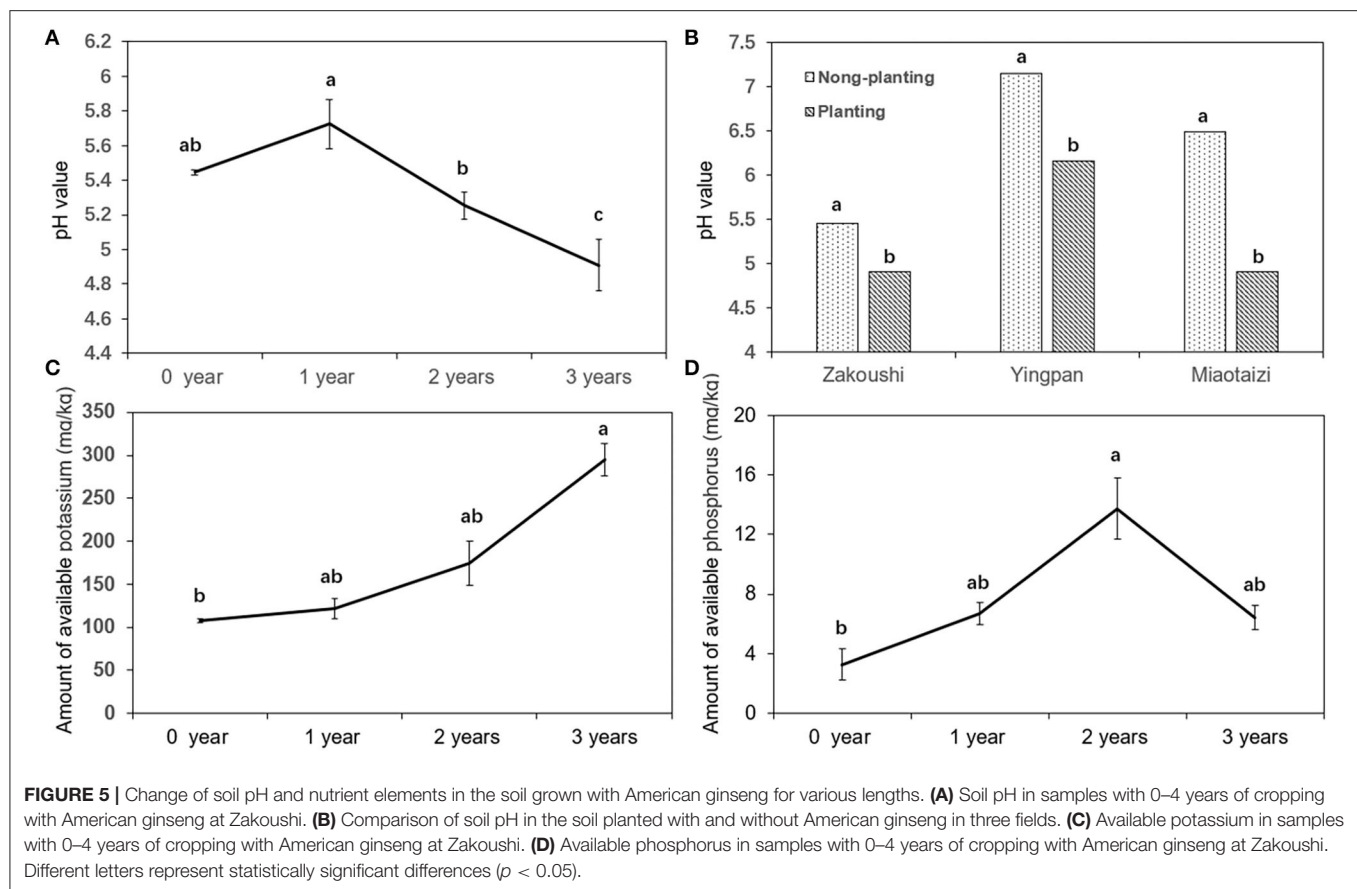


FIGURE 4 | Relative abundance of *Variovorax* spp. (A) and *Rubrivivax* spp. (B) in the soil from three fields. Different letters represent statistically significant differences ($p < 0.05$).



abundance of two bacteria, *Variovorax* spp. and *Rubrivivax* spp., decreased with the increase of cropping years (Figure 3). The amount of *Variovorax* spp. declined from 0.39% in non-planting soil to 0.14% in planting soil at Zakoushi, dropped from 0.59 to 0.14% at Yingpan, and dropped from 0.69 to 0.02% at Miaotaizi (Figure 4A). The abundance of *Rubrivivax* spp. decreased from 0.36 to 0.14% at Zakoushi, dropped from 0.46 to 0.16% at Yingpan, and fell from 0.56 to 0.05% at Miaotaizi (Figure 4B).

Changes of pH value and Concentration of Potassium and Phosphorus Elements in Soil

The soil pH decreased with the increasing year of cropping with American ginseng at Zakoushi (Figure 5A). At the other two fields (LZA2 and LZA3), soil pH was also reduced ($P < 0.05$) from 7.2 (control) to 6.2 at Yingpan and decreased from 6.5 (control) to 4.9 at Miaotaizi (Figure 5B). The available potassium increased ($P < 0.05$) with the continuous planting years (Figure 5C), but there was no linear trend in the available phosphorus (Figure 5D).

DISCUSSION

Soil sickness is a phenomenon that causes the reduction in both crop yield and quality when the same crop or its relative

species are cultivated on the same soil successively (Yu, 2008). Cultivation of American ginseng for one crop season usually takes 4 years in the soil until harvesting. Through analysis of soils grown with American ginseng for a number of years, we identified several possible causes that could contribute to the replant issue in American ginseng: (1) accumulation of several plant fungal root pathogens (e.g., *Fusarium oxysporum*, *F. solani*, *F. proliferatum*, and *Gibberella baccata*); (2) reduction of some potential beneficial bacteria, such as *Methylibium* spp., *Sphingomonas* spp., *Variovorax* spp., and *Rubrivivax* spp.; and (3) gradual decreasing soil pH value.

Fusarium spp. is a damaging plant pathogen, infecting many plant species and causing root rot and wilt diseases (Huang et al., 1998; Bi et al., 2011). In the diseased roots of American ginseng, *F. oxysporum* and *F. solani* were commonly isolated (data not shown). Punja (1997) isolated *Fusarium solani*, *F. oxysporum*, *F. avenaceum*, and *F. equiseti* from the diseased roots of American ginseng. *Fusarium solani*, *F. oxysporum*, *F. tricinatum*, *F. proliferatum*, and *Cylindrocarpum destructans* were all isolated from the diseased roots of American ginseng, and among these, *F. solani* and *F. oxysporum* were mainly responsible for root rot diseases (Bi et al., 2011). The present finding agrees with those previous findings as the relative abundance of *Fusarium* spp. in the soil grown with American ginseng increased considerably. The present study also suggests other

common root pathogens in the *Nectriaceae* family may also contribute to the replant issue of American ginseng, including *Ilyonectria robusta*, *Dactylonectria anthuriicola*, and *I. mors-panacis*, which causes red-skin root in *Panax ginseng* (Lu et al., 2019). Therefore, accumulation of pathogens is likely to be one of the reasons for the reduced productivity of American ginseng in continuous monocropping.

Mortierella spp. has been widely used in biosynthesis. Certik and Shimizu (1999) report that arachidonic acid, dihomog-linolenic acid, and mead acid are produced by *Mortierella* spp. In the present study, *Mortierella* spp. is the dominant fungal group in all soil samples. Although, Lu et al. (2014) isolated *Mortierella* sp. from the roots of red-skin Asian ginseng (*P. ginseng*), it has not yet been demonstrated whether *Mortierella* spp. is responsible for red-skin disease. As *Mortierella* spp. constituted such a high proportion in all the ginseng soil, further research is needed to ascertain whether ginseng health is affected by *Mortierella* spp.

In the soils grown with American ginseng for 1–4 years, the abundance of two genera, *Variovorax* spp. and *Rubrivivax* spp., declined with increasing cropping years. Some species of *Variovorax* are able to metabolize a large variety of different substrates (Satola et al., 2013). *V. paradoxus* showed great capabilities in increasing the root and shoot biomass of pea and the uptake of N, P, K, Ca, and Mg (Jiang et al., 2012) and degrading lots of compounds, such as isoproturon (Hussain et al., 2011), linuron (Sorensen et al., 2005), atrazine (Smith et al., 2005), polycyclic aromatic hydrocarbons (Eriksson et al., 2003; Young et al., 2006), chlorinated hydrocarbons (Humphries et al., 2005), and methyl tertiary butyl ether (Zaitsev et al., 2007). *Rubrivivax gelatinosus* can degrade pollutants in fish industry effluent (Leandro et al., 2011). Hence, we speculate that *Variovorax* spp. and *Rubrivivax* spp. may play a role in degrading toxicants in the soil growing with American ginseng for multiple years. Their decrease might lead to accumulation of some toxic substances, which might impact negatively on American ginseng growth. Further research is needed to support or disprove our speculation.

Soil physicochemical property deterioration is believed to be a factor responsible for reduced cropping potentials in the continuous cropping of *P. ginseng*. Specific gravity and bulk density of the soils grown with ginseng increased, and soil porosity decreased compared to the soil without ginseng (Laura et al., 2000; Wu et al., 2008). reports that low pH restricts nitrification rates and increases concentrations of certain elements known to be toxic to many plants (e.g., aluminum). Soil pH affected root length, plant height, nodule, and pod number of cowpea (Joe and Allen, 1980). Our results show that the pH value in American ginseng cropping soil declined significantly from 5.72 in the first year to 4.91 in the third year at Zakoushi, from 7.16 to 6.16 in the soil at Yingpan, and from 6.49 to 4.92 in the soil at Miaotaizi. The soil pH 4.91 is outside the pH range of 5.5–6.0 suitable for American ginseng (Hong, 1978). Interestingly, the present study revealed that the relative abundance of an alkali-tolerant bacterium, *Ramlibacter*,

decreased, but the phylum of Acidobacteria increased in the soils grown with American ginseng. Low pH (and associated changes in specific microorganisms in the soil) may be another factor contributing to reduced growth in continuous monocropping of American ginseng.

There are other factors that could contribute to reduced crop productivity in monocropping. Root exudates can lead to changes in soil physicochemical properties and the microbial community; the microbial community may alter the degradation of root exudates and further affect the physicochemical properties of the soil; the change of soil physicochemical properties can, in turn, influence microbial community structures. Further research is needed to study the cross-talk among the microbial community, root exudates, and soil physicochemical properties in American ginseng monocropping.

DATA AVAILABILITY STATEMENT

Raw sequences were uploaded to the NCBI BioProject database (Accession: PRJNA640251).

AUTHOR CONTRIBUTIONS

XH, XX, and SF planned and designed the research. JZ, FZ, JQ, WS, JD, SE, and XL performed the experiments. SE, JD, JZ, and XH analyzed the data. JZ, JQ, XX, and XH wrote 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/fpls.2020.572199/full#supplementary-material>

Supplementary Figure 1 | Number of sequences plotted against the coverage of OTUs; each line is standard for one of the 31 samples.

Supplementary Table 1 | The OTU number with taxon for fungi in the soil of different American ginseng cropping years.

Supplementary Table 2 | Fungal relative abundance in American ginseng cropped soil for 0–4 years.

Supplementary Table 3 | Relative abundance of fungi in the soil planted and non-planted with American ginseng at Yinpan and Miaotaizi.

Supplementary Table 4 | The OTU number with taxon for bacteria in the soil of different American ginseng cropping years.

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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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Dark Septate Endophytes Isolated From Wild Licorice Roots Grown in the Desert Regions of Northwest China Enhance the Growth of Host Plants Under Water Deficit Stress

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This study aimed to explore dark septate endophytes (DSE) that may improve the cultivation of medicinal plants in arid ecosystems. We isolated and identified eight DSE species (*Acremonium nepalense*, *Acrocalymma vagum*, *Alternaria chartarum*, *Alternaria chlamydospora*, *Alternaria longissima*, *Darksidea alpha*, *Paraphoma chrysanthemicola*, and *Preussia terricola*) colonizing the roots of wild licorice (*Glycyrrhiza uralensis*) in the desert areas of northwest China. Moreover, we investigated the osmotic stress tolerance of the DSE using pure culture, along with the performance of licorice plants inoculated with the DSE under drought stress in a growth chamber, respectively. Here, five species were first reported in desert habitats. The osmotic-stress tolerance of DSE species was highly variable, *A. chlamydospora* and *P. terricola* increased the total biomass and root biomass of the host plant. All DSE except *A. vagum* and *P. chrysanthemicola* increased the glycyrrhizic acid content; all DSE except *A. chartarum* increased the glycyrrhizin content under drought stress. DSE × watering regimen improved the glycyrrhizic acid content, soil organic matter, and available nitrogen. Structural equation model analysis showed that DSE × watering regimen positively affected soil organic matter, and total biomass, root length, glycyrrhizic acid, and glycyrrhizin (Shapotou site); and positively affected soil organic matter, available phosphorus, and glycyrrhizin (Minqin site); and positively affected the root length (Anxi site). DSE from the Shapotou site accounted for 8.0, 13.0, and 11.3% of the variations in total biomass, root biomass, and active ingredient content; DSE from the Minqin site accounted for 6.6 and 8.3% of the variations in total biomass and root biomass; DSE from the Anxi site accounted for 4.2 and 10.7% of the variations in total biomass and root biomass. DSE × watering regimen displayed a general synergistic effect on plant growth and active ingredient contents. These findings suggested that the DSE–plant interactions were affected by both DSE species and DSE originating habitats. As *A. chlamydospora* and *P. terricola* positively affected the total biomass, root biomass, and active ingredient content of host plants under drought stress, they may have important uses as promoters for the cultivation of licorice in dryland agriculture.

Keywords: licorice, dark septate endophytes, desert ecosystem, drought stress, inoculation

INTRODUCTION

Licorice (*Glycyrrhiza uralensis* Fisch.) belongs to the Leguminosae family, and it has been widely used in the medicine and food processing industries (Michielsen et al., 2018). In China, licorice plants are mainly distributed in the northern regions. Licorice has been formally listed in Chinese Pharmacopeia due to its pharmacological ingredients such as glycyrrhizin and glycyrrhizic acid and associated biological functions (Kao et al., 2014; He et al., 2019a). In addition, licorice has been used to restore degraded soil in China because of its strong adaptability to low-fertility and arid soil (Chen et al., 2017). For decades, licorice cultivation in China has been used as a source of eco-economic value. However, the production and quality of cultivated licorice remain inadequate (Li et al., 2012).

The rhizosphere of plants usually contains diverse microorganisms, including those related to drought stress tolerance and plant growth promotion (Li et al., 2015; Lata et al., 2018; Suleiman et al., 2019). For example, rhizobia, symbiotic nitrogen-fixing bacteria, are widely found in arid soil and can significantly increase the plant biomass of *G. uralensis* (Hao et al., 2019). Fungal endophytes can enhance the tolerance of plants to drought stress, salt, and high temperature stress (Sarkar et al., 2015; Aghai et al., 2019; He et al., 2019b). These host-related endophytic fungi, such as arbuscular mycorrhizal fungi, *Trichoderma* spp., and dark septate endophytes (DSE), can be used as growth promoters or biofertilizers, in line with modern trends in crop management, environmental conservation, and functional food production (Acuna-Rodriguez et al., 2019; He et al., 2019a; Szczalba et al., 2019; Xie et al., 2019).

Dark septate endophytes can colonize the roots of mycorrhizal and non-mycorrhizal plants in a variety of extreme environments (Mandyam and Jumpponen, 2005; Hou et al., 2019). After successful colonization, typical dark septate hyphae and microsclerotia grow inter- and intra-cellularly in the plant root tissues (Xie et al., 2017). Moreover, the varied extensive characteristics of DSE in different habitats highlight their ecological roles, especially under stress environments, which are important for plant survival, biodiversity conservation, and ecosystem function stability (Pennisi, 2003; Lugo et al., 2015; Xu et al., 2015; Hou et al., 2019).

Although DSE species are commonly associated with plants throughout ecosystems, their germplasm resources and ecological roles need to be further clarified. At present, more than 600 plant species from 114 families associating with DSE colonizing their roots have been confirmed (Yuan et al., 2010; Tienaho et al., 2019). Novel DSE species and DSE-plant symbionts are constantly being discovered (Mahmoud and Narisawa, 2013; de Marins and Carrenho, 2017; Xie et al., 2017; Hou et al., 2019). The effects of DSE inoculation on plants range from positive to negative (Tellenbach et al., 2011; Li et al., 2019a). As an accelerator, DSE can promote plant growth by increasing nutrient and water absorption (Li et al., 2018; Yakti et al., 2018; He et al., 2019a), and by protecting plants from biotic and abiotic stresses (Likar and Regvar, 2013; dos Santos et al., 2017). Some studies have shown that inoculation with DSE can improve the

production and quality of medicinal plants (Zhu et al., 2015; He et al., 2019a).

Water deficiency is one of the important abiotic factors that affect the sustainable development of agriculture (Lopes et al., 2011; Li et al., 2019b). Although host plants exhibit many adaptive strategies to mitigate or overcome the effects of stress, mycorrhiza are becoming a key highly effective approach to combat the effective ways of stress and thereby improve plant growth in arid environments (Gianinazzi et al., 2010; Li et al., 2019b). In fact, several studies have shown the importance of DSE inoculants in alleviating drought stress in plants (Nagabhyru et al., 2013; He et al., 2019b). Our previous study showed that inoculation of DSE species (*Acrocalymma vagum* and *Paraboeremia putaminum* from cultivated licorice plants in a farmland plot) improve licorice plant growth after sterilization treatment (He et al., 2019a).

The current study aimed to explore the diversity of DSE from wild licorice in the northwest deserts of China; to assess their osmotic stresses tolerance *in vitro*; and to evaluate the effects of DSE species from different desert habitats on the growth of licorice seedlings under drought conditions. First, DSE species were isolated and identified from the root of wild licorice in the desert area of northwest China. Second, the DSE species were exposed to low osmotic potential induced by polyethylene glycol (PEG) 6000, and their tolerance to osmotic stress was compared. Third, the effects of DSE on the growth performance of licorice plants under drought stress was studied. We mainly focused on the following questions: (1) What is the diversity of DSE species from wild licorice plants in northwest desert regions of China? (2) Do DSE species from desert habitats show high tolerance to osmotic stress *in vitro*? (3) Does inoculation of DSE improve the performance of licorice plants under drought stress? And (4) does drought stress affect the interaction between DSE and medicinal plants?

MATERIALS AND METHODS

Study Sites and Sampling

Wild licorice plants were collected from the Shapotou Desert Research and Experiment Station of the Chinese Academy of Sciences (37°46'N, 105°01'E), Ningxia Province; Minqin Liangucheng National Nature Reserve (38°84'N, 103°27'E), and Anxi Extra-Arid Desert National Nature Reserve (40°43'N, 96°32'E), Gansu Province. These three areas are typical desert ecosystems in northwest China, with remarkable seasonal and diurnal temperature variations. Licorice is always a dominant species there. The mean annual precipitation at the Shapotou, Minqin, and Anxi sites is 186.6 mm, 113.9 mm, and 45.7 mm, respectively. The soils in the three sites are composed of Entisols and Aridisols (Soil Survey Staff, 2014). The soil physicochemical properties of the Shapotou, Minqin, and Anxi sites are as follows: pH: 8.15, 8.16, and 8.18; organic matter: 1.11, 0.91, and 2.05%; available nitrogen (N): 0.034, 0.037, and 0.027 g/kg; and available phosphorus (P): 0.006, 0.005, and 0.001 g/kg, respectively.

Three sample plots were chosen at each site in July 2018. Five replicate soil samples (at a depth of 30 cm) containing

the fine roots of wild licorice were randomly selected from the rhizospheres of native wild licorice plants in each plot. The distance between the plants that were sampled was 100 m. We collected a total of 30 samples, stored them in sealed plastic bags, and transported them to the laboratory in an insulated container within 48 h. Before processing, the soil samples were sieved (2-mm mesh size) and fine roots were collected from each sample. Subsamples from each replicate were used to determine the physicochemical properties of the soils.

Mature seeds of licorice were collected from natural populations in Gansu Province and stored at 4°C.

Quantification of Fungal Colonization

The roots were cut into 0.5-cm-long segments and stained with 0.5% (w/v) acid fuchsin after washing them under tap water and subjecting them to clearing using 10% (w/v) KOH (Phillips and Hayman, 1970). The glass slide method was used to assess fungal colonization status, with 20 random root segments being examined under microscope at 200× and 400× magnification (Biermann and Linderman, 1980). The value of colonization rate by DSE (hyphal, microsclerotial, and total) was calculated as the percentage of infected root segments in each root sample.

Endophytic Fungal Morphological and Genetic Characterization

Root samples were surface-sterilized by sequential washes in 75% ethanol for 5 min and 10% NaClO for 5 min, followed by washing three times in deionized water. Finally, root segments were placed on potato dextrose agar (PDA) culture plates with ampicillin and streptomycin sulfate and cultured at 27°C with daily observation. Colonies with dark mycelium were isolated for further growth and fungal characterization, involving examination of the colony morphology and microscopic morphological traits were observed. Moreover, each isolate was cultured three times for 2 months at 10°C to induce sporulation (He et al., 2019a).

Fresh mycelia (approximately 50 mg) were scraped from the surface of PDA plates, and DNA was extracted using a genomic DNA extraction kit (Solarbio, China). Two primers, ITS4 (5'-TCCTCCGCTTATTGATATGC-3') and ITS5 (5'-GGAA GTAAAAGT CGTAAACAAGG-3') were used for all isolates (Xie et al., 2017). PCR was conducted in a Life ECO™ system (BIOER, China) in a reaction system (40 µL) that comprised 7 µL of DNA template, 1 µL of each primer, 20 µL of 2 × Es Taq Master Mix, and 11 µL of ddH₂O. The PCR program was as follows: initial denaturation for 5 min at 94°C, 35 cycles of denaturation for 1 min at 94°C, primer for 1 min at 55°C, and extension for 1 min at 72°C, followed by final extension for 10 min at 72°C (He et al., 2019a). Finally, the PCR products were purified using Exonuclease I (20 U µL⁻¹) and FastAP Thermosensitive Alkaline Phosphatase (1 U µL⁻¹). Sequencing reactions were performed in a 3500 Series Genetic Analyzer using a BigDye™ Terminator Cycle Sequencing Kit v.3.1 (Applied Biosystems™). Sequences were stored in GenBank under accession numbers MN517851 (DSE1), MN517852 (DSE2), MN517853 (DSE3), MN517854 (DSE4), MN517855 (DSE5), MN517856 (DSE6), MN517857 (DSE7), and MN517858 (DSE8).

Sequence alignment was completed using Clustal X (v.1.81), and MEGA 6 was used to construct a maximum likelihood phylogenetic tree (Tamura et al., 2013).

Osmotic Stress Tolerance of DSE *in vitro*

Osmotic stress was brought about by adding PEG 6000 to pure modified Melin–Norkrans (MMN) medium (pH 5.5) (Hosseini et al., 2019) to achieve osmotic potentials of 0, −0.45, −1.34, and −1.79 MPa (Chen et al., 2003). A 5-mm disc of inoculum was cut from each isolate and placed in a 250-mL Erlenmeyer flask with 100 mL of liquid MMN. The flasks were cultured for 10 days in the dark with continuous shaking. The fungal mycelium was rinsed with distilled water several times before harvesting. There were five replicates for each osmotic stress level. Superoxide dismutase (SOD) activity, and malondialdehyde (MDA) and melanin content were directly determined using a portion of each fungal sample from each replicate. Thereafter, the other portions were weighed before drying to constant weight at 80°C. The biomass of each repeated culture was the sum of the dry weight of two parts.

Superoxide Dismutase Activity and MDA Content Determination

Fresh hyphae (0.2 g) were homogenized in 5 mL of reaction mixture (extracted in 50 mM phosphate buffer with 0.2 mM EDTA, and 2% polyvinylpyrrolidone); pH 7.8 at 4°C in a precooled mortar. The homogenate was centrifuged for 30 min at 15000 × g and 4°C, and then the supernatant was collected for SOD activity analysis. The SOD activity was determined using the photochemical method described by Elavarthi and Martin (2010), based on the decrease in the absorbance by a nitroblue tetrazolium (NBT) complex, which is related to SOD activity. One unit of SOD activity indicates the quantity of enzyme needed to inhibit NBT reduction by 50%, assessed based on absorbance at 560 nm.

The thiobarbituric acid (TBA) method was used to assess the MDA content (Peever and Higgins, 1989). Briefly, fresh hyphae (0.2 g) from each isolate were homogenized in 10% trichloroacetic acid (TCA; 5 mL), and then centrifuged for 10 min at 12000 × g. Next, 2 mL of supernatant and 2 mL of 0.5% thiobarbituric acid (TBA) were mixed in a boiling water bath. After 15 min, the mixture was swiftly cooled and then centrifuged for 10 min at 12000 × g. Absorbance of the supernatant was measured using a spectrophotometer at 450, 532, and 600 nm. The MDA content was calculated using the following formula:

$$C (\mu\text{mol/L}) = 6.45(\text{OD}_{532} - \text{OD}_{600}) - 0.56(\text{OD}_{450})$$

Melanin Content Determination

To determine the melanin content in mycelia, an extraction protocol was carried out as described by Ellis and Griffiths (1974). Briefly, melanin was extracted from hyphae with hot alkali solution (1 M NaOH at 100°C) for 4 h in a water bath. The cooled cell extract was filtered through a double layer of filter paper and acidified with concentrated HCl (7 M) until precipitation at pH 2.0. The resulting dark brown precipitate was recovered by centrifugation at 10,000 × g for 15 min and washed with

distilled water. The coagulated melanin was then dissolved in 1 M NaOH, and the yield of melanin was determined. The melanin content (expressed as mg/g) was measured using a standard curve showing absorbance at 459 nm.

Dark Septate Endophytes Inoculation Experiment

The experiment was conducted in a growth chamber using a completely randomized factorial design (9 inoculation treatments \times 2 watering regimes) with five replicates. A total of 90 pots were prepared. The inoculation treatments comprised DSE1, DSE2, DSE3, DSE4, DSE5, DSE6, DSE7, DSE8, and a non-inoculated control. The watering regimes involved well-watered (WW) and drought stress (DS) conditions.

Licorice seeds were surface-disinfected with 70% ethanol for 3 min and with 2.5% sodium hypochlorite for 10 min, and then thoroughly washed with sterile water. Following germination on water agar medium (containing 10 g/L agar) at 27°C, the seedlings were cultivated in sterile pots (13 cm diameter, 12 cm height, 2 seedlings/pot) containing an 800 g sterilized culture substrate. For the inoculation treatments, two 5-mm disks were cut from a 14-day-old PDA culture medium and placed at 1 cm below the plant roots (He et al., 2019a). For the non-inoculated controls, two 5-mm disks were cut from the PDA culture medium without fungi and placed in the pot. All the inoculation procedures were conducted on a clean bench. Each pot was cultured in a growth chamber for 90 days, with a 14 h/10 h light/dark photoperiod, at 27°C/22°C (day/night) and a mean relative air humidity of 60%.

One month after sowing, half of the seedlings (inoculated and control) were subjected to WW treatment (70% of the field water capacity), and the rest were subjected to DS treatment (30% of field water capacity; the DS treatment was based on the median value in the natural habitat of licorice plants in northwest China). A soil humidity recorder (L99-TWS-2; China) was used to assess soil moisture. Sterile distilled water was added daily to maintain the designated percentage of field water capacity based on regular weighing.

Plant Biomass and Morphological Traits

Plant height and leaf number from each pot were recorded before plant shoots and roots were separately harvested. The root system was gently rinsed with tap water to remove adherent sandy soil. Fresh roots were randomly selected and cut into 0.5-cm segments for DSE colonization assessment as described above. Individual root sections were floated in approximately 1-cm-deep water in a plexiglass tray and scanned using a desktop scanner (EPSON Perfection V800 Photo; Suwa, Japan). Root morphological traits, comprising total root length, root surface area, and mean root diameter, were assessed using a WinRHIZO image analysis system (Regent Instruments, Quebec, QC, Canada). The root sections were collected after scanning. Fresh shoots were dried at 70°C for at least 48 h prior to weighing.

Determination of Active Component Content

Dried roots from each pot were ground with a mortar and sieved with a 40-mesh sieve. An approximately 0.05 g sample was accurately weighed and subjected to extraction using 10 mL of 70% methanol in an ultrasonic bath for 30 min, then cooled to 25°C and filtered through a 0.45- μ m filter. Next, 10 μ L of filtrate was separated via high-performance liquid chromatography using a reverse-phase C₁₈ symmetry column (4.6 \times 250 mm, pore size: 5 μ m; Waters Corp., Milford, MA, United States). Separation was carried out at 25°C using a gradient elution mode (**Supplementary Table 1**), where the mobile phase consisted of deionized water: phosphoric acid (100:0.05, v/v) and acetonitrile. The flow rate was 1.0 mL/min. The levels of the eluted compounds were determined at 237 nm using a 2998 PDA photodiode array detector (Waters Corp.). Standard substances (glycyrrhizic acid and glycyrrhizin) were purchased from China National Institutes for Food and Drug Control (Zhang et al., 2013; He et al., 2019a).

Soil Parameters

Rhizospheric soil samples that strongly adhered to the roots were collected, sieved (2-mm sieve), and dried at room temperature. Each dried soil sample (0.2 g) was digested in 10 mL of a 10:1:2 mixture of perchloric acid (12.7 mol/L), sulfuric acid (18 mol/L), and water using a Mars 6 microwave digestion system (CEM Corporation, Matthews, NC, United States) until a clear liquid was obtained. Soil organic matter was measured as the percentage of organic carbon using dichromate oxidation in H₂SO₄ (Rowell, 1994). Available N and P were determined by the alkaline hydrolysis diffusion method (Rowell, 1994) and the chlorostannous-reduced molybdophosphoric acid blue color method (Olsen et al., 1954), respectively.

Statistical Analysis

All statistical analyses were performed in SPSS 21.0 (SPSS Inc., Chicago, IL, United States). One-way analysis of variance (ANOVA) was performed to analyze the effects of osmotic stress on the biomass, SOD activity, and MDA and melanin content of each DSE species. Two-way ANOVA was performed to examine the effects of DSE inoculation, watering regime, and DSE \times watering regimen on plant biomass, morphological traits, glycyrrhizic acid content, glycyrrhizin content, and soil parameters. The pairwise differences in means were analyzed by Duncan's multiple range test. $P < 0.05$ indicated statistical significance. The values in the figures are the means of at least three replicates. The Mantel test and structural equation modeling (SEM) were used to test the effects of DSE species, watering regimen, and soil parameters on the growth and active component content of licorice plants using R-3.2.2 package ecodist (Goslee and Urban, 2007) and AMOS 21.0 (maximum likelihood). The effect sizes of DSE from different sites, watering regime, and soil variables on the total biomass, root biomass, and the active component content of host plants were estimated by variation partitioning.

RESULTS

Characterization and Identification of DSE Species in Wild Licorice Roots

In the roots of the licorice plants, we observed dark septate hyphae, ranging from brown to dark brown, and microsclerotia structures (Supplementary Figure 1). Specifically, the hyphae invaded the epidermal cells, cortical cells, or vascular tissue. The microsclerotia were chainlike and formed conglomerates, and they colonized single or multiple cortical cells. The DSE

colonization rates for licorice plants from Shapotou, Minqin, and Anxi were as follows: hyphae: 72.2%, 71.7%, and 40.5%; microsclerotia: 32.8%, 40.6%, and 3.3%; and total: 78.3%, 80.3%, and 43.3%, respectively.

In vitro, DSE species varied in color from ashen to gray to dark brown (Figure 1). They had linear growth curves, and the mean growth rates of DSE1–8 were 0.29, 0.33, 0.21, 0.12, 0.29, 0.33, 0.21, and 0.26 cm/d, respectively. Although DSE1, DSE2, DSE5, DSE6, and DSE8 produced chlamydospores *in vitro*, neither conidia nor reproductive structures of other DSE species were observed.

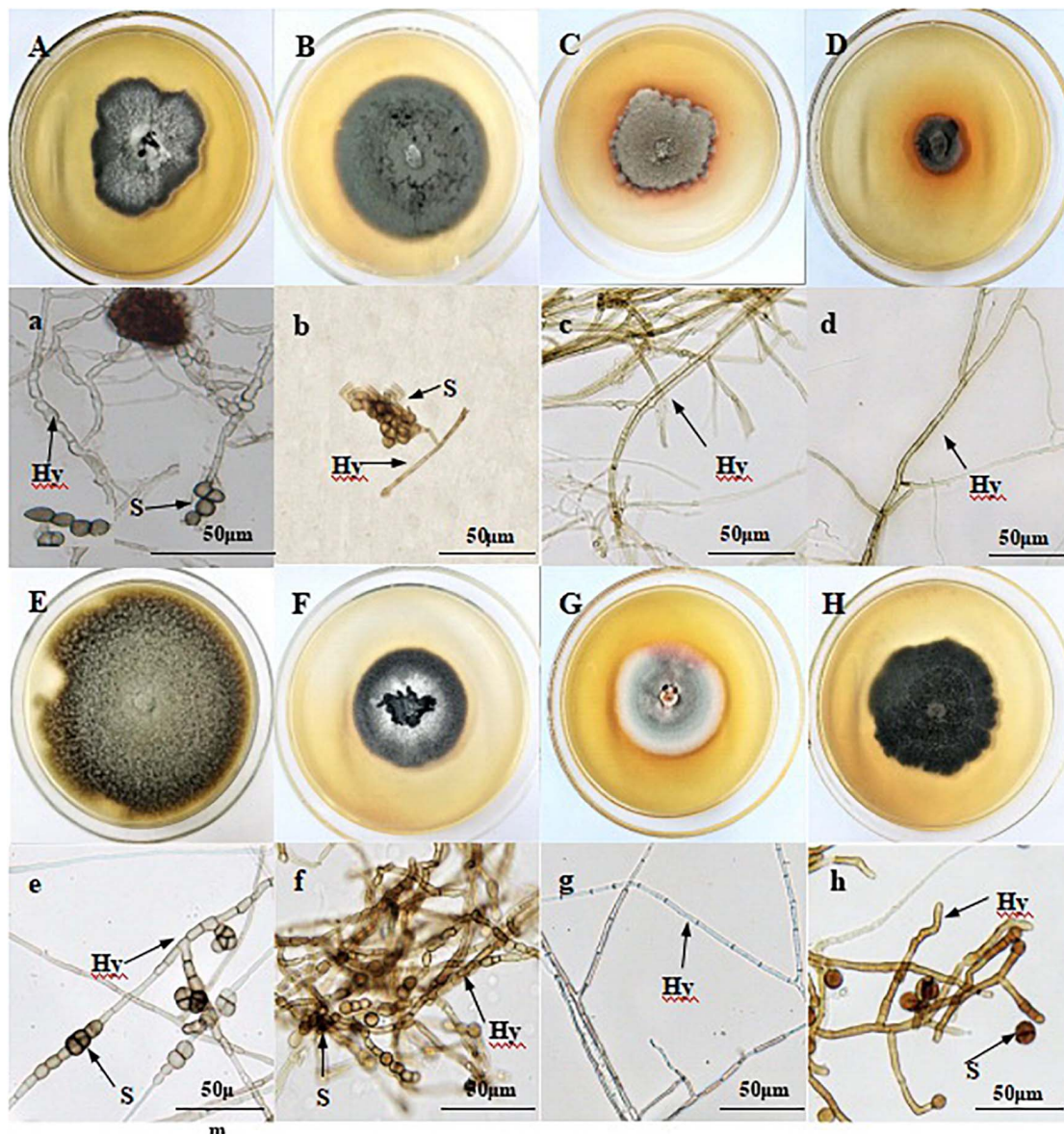


FIGURE 1 | Colonies of endophytic fungi isolated from the roots of wild licorice (A–H). Microscopic morphology of endophytic fungi (a–h) (Bars = 50 μm). (A) a: *Acrocalymma vagum* (DSE1); (B) b: *Paraphoma chrysanthemicola* (DSE2); (C) c: *Alternaria longissima* (DSE3); (D) d: *Darksidea alpha* (DSE4); (E) e: *Alternaria chlamydospora* (DSE5); (F) f: *Acremonium nepalense* (DSE6); (G) g: *Preussia terricola* (DSE7); (H) h: *Alternaria chartarum* (DSE8). Arrows indicate: Hy, DSE hyphae; S, DSE spores.

DSE1, DSE2, DSE3, and DSE4 were isolated in Sapotou; DSE5, DSE6, and DSE7 in Anxi; and DSE8 in Minqin.

Molecular Phylogeny of DSE Species

A phylogenetic tree based on ITS4-5.8S-ITS5 rDNA is shown in **Figure 2**. According to the morphological characteristics

and ITS sequence analysis, the DSE species were identified as *Acrocalymma vagum* (DSE1), *Paraphoma chrysanthemicola* (DSE2), *Alternaria longissima* (DSE3), *Darksidea alpha* (DSE4), *Alternaria chlamydospora* (DSE5), *Acremonium nepalense* (DSE6), *Preussia terricola* (DSE7), and *Alternaria chartarum* (DSE8).

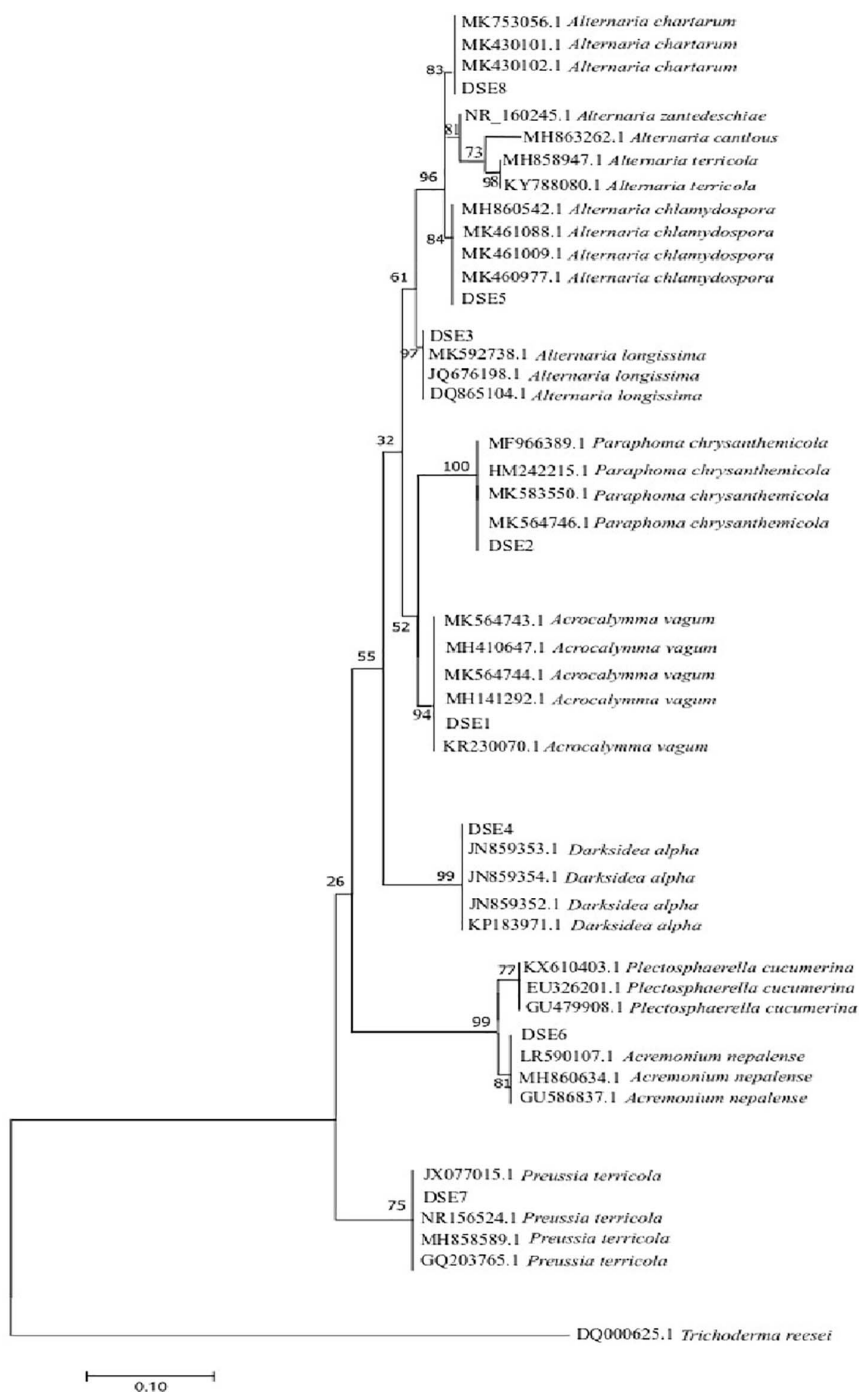


FIGURE 2 | Maximum parsimony tree generated from ITS (ITS4 and ITS5) sequences of the isolate strains and their closest matches, followed by GenBank accession number.

Dark Septate Endophytes Osmotic Stress Tolerance *in vitro*

All eight DSE species showed relatively high osmotic tolerance *in vitro* (Figure 3 and Table 1). However, the various DSE species exhibited different biomass growth (after 10 days of culturing) under osmotic stress relative to the unstressed condition (0 MPa). For instance, DSE6 exhibited higher biomass at -0.45, -1.34, and -1.79 MPa relative to the unstressed condition (0 MPa), whereas DSE7 exhibited lower biomass at these osmotic stress levels (Figure 3A).

The various DSE species exhibited different SOD activity under osmotic stress relative to the unstressed condition (0 MPa). For instance, DSE5 exhibited higher SOD activity at -0.45 MPa, and DSE7 exhibited higher SOD activity at -0.45, -1.34, and -1.79 MPa, whereas DSE3 and DSE6 exhibited lower SOD activity at -1.34 MPa (Figure 3B).

The various DSE species showed different MDA content under osmotic stress relative to the unstressed condition (0 MPa). For example, DSE3, DSE4, and DSE7 exhibited higher MDA content

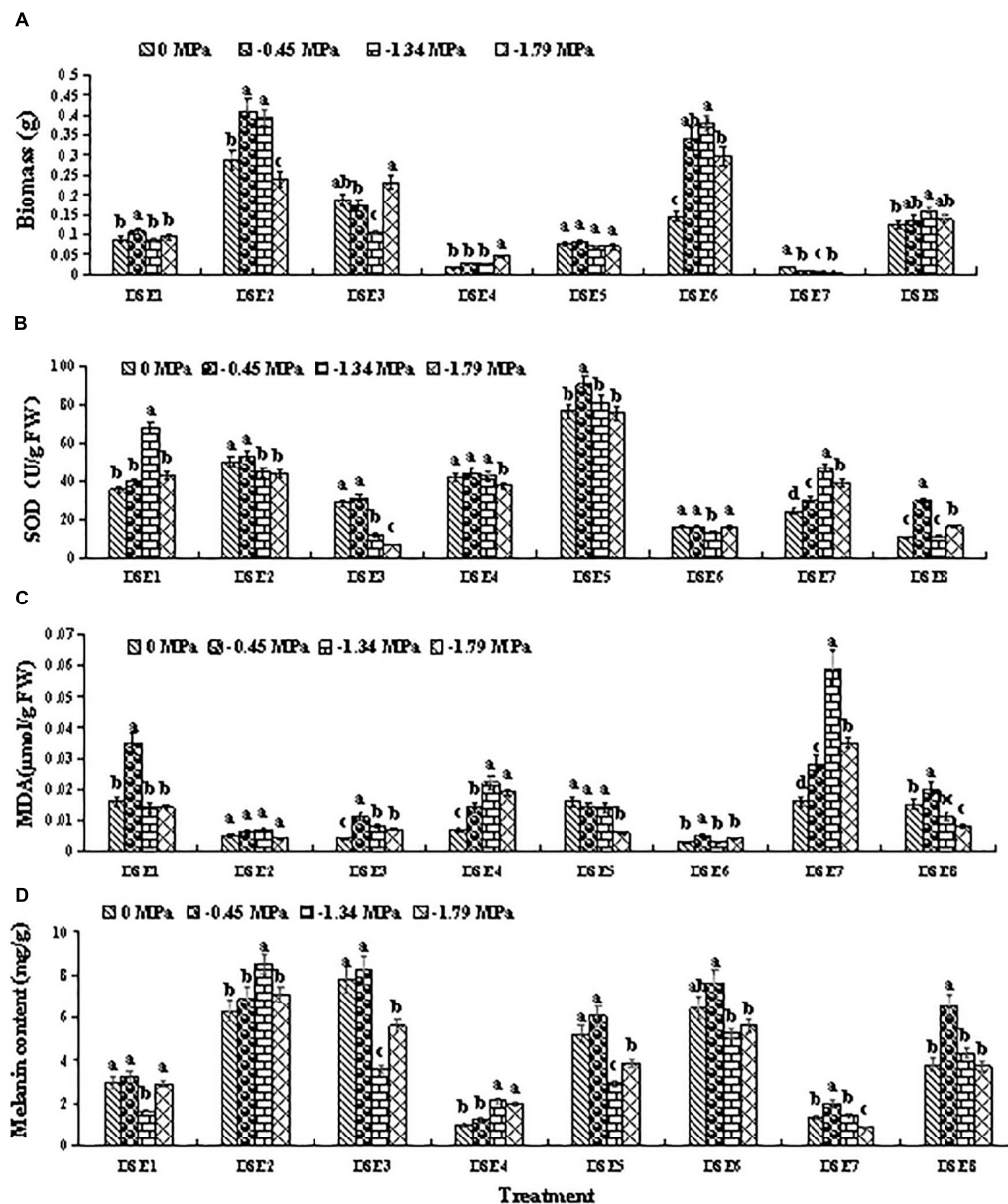


FIGURE 3 | Response of the biomass (A), superoxide dismutase (SOD) activity (B), and malondialdehyde (MDA) (C), and melanin content (D) in the eight dark septate endophytes (DSE) to different concentrations of polyethylene glycol (PEG) 6000. Different letters above the error bars indicate significant differences at $P < 0.05$.

TABLE 1 | Analysis of variance (ANOVA) for the effects of dark septate endophytes and osmotic stress on the biomass, superoxide dismutase activity (SOD), and malondialdehyde (MDA), and melanin content of DSE species.

	Biomass (g)		SOD (U/g FW)		MDA ($\mu\text{mol/g FW}$)		Melanin (mg/g)	
	F	P	F	P	F	P	F	P
DSE	35.431	<0.001	232.357	<0.001	48.349	<0.001	73.775	<0.001
Osmotic stress	1.794	0.157	3.572	0.019	7.747	<0.001	12.767	<0.001
DSE \times osmotic stress	1.617	0.073	10.308	<0.001	8.341	<0.001	4.134	<0.001

Significant *P*-values are in bold.

at all osmotic stress levels, whereas DSE5 and DSE8 exhibited lower MDA content at -1.79 MPa (**Figure 3C**).

Lastly, the various DSE species showed different melanin content under osmotic stress relative to the unstressed condition (0 MPa). For example, DSE7 and DSE8 exhibited higher melanin content at -0.45 MPa, whereas DSE3 and DSE5 exhibited lower melanin content at -1.34 and -1.79 MPa (**Figure 3D**).

Effects of DSE on Plant Morphological Parameters

All seedlings exhibited healthy growth throughout the experimental period. After harvesting, microscopic observation revealed that all DSE isolates successfully colonized the roots of licorice seedlings. The morphological parameters of the inoculated plants relative to the control plants are shown in **Figure 4** and **Table 2**. In general, DSE significantly affected the height, leaf number, and root morphology of licorice plants, regardless of the watering regime.

Under WW conditions, DSE6 reduced plant height relative to the control plants, whereas DSE3 and DSE5 increased it. Under DS conditions, DSE7 reduced plant height relative to the control plants.

Under WW conditions, DSE2, DSE4, and DSE8 increased the number of leaves relative to the control plants, whereas DSE1 reduced it. However, under DS conditions, all DSE increased the number of leaves relative to the control plants.

Regarding root morphology, DSE colonization affected the roots relative to control roots under both watering regimens. For instance, DSE1, DSE2, and DSE4 increased the root length, whereas DSE6 reduced it, regardless of the watering regime. Under WW conditions, DSE1 increased the root diameter, whereas DSE2 and DSE4 reduced it; under DS conditions, only DSE8 increased it. Under WW conditions, DSE2, DSE5, and DSE8 increased the root surface area, whereas DSE3 and DSE6 reduced it; under DS conditions, DSE1, DSE2, DSE4, DSE5, DSE7, and DSE8 increased it, whereas DSE6 reduced it (**Figure 4**). Notably, DSE \times watering regimen significantly affected the leaf number and root diameter of host plants (**Table 2**).

Effects of DSE on Plant Biomass

Dark septate endophytes colonization significantly affected the biomass and root:shoot ratio of licorice plants. In particular, DSE5 increased the total, shoot, and root biomass relative to the control plants, whereas the other DSE species had no obvious

effect on the shoot biomass, regardless of the watering regime. Under WW conditions, DSE1 increased the total biomass, and DSE2 increased the total and root biomass, whereas DSE3, DSE4, DSE6, DSE7, and DSE8 reduced these parameters, relative to the control plants. Under DS conditions, DSE7 increased the total and root biomass, whereas DSE1 and DSE4 reduced the total and root biomass, and DSE3 reduced the root biomass, relative to the control plants. Under WW conditions, DSE1, DSE3, DSE4, DSE6, DSE7, and DSE8 decreased the root:shoot ratio, whereas DSE1 and DSE4 decreased it; under DS conditions, DSE2, DSE5, and DSE7 increased it (**Figure 5**). DSE \times watering regimen significantly affected the root biomass, total biomass, and root:shoot ratio (**Table 2**).

Effects of DSE on Active Ingredient Contents

Regarding the glycyrrhizic acid content in the roots, DSE colonization had significant effects relative to the control plants, regardless of the watering regime (**Figure 6**). Under WW conditions, all DSE reduced the glycyrrhizic acid content, relative to the control plants; under DS conditions, DSE3, DSE4, DSE5, DSE6, DSE7, and DSE8 increased its content, while DSE1 and DSE2 had no significant effects (**Figure 6**). Under WW conditions, all DSE reduced the glycyrrhizin content, relative to the control plants; under DS conditions, DSE1, DSE2, DSE3, DSE4, DSE5, DSE6, and DSE7 increased it (**Figure 6**). DSE \times watering regimen significantly affected the glycyrrhizic acid content (**Table 2**).

Effects of DSE on Soil Parameters

Regarding the soil parameters, DSE colonization had significant effects relative to the control plants, regardless of the watering regimen (**Figure 7**). Under WW conditions, DSE1, DSE2, DSE3, DSE4, DSE5, DSE6, and DSE8 increased the organic matter content in the rhizopsheric soil, relative to the control plants; DSE2, DSE3, and DSE6 increased the available P, whereas DSE4 and DSE7 decreased it; DSE1, DSE2, DSE3, DSE4, DSE7, and DSE8 increased soil available N, while DSE5 decreased it. Under DS conditions, all DSE increased soil organic matter, relative to the control plants; only DSE1 increased soil available P, while DSE2, DSE3, DSE4, DSE5, and DSE8 reduced it; DSE5 and DSE6 increased soil available N, while DSE1, DSE2, DSE3, DSE4, DSE7, and DSE8 decreased it (**Figure 7**). DSE \times watering regimen significantly affected soil organic matter and available N (**Table 3**).

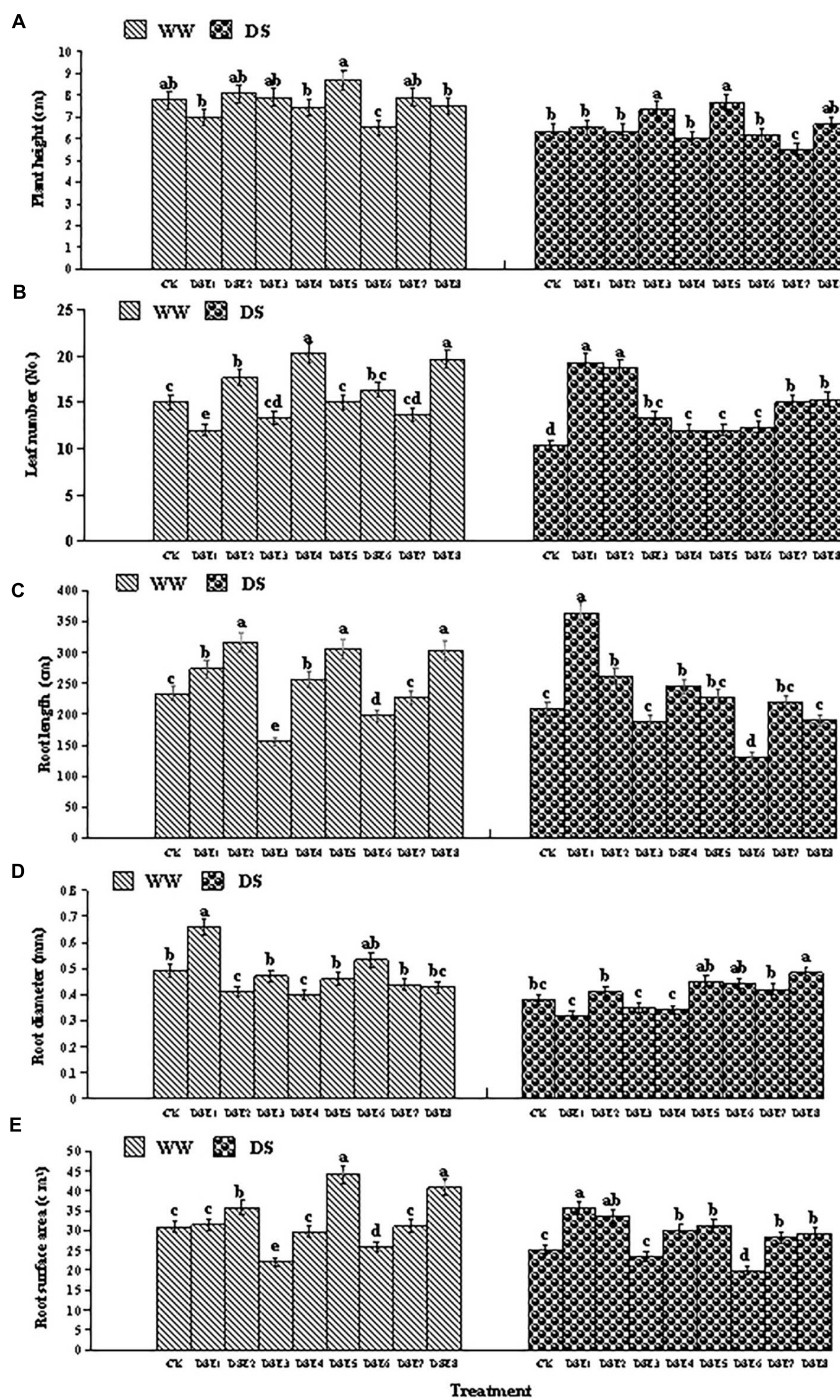


FIGURE 4 | (A–E) Effects of dark septate endophytes (DSE) and water treatment on the morphological parameters of licorice plants. Different letters above the error bars indicate significant difference at $P < 0.05$. CK indicates non-inoculated plants. DSE1–DSE8, indicate plants inoculated with different DSE species. WW, DS, indicate well-watered and drought stress treatment, respectively.

Correlation Analyses

To assess the impacts of DSE from different sites, watering regime, DSE \times water, and soil parameters on the plant characteristics (root length, biomass, and active component content), a SEM model and the Mantel test were conducted.

The Mantel test revealed notable correlations among DSE, watering regime, root length, root biomass, total biomass, glycyrrhizin, glycyrrhizic acid, and soil components (**Supplementary Tables 2–4**). Combined with the correlation coefficients (R -values), a SEM model was made to evaluate the

TABLE 2 | Two-way analysis of variance for the effects of dark septate endophytes (DSE) and water condition on the growth and active ingredient content of licorice plants.

	DSE		Water		DSE × water	
	F	P	F	P	F	P
Plant height (cm)	1.853	0.099	17.567	<0.001	0.69	0.697
Leaf number (No.)	5.363	<0.001	8.721	0.006	7.646	<0.001
Root length (cm)	4.436	<0.001	2.675	0.111	1.637	0.149
Root surface area (cm ²)	2.284	0.043	2.851	0.1	0.677	0.708
Root diameter (cm)	2.648	0.022	24.049	<0.001	5.82	<0.001
Shoot biomass (g/pot)	1.235	0.307	24.454	<0.001	0.809	0.599
Root biomass (g/pot)	7.484	<0.001	99.743	<0.001	3.793	0.003
Total biomass (g/pot)	5.472	<0.001	86.486	<0.001	2.515	0.028
Root: shoot ratio	3.848	0.002	34.195	<0.001	3.156	0.008
Glycyrrhizic acid (%)	2.089	0.063	1.958	0.170	4.576	<0.001
Glycyrrhizin (%)	0.719	0.673	0.797	0.378	1.01	0.446

Significant *P*-values are in bold.

relationship between DSE and all measured parameters for each of the studied sites. From this analysis, it emerged that DSE positively affected the root length, total biomass, glycyrrhizic acid, and soil organic matter, and negatively affected glycyrrhizin and soil available N (Shapotou site) (**Figure 8A**); and DSE positively affected the root length, whereas adversely affected total biomass, glycyrrhizin, and soil available P (Minqin site) (**Figure 8B**); and DSE positively affected root length and soil available N (Anxi site) (**Figure 8C**). Watering regime commonly positively affected the total biomass and glycyrrhizic acid; positively affected soil parameters (Shapotou site); and the root length and glycyrrhizin (Minqin site); and soil available P (Anxi site). DSE × watering regimen showed positive effects on the root length, total biomass, glycyrrhizin, glycyrrhizic acid, and soil organic matter (Shapotou site); and positive effects on glycyrrhizin, soil organic matter, and available P (Minqin site); and positive effects on the total biomass (Anxi site). Soil organic matter positively affected the root length, and soil available P positively affected glycyrrhizic acid, and soil available N positively affected the glycyrrhizin (Shapotou or Minqin site) (**Figures 8A,B**); soil organic matter positively affected the root length and total biomass, and soil available P positively affected the total biomass and glycyrrhizin, and soil available N positively affected the total biomass (Anxi site) (**Figure 8C**).

Variation Partitioning Analysis

Variation partitioning analysis was used to evaluate the contributions of DSE from different sites, watering regime, and soil parameters to the total biomass, root biomass, and active component content (**Figure 9**). This analysis showed that DSE from Shapotou significantly affected the root biomass and active component content, while watering regimen might be a key factor influencing the total biomass and active component content (**Figures 9A–C**). DSE from Minqin had a weaker effect on the total biomass, root biomass, and active component content, and soil parameters mainly affected the total biomass and active component content, while watering regimen might

be the main factor affecting the total biomass, root biomass, and active component content (**Figures 9D–F**). DSE from Anxi mainly affected the root biomass, and watering regimen and soil parameters overall affected the total biomass, root biomass, and active component content (**Figures 9G–I**). Moreover, the analysis showed that the combination of DSE, watering regimen, and soil parameters might be the main contributor to the total biomass and active component content (**Figures 9A–I**).

DISCUSSION

Identification of the DSE Species

In the present study, we found that typical DSE structures, such as dark septate hyphae and microsclerotia, colonized the licorice roots, with a mean colonization rate of 67.3%. The dark septate hyphae of DSE are important for host survival in stressful environments, mainly because oxygen radicals can be trapped and eliminated by melanin in the cell walls during abiotic stress (Yu et al., 2015). In addition, the chlamydospore-like microsclerotia observed in licorice roots may be related to the higher drought stress tolerance among plants (Xie et al., 2017; Hou et al., 2019). Based on morphological and molecular identification, we identified the eight DSE species isolated from licorice roots collected from pedo-climatically different locations as *A. nepalense* (Hypocreales), *A. vagum*, *A. chartarum*, *A. chlamydospora*, *A. longissima*, *D. alpha*, *P. chrysanthemicola*, and *P. terricola* (Pleosporales). Among these DSE species, the last three have been reported as DSE species in desert habitats (Massimo et al., 2015; Li et al., 2018). Regarding the first five DSE species, this is the first report of these species in desert areas in northwest China.

In previous studies, *D. alpha*, a common member of the core DSE community distributed in semiarid grasslands worldwide, was proposed to play a key role as a decomposer of dead roots due to the general increase in carbohydrate active enzymes in DSE fungi (Knapp et al., 2018). The pathogen *P. chrysanthemicola* has been shown to cause root and basal rot, resulting in wilting seedlings (Hay et al., 2015). *Preussia* spp. such as *P. terricola* have been reported to improve plant growth and produce phytohormones (Al-Hosni et al., 2018). *A. nepalense*, a manganese oxide-depositing fungus, can convert soluble Mn (II) into insoluble Mn (III, IV) oxides (Saiz-Jimenez et al., 2012). *A. vagum* from field-grown licorice has been shown to reduce heavy metal content in tobacco leaves (Jin et al., 2018), and to improve the growth and active component content of licorice plants (He et al., 2019a). *Alternaria* spp. are known to be highly pathogenic to plants, causing great losses to many crops (Woudenberg et al., 2013).

Dark Septate Endophytes Biochemical Characterization

The different DSE species exhibited very diverse growth in response to osmotic stress and to the level of the stress. In general, the cells of stressed organisms produce reactive oxygen species (ROS), and the elevated ROS levels may lead to severe oxidative damage to the cellular biomolecules

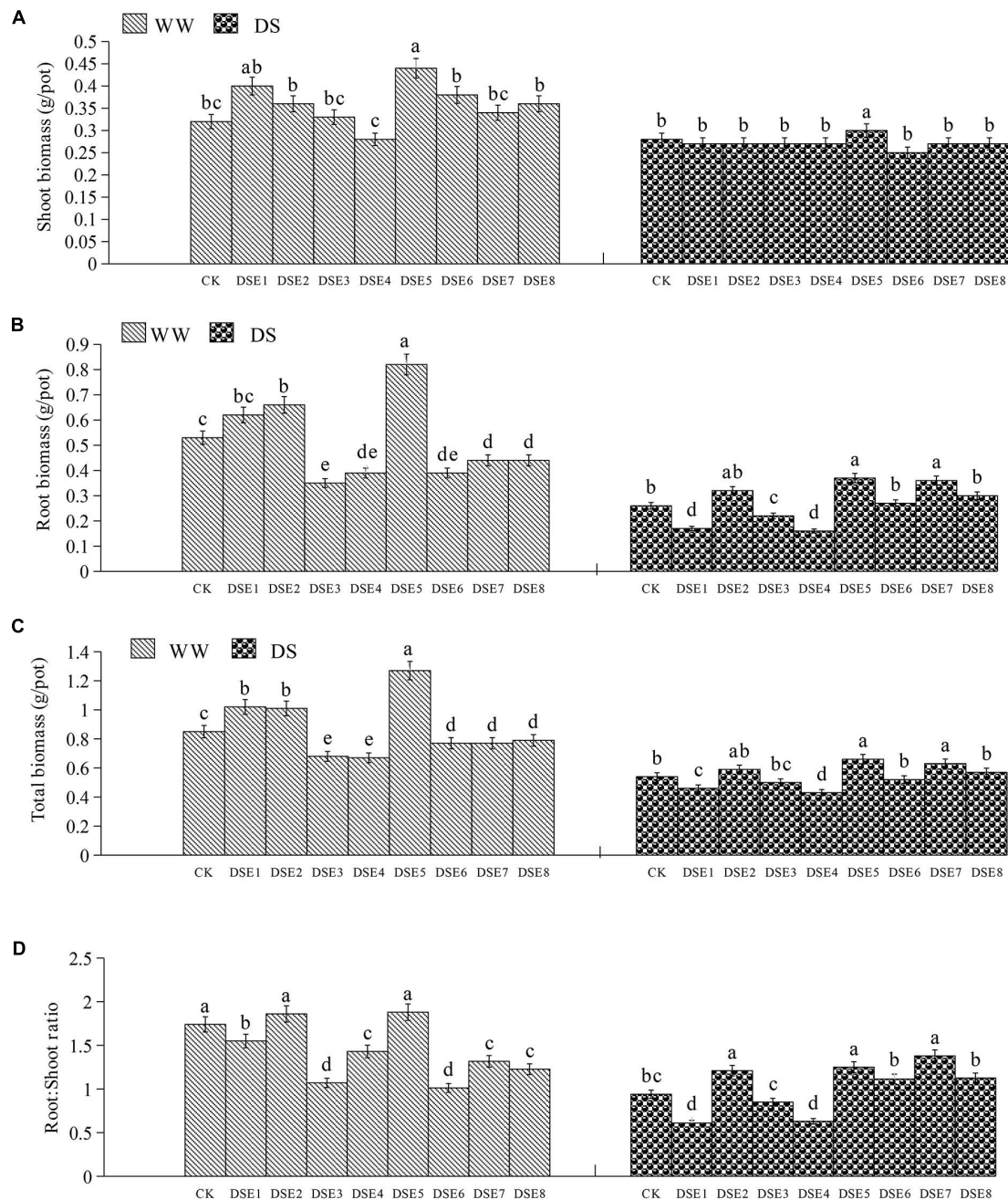


FIGURE 5 | (A–D) Effects of dark septate endophytes (DSE) and water treatment on the biomass production of licorice plants. Different letters above the error bars indicate significant difference at $P < 0.05$. CK indicates non-inoculated plants. DSE1–DSE8, indicate plants inoculated with different DSE species. WW, DS, indicate well-watered and drought stress treatment, respectively.

(Hernández-Pérez et al., 2021). To counteract such damage, the organisms grew more as a part of their defense mechanism (Baxter et al., 2014). Previous research reported that DSE from wild rice grew more at -0.8 MPa relative to 0 MPa (dos Santos et al., 2017). Li et al. (2019a) reported that the intermediate stress conditions (-1.34 or -2.24 MPa) were more suitable, relative to other osmotic stresses, for the growth of DSE obtained from *Hedysarum scoparium*. We conjecture that the osmotic stress

tolerance of DSE may be related to the DSE species and their ecological adaptability (He et al., 2019b; Li et al., 2019a).

Osmotic stress usually negatively affects organisms and causes oxidative damage to cells (Liu et al., 2010). In this study, we determined the SOD activity and MDA and melanin content in the eight DSE to assess antioxidant responses to osmotic stress. SOD is one of the most important enzymes for the removal of reactive oxygen species (ROS) (Hosseini et al., 2019).

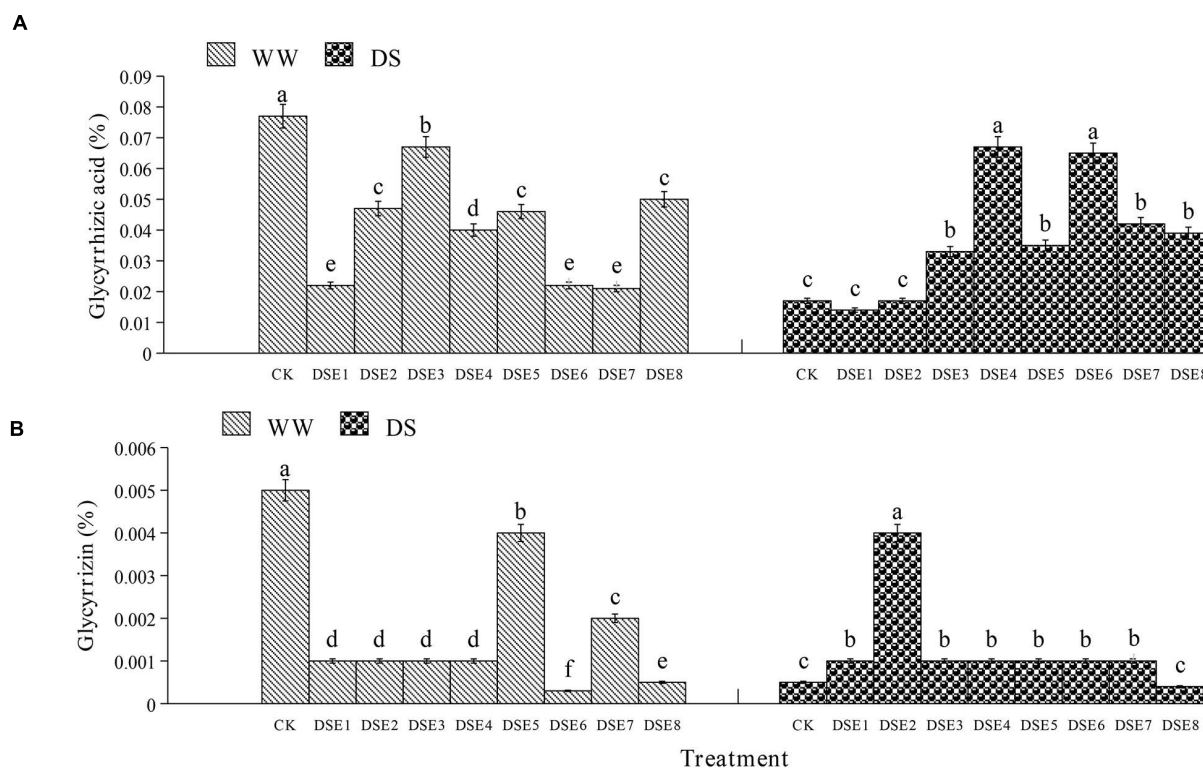


FIGURE 6 | (A,B) Effects of dark septate endophytes (DSE) and water treatment on the active ingredient content of licorice plants. Different letters above the error bars indicate significant difference at $P < 0.05$. CK indicates non-inoculated plants. DSE1–DSE8, indicate plants inoculated with different DSE species. WW, DS, indicate well-watered and drought stress treatment, respectively.

TABLE 3 | Two-way analysis of variance for the effects of dark septate endophytes (DSE) and water condition on soil parameters in the rhizosphere of licorice plants.

	Soil organic matter (%)		Soil available P (g/kg)		Soil available N (g/kg)	
	F	P	F	P	F	P
DSE	1.536	0.169	0.96	0.482	0.965	0.483
Water	14.981	<0.001	4.396	0.042	6.017	0.019
DSE × water	2.437	0.032	1.183	0.336	5.575	<0.001

Significant P -values are in bold.

The SOD activity in DSE1, DSE5, DSE7, and DSE8 increased under specific osmotic stress conditions, indicating that SOD was synthesized to remove ROS under intensified stress. In contrast, the reduced SOD activity in DSE2 and DSE3 at -1.34 and -1.79 MPa indicated that other components might contribute to the DSE response to enhanced osmotic stress. In addition, as a biomarker of environmental stress, the MDA content of DSE5 and DSE8 decreased at -1.79 MPa, which indicated that these DSE could resist the detrimental effects of drought stress (Khan et al., 2012; Xu et al., 2017). In contrast, the increased MDA content in DSE3, DSE4, and DSE7 under various osmotic stress conditions implies that osmotic stress enhanced the membrane lipid peroxidation in DSE, as these DSE had decreased tolerance to osmotic stress (Suleiman et al., 2019).

The effect of osmotic stress on the melanin content of DSE depended on the DSE species. Melanin, an antioxidant

agent that reduces oxidative damage, protects organisms from environmental stress (Zhan et al., 2011; Liu et al., 2018; Hosseini et al., 2019). Eisenman et al. (2020) also reported that melanin protects fungi from a range of stresses in the environment.

Effects of DSE Inoculation on Plant and Rhizospheric Soil

Hyphae and microsclerotia were detected in licorice roots inoculated with the eight DSE under drought stress, which indicated that the eight DSE are effective root colonizers even under drought stress. In addition, the response of licorice plants to DSE colonization was strain-dependent. Specifically, licorice plants inoculated with DSE5 had significantly increased root and total biomass compared to the control plants, whereas DSE4 had significant negative effects on the root and total biomass, regardless of the watering regimen. Our observations

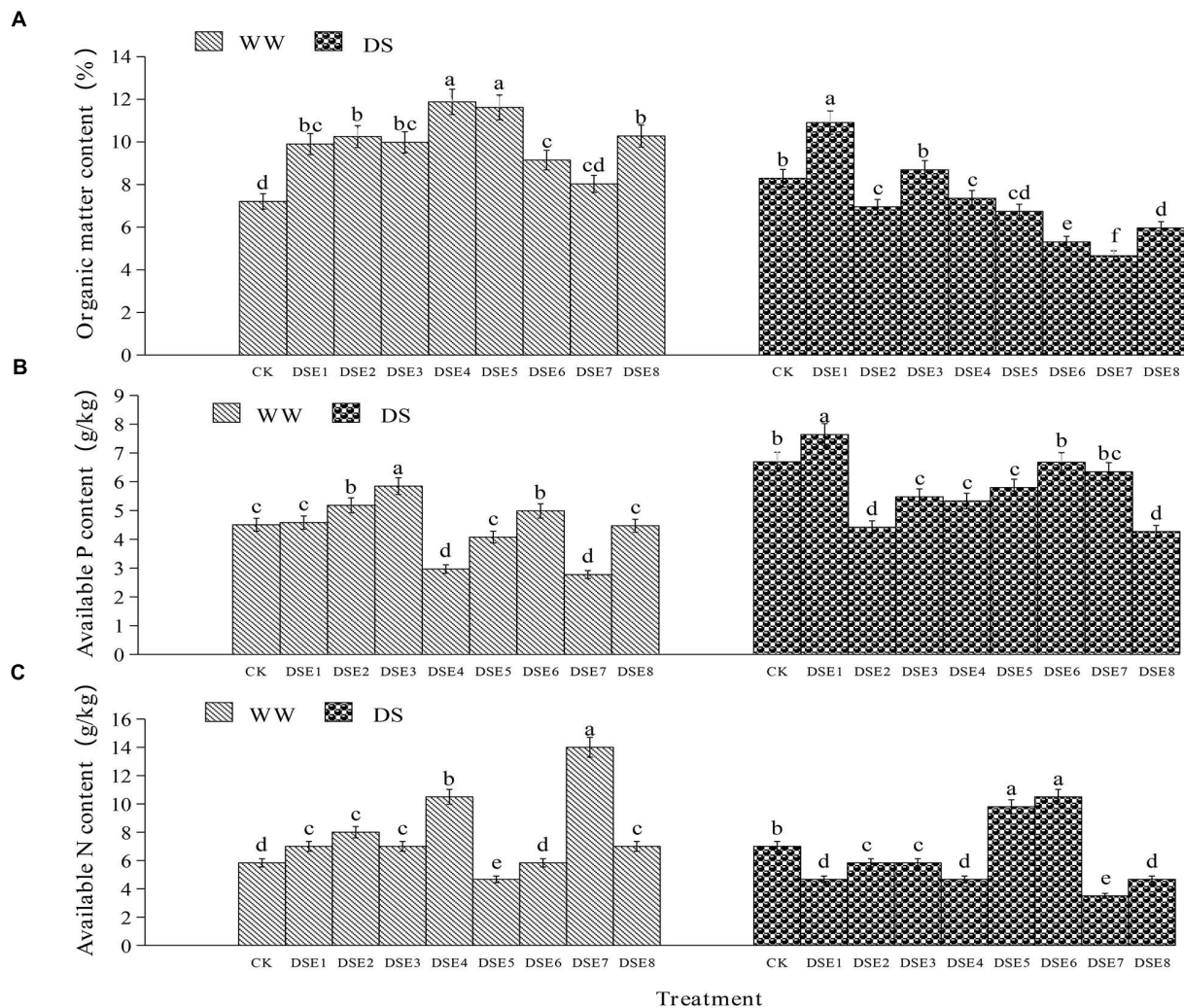


FIGURE 7 | (A–C) Effects of dark septate endophytes (DSE) and water treatment on soil parameters. Different letters above the error bars indicate significant difference at $P < 0.05$. CK indicates non-inoculated plants. DSE1–DSE8, indicate plants inoculated with different DSE species. WW, DS, indicate well-watered and drought stress treatment, respectively.

are consistent with those of previous studies showing that the DSE species influences the DSE–plant interaction (He et al., 2019b; Li et al., 2019a,b). The SEM analysis showed that DSE from various desert habitats directly affected the root length, active component content of roots, and plant biomass. This study is the first to report the neutral and positive effects of *Alternaria* (i.e., *Alternaria chartarum*, DSE8), which is considered a plant pathogen (Woudenberg et al., 2013). Moreover, although no DSE except DSE5 affected the shoot biomass, DSE improved the root biomass under drought stress. Thus, our results suggested that DSE promoted the growth of licorice plants under drought stress, potentially by altering the osmotic stress tolerance or the nutrient solubilization capacity regarding the rhizospheric soil (Porras-Alfaro et al., 2008; Li et al., 2018). Furthermore, our results revealed that DSE \times watering regimen significantly affected the glycyrrhizic acid content in the host plants. These findings suggested that DSE colonization could alleviate the negative

effects of drought stress on the active component content and plant growth (He et al., 2019b).

Both the DSE species and watering regimen affected the root morphological traits. Specifically, DSE1, DSE2, DSE4, DSE5, DSE7, and DSE8 positively affected the root surface area under drought stress, whereas DSE6 negatively affected it under drought stress. Enhanced root growth (e.g., root biomass, length, and surface area) is beneficial for plant roots as it allows them to extend into deeper soil layers and improve water extraction from the soil, thereby increasing plant survival and resistance to ground evaporation in desert ecosystems (Schulze et al., 1996).

Soil organic matter and available N in the rhizospheric soil were positively affected by DSE \times watering regimen. The SEM analysis and variation partitioning also indicated that DSE directly influenced soil nutrient properties. As a bridge between the plant and soil environment, DSE are a key element of plant nutrient uptake; their underground mycelium network facilitates

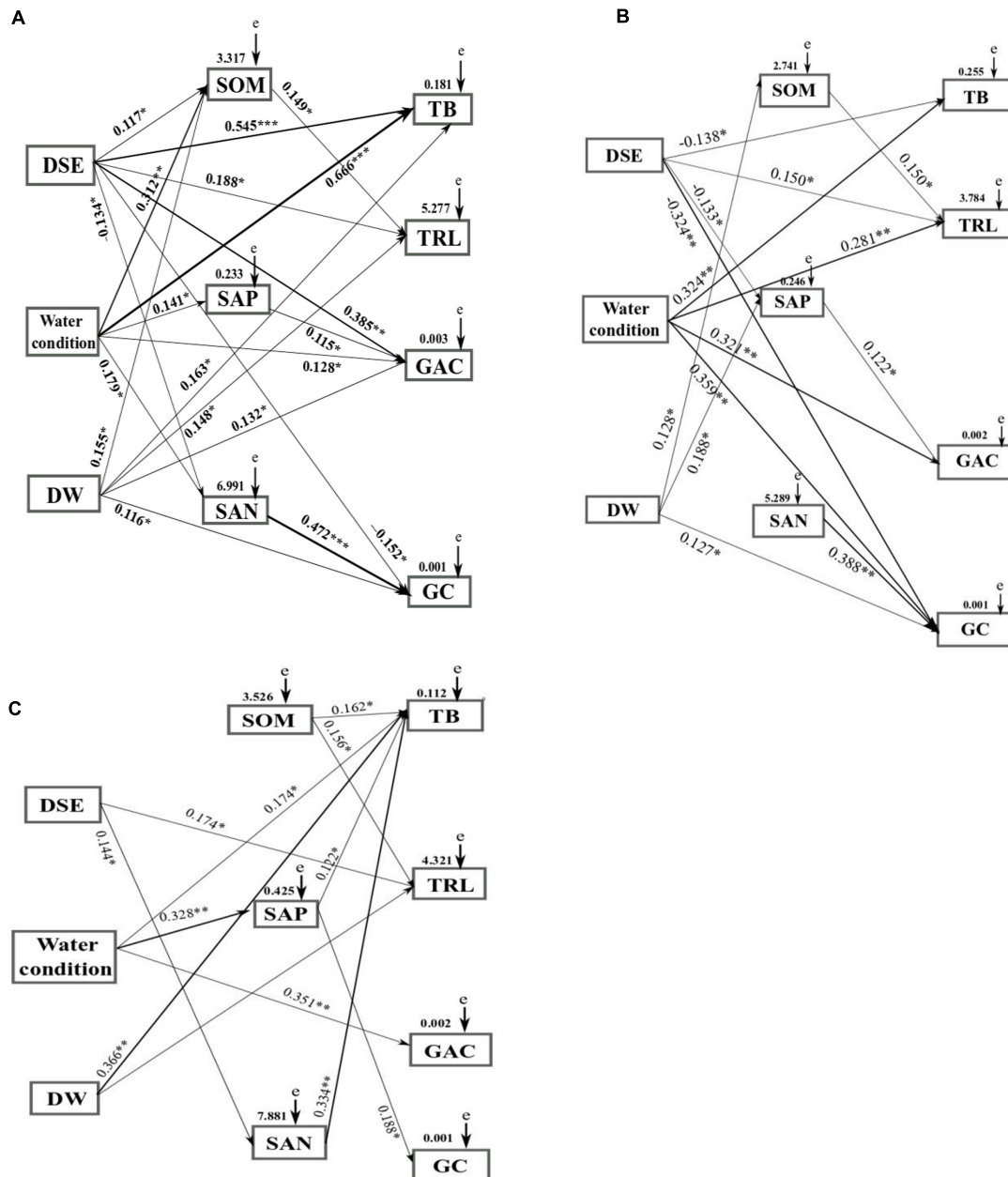


FIGURE 8 | Structural equation model showing the causal relationships among DSE from different sites, water condition, DSE \times water, soil parameters, and growth indicators and active ingredients. The final model fitted the data well: maximum likelihood, Shapotou site **(A)** $\chi^2 = 43.513$, $df = 10$, $P = 0.014$, root mean square error of approximation = 0.253, goodness-of-fit index = 0.613, Akaike information criteria = 148.468; Minqin site **(B)** $\chi^2 = 69.482$, $df = 12$, $P = 0.005$, root mean square error of approximation = 0.267, goodness-of-fit index = 0.571, Akaike information criteria = 161.992; Anxi site **(C)** $\chi^2 = 82.476$, $df = 13$, $P = 0.001$, root mean square error of approximation = 0.323, goodness-of-fit index = 0.538, Akaike information criteria = 172.965. Solid lines and dashed lines indicate significant and non-significant pathways, respectively. The width of the solid lines indicates the strength of the causal effect, and the numbers near the arrows indicate the standardized path coefficients (* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$). DW, combination of DSE and water; SOM, soil organic matter; SAP, soil available P; SAN, soil available N; e, the values of residuals; TB, total biomass; TRL, root length; GAC, glycyrrhizic acid content; GC, glycyrrhizin content.

nutrient transfer from the soil to colonized roots (Usuki and Narisawa, 2007; Alberton et al., 2010; Vergara et al., 2019). For example, there were some positive correlations between a specific DSE and the total biomass and root length in this study. Barrow (2003) also suggested that septate endophytic hyphae

improved nutrient and water transport by extending $> 300 \mu\text{m}$ from the root matrix during extended DS. Additionally, DSE can play important roles in host nutrition via complex substrate degradation mechanisms; secreted enzymes from DSE can degrade organic matter and mineralize organic N and insoluble

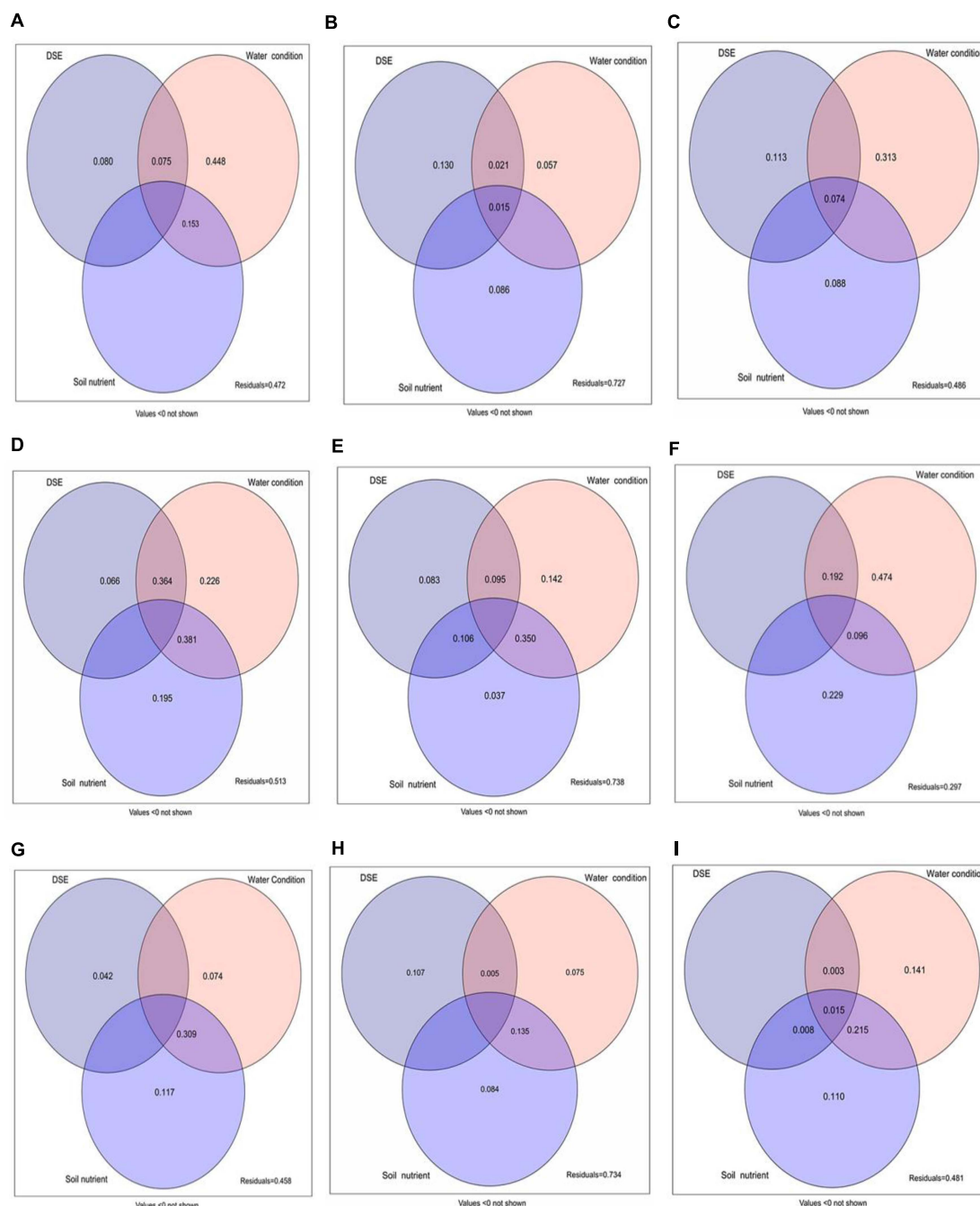


FIGURE 9 | Variation partitioning of DSE from different sites, water condition, and soil parameters on the total biomass (**A,D,G**), root biomass (**B,E,H**), and active ingredient contents (**C,F,I**) of licorice plants. DSE, DSE species. Soil nutrient, nutrient content in soil (including soil organic matter, available N, and available P). Values below 0 are not shown. (**A–C**) DSE from Shapotou; (**D–F**) DSE from Minqin; (**G–I**) DSE from Anxi.

P into available forms; thus, plant growth and tolerance can be promoted under stress (Caldwell et al., 2000; Surono and Narisawa, 2017; He et al., 2019a).

Some studies have found that different DSE and DSE–plant interactions respond differently to abiotic stresses (Elvira-Recueno et al., 2014; Kia et al., 2017; Bragulat et al., 2019). For instance, under drought stress, *A. vagum* from cultivated

licorice in arid farmland increased the total biomass, root biomass, and glycyrrhizic acid content of the licorice plant (He et al., 2019b). In the current study, under drought stress, the total biomass and root biomass were reduced, and the glycyrrhizin content was increased, for licorice plants inoculated with *A. vagum* from wild licorice in a desert environment. These findings regarding the different effects of DSE isolated from

different habitats on plant growth and compound production confirm the specific nature of DSE–plant interactions, as proposed by other authors (Mandyam and Jumpponen, 2005; Li et al., 2019a). To our knowledge, the increase in licorice plant growth and drought stress tolerance might be attributable to improved soil nutrition, microbiota, and root structure (González-Teuber et al., 2018; Changey et al., 2019; He et al., 2019b). According to the variation partitioning analysis, there were certain degrees of unexplained variations in the total biomass, root biomass, and active component content, which indicates that unexplored factors (such as the DSE inoculation volume and degree of drought stress) affect licorice plant growth and active component accumulation.

CONCLUSION

In the present study, we first characterized eight DSE species from wild licorice plants in desert habitats and investigated the performance of licorice plants after DSE inoculation under drought stress. The osmotic stress tolerance of the eight DSE species was highly variable and there were obvious functional differences in the performance of inoculated licorice plants. The response of licorice plants to DSE varied from neutral to beneficial depending on both the DSE species and watering regimen. Specifically, *A. chlamydospora* and *P. terricola* increased the total biomass and root biomass of licorice plants under drought stress; all DSE except *A. vagum* and *P. chrysanthemicola* increased the glycyrrhizic acid content under drought stress; and all DSE except *A. chartarum* increased the glycyrrhizin content under drought stress. Additionally, DSE \times watering regimen increased the root glycyrrhizic acid content and the available N and organic matter in the rhizospheric soil. Furthermore, the DSE–plant interaction was affected by DSE species and DSE originating habitat. As *A. chlamydospora* and *P. terricola* positively affected the total biomass, root biomass, and active component content under drought stress, they may have important uses as promoters in the cultivation of licorice plants in arid areas.

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DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the sequences were stored in GenBank with accession numbers of MN517851 (DSE1), MN517852 (DSE2), MN517853 (DSE3), MN517854 (DSE4), MN517855 (DSE5), MN517856 (DSE6), MN517857 (DSE7), and MN517858 (DSE8).

AUTHOR CONTRIBUTIONS

CH and WW conceived and designed the experiments. CH and JH performed the experiments. CH, JH, and XL analyzed the data. CH, WW, and XL wrote 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/fmicb.2021.522449/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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