THE PLANT HOLOBIONT VOLUME I: MICROBIOTA AS PART OF THE HOLOBIONT; CHALLENGES FOR AGRICULTURE

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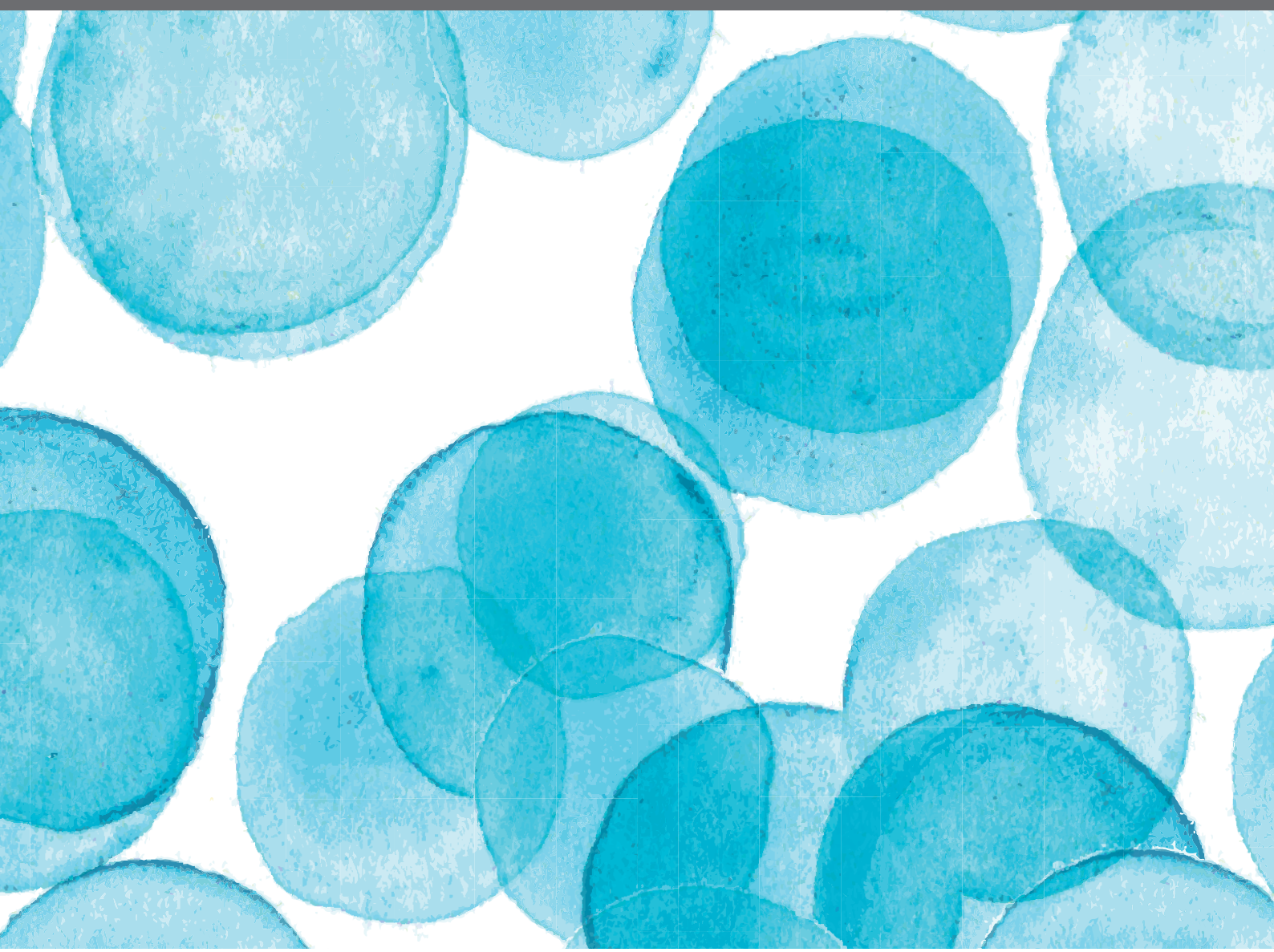

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THE PLANT HOLOBIONT VOLUME I: MICROBIOTA AS PART OF THE HOLOBIONT; CHALLENGES FOR AGRICULTURE

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EDITORIAL
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Editorial: The Plant Holobiont Volume I: Microbiota as Part of the Holobiont; Challenges for Agriculture

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Editorial on the Research Topic

The Plant Holobiont Volume I: Microbiota as Part of the Holobiont; Challenges for Agriculture

INTRODUCTION

A possible approach to ensure the sustainability of cropping systems in agroecology is to promote beneficial interactions between plants and their associated microbiota. Plant-microbe interactions in the rhizosphere are often described as a feedback-loop in which plants recruit, through the release of exudates, specific rhizosphere microbiota that, in turn, affect plant growth and health. Furthermore, these interactions can change during the different phases of the life cycle of the organisms.

Recently, the concept of holobiont has been introduced for crop plants. The term holobiont was first defined by Margulis (1991), as a simple biological unit involving a host and a single inherited symbiont. Then, Zilber-Rosenberg and Rosenberg (2008) have expanded this definition to the entire microbiota and proposed the holobiont to be a selection unit, which underlies the hologenome based theory of evolution.

The added value of the hologenomic view of crop-microbe interactions in agroecology is to better take into account plant-microbe interactions and their results on plant biology, ecology and evolution. This represents a major issue in the conception of the so-called plant ideotype, defined as a plant genotype chosen for its capacity to better exploit a given environment and to adapt to abiotic and biotic stresses.

The next step will be to consider interactions between plants within a plant canopy (mono- or pluri-species) as interactions between holobionts when taking into account ecosystem services expected from agroecosystems (e.g., food production, climate mitigation, water biofiltration).

Thus, new plant genotypes need to be selected in order to better value rhizodeposits and plant-microbiota interactions in the rhizosphere. This will require the identification of plant traits involved in the recruitment of beneficial microbial populations/functions.

For reaching that target, further research is needed to (i) identify plant and microbiota determinants for a beneficial interaction, (ii) define functional crop holobionts, (iii) design cropping systems that value the best the corresponding holobionts, and (iv) understand and predict the eco-evolution of the beneficial plant-microbe interactions.

We host in this Research Topic, "The Plant Holobiont Volume I: Microbiota as Part of the Holobiont; Challenges for Agriculture," 14 articles (12 research articles, 1 method article and

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1 review article) enhancing our knowledge on agricultural practices (organic and conventional farming, intercropping) and plant traits (exudates, genes) modulating rhizosphere microbiota (bacterial and fungal communities) under different environments and biotic (e.g., *Rhizoctonia solani*) and abiotic (i.e., drought, heavy metal) stresses. On the other way round, the beneficial effect of some rhizospheric strains on plant growth and health, and the transmission of these beneficial strains from one plant generation to the following are also described. Sixteen plant species were used to perform these studies: 10 crop plants (grapevine, four leafy green crops, maize, pea, pumpkin, rice, sugar beet, sugarcane, and wheat), two trees (olive and poplar), a model (*Brachypodium distachion*), and a grass (ryegrass). Some of these studies characterized the impact of different genotypes/cultivars (maize, pea, pumpkin, rice, sugar beet, and wheat) on the microbiota and one (Zhao et al.) assessed the impact of a mutant plant. Finally, the study of Zhou et al. made a first attempt to correlate microbial community composition to grapevine berries and wine properties.

CONTENT COLLECTION

Original Research Articles

Several articles confirmed the well-known plant species effect on rhizosphere microbiota. This rhizosphere effect was shown to be modulated by the soil type when considering saprophytic and symbiotic fungi (Zhou et al.) and by the plant species (Cesaro et al.; Pivato et al.), but did not differ upon the developmental stage of grapevine (Cesaro et al.).

Beyond soil physico-chemical parameters and plant species, the farming system deeply affects rhizosphere microbiota. Bacterial diversity in maize rhizosphere was higher when cultivated in organic than in conventional farming (Ares et al.). Organic amendment increased only the bacterial but not the fungal diversity in poplar rhizosphere; however, this greater microbiome diversity does not imply a better plant wellness or phytoremediation ability in zinc-polluted soil (Guarino et al.).

Intercropping agricultural practice tested by Pivato et al. affected the co-occurrence network of rhizosphere bacterial communities but not their diversity. However, the variations of the microbiota (diversity and network of co-occurrence) mediated by the agricultural practices on the host-plant mostly remain untapped and further studies are needed to elucidate these effects in order to identify the practices that allow to better value plant-microbe interactions.

Beside agricultural practices, the impact of the plant genotypes/cultivars on rhizosphere microbiota was also evaluated. Two maize genotypes ("Pigarro," improved landrace and "SinPre," a composite cross population), did not affect bacterial microbiota, although they shared a core bacteriome (Ares et al.). Chang et al. characterized the impact of 12 rice accessions (five wild and seven domesticated) on the diversity, structure and co-occurrence network of fungal communities. Domestication increased the alpha-diversity of the rice rhizosphere fungal communities, however, the co-occurrence network of symbiotic and saprophytic fungi was more developed in the wild rice rhizosphere. Pivato et al. showed differences in the bacterial diversity between pea hr and Hr genotypes, whilst no differences were found between wheat genotypes. The impact of plant genotype/cultivar on microbiota seems not to be consistent and to vary according plant genotypes/cultivars. Further research will have to understand the rationale for these variations.

Some works hosted in this Research Topic made attempts to identify plant genotypes able to select beneficial microbiota. Dumigan et al. characterized the impact of the wild maize ancestor *Zea mays* ssp. *parviglumis*, and an ancient mexican landrace (*Z. mays* spp. *mays*) on diazotrophs endophytes. The authors identified four strains able to growth in N-free media and to increase annual ryegrass biomass, that seem to come from the seeds and not from the soil. By using both molecular and culturomic approaches, Taffner et al. could characterize the diversity of bacterial communities of four indigenous leafy green vegetables but also their antagonistic activities against phytopathogenic fungi. A large core microbiome common in the four plants comprising 18 prokaryotic families was found, and the bacterial culture approach allowed to identify Sphingomonadaceae and Bacillaceae as key candidates for sustainable biocontrol agents. Wolfgang et al. used five different cultivars of sugar beet in order to characterize microbial populations, which are involved in plant protection against *R. solani*. Three bacterial genera preferentially associated to *Rhizoctonia*-tolerant cultivars are expected to contribute to this tolerance. Taken together, these results show that cultivars that are tolerant to this soilborne disease host microbial communities that account for the low disease expression, thus clearly supporting the reciprocal beneficial effect of plants and associated microbiota.

The legacy effect of the rhizosphere effect has been further explored by testing the possible transmission of rhizosphere microbiota (fungi and bacteria) of *Cucurbita pepo* to their progeny seeds (Kusstatscher et al.). Beneficial bacterial taxa appeared to be enriched in progeny seeds, indicating that beneficial microorganisms recruited from soil may be transmitted to the following plant generation.

However, if plants actively recruit beneficial microbiota, many aspects of this process remain to be untapped. Two articles in this Research Topic focused on the identification of plant traits mediating the recruitment and activities of beneficial microbial populations. Mavrodi et al. identified plant phenotypic traits that modulate microbial functioning. Characterization of the RNA-seq profiling of *Pseudomonas* cultures in the presence of root exudates of *Brachypodium dystachyon* stressed the impact of these exudates on the expression of genes encoding numerous catabolic and anabolic enzymes, transporters, transcriptional regulators, stress response, and conserved hypothetical proteins. Zhao et al. described the fungal community of the transgenic (TG) sugarcane variety GN18, harboring the drought-tolerant gene *Ea-DREB2B* and its corresponding non-TG wild-type (WT) variety, FN95-1702, suggesting this gene as plant determinant able to modulate the microbial community. These studies open future perspectives to identify plant determinants structuring rhizospheric communities.

Finally, rhizospheric microbiota on plant were reported to not only promote growth and health, but also nutritional quality of edible parts. A possible relation between bacterial community and fruit quality was assessed (Zhou et al.). The strategy followed consisted in comparing fruit/wine quality and bacterial communities from 22 different sites. However, differences in wine quality between these sites not only rely on the bacteriome but also on a series of parameters involved in the so-called "terroir."

Method Article

Articles in the Research Topic used amplicon metagenomics sequencing to characterize genetic diversity and structure of microbial communities and culturomic (sometimes coupled with genomics, as in Mavrodi et al.) to characterize their functionality. However, even if NGS are widely used, developments still need to be made specially when targeting plant niches. Haro et al. assessed the influence of commercially available DNA extraction kits and different primer pairs to produce a non-biased version of the composition of bacterial communities present in olive xylem sap. They showed a most accurate depiction of a bacterial mock community artificially inoculated on sap samples when using the PowerPlant DNA extraction kit, the combination of 799/1193R primers amplifying the hypervariable V5-V7 region and the Silva 132 database for taxonomic assignment. Moreover, Li et al. focused the first part of their review article on methodological approaches in order to characterize orchid mycorrhizal fungi and they identified the method that they consider as the most efficient. Progress is constantly been made also in bioinformatics analysis; for instance the use of Amplicon Sequence Variants (ASV, here used in Wolfgang et al.) instead of OTUs allows a better control of DNA extraction and PCR bias (Callahan et al., 2017).

Review Articles

Li et al. concerns in mycorrizal diversity in Orchids made the only review article of the Research Topic. Orchids have complex symbiotic relationships with fungi at various stages of their life cycle. Environmental conditions significantly affect Orchid Mycorrhizal Fungi (OMF) diversity and abundance and plant deterministic processes seem to contribute to the construction of OMF communities. Methodological improvement and standardization could facilitate better comparability of core fungal taxa of different or the same orchid species from different case studies. Clarifying the architecture of the OMF networks formed by co-existing orchids can help to comprehend how these hyper-diverse interacting guilds are maintained and co-evolve in their habitats. This can further reveal the species, lineages, or functional taxa that are significant to ecosystem services.

AUTHOR CONTRIBUTIONS

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

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ORIGINAL RESEARCH
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Effects of Zinc Pollution and Compost Amendment on the Root Microbiome of a Metal Tolerant Poplar Clone

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Until recently, many phytoremediation studies were focused solely on a plants ability to reclaim heavy metal (HM) polluted soil through a range of different processes, such as phytoextraction and phytostabilization. However, the interaction between plants and their own rhizosphere microbiome represents a new research frontier for phytoremediation. Our hypothesis is that rhizomicrobiome might play a key role in plant wellness and in the response to external stimuli; therefore, this study aimed to shed light the rhizomicrobiome dynamics after an organic amendment (e.g., compost) and/or HM pollution (e.g., Zn), and its relation with plant reclamation ability. To reach this goal we set up a greenhouse experiment cultivating in pot an elite black poplar clone (N12) selected in the past for its excellent ability to reclaim heavy metals. N12 saplings were grown on a soil amended with compost and/or spiked with high Zn doses. At the end of the experiment, we observed that the compost amendment strongly increased the foliar size but did not affect significantly the Zn accumulation in plant. Furthermore, the rhizomicrobiome communities (bacteria and fungi), investigated through NGS, highlighted how α diversity increased in all treatments compared to the untreated N12 saplings. Soil compost amendment, as well as Zn pollution, strongly modified the bacterial rhizomicrobiome structure. Conversely, the variation of the fungal rhizomicrobiome was only marginally affected by soil Zn addition, and only partially impaired by compost. Nevertheless, substantial alterations of the fungal community were due to both compost and Zn. Together, our experimental results revealed that organic amendment increased the bacterial resistance to external stimuli whilst, in the case of fungi, the amendment made the fungi microbiome more susceptible. Finally, the greater microbiome biodiversity does not imply, in this case, a better plant wellness or phytoremediation ability, although the microbiome plays a role in the external stimuli response supporting plant life.

Keywords: phytoremediation, metals, compost, microbiome, bacteria, fungi, NGS

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INTRODUCTION

Heavy metal (HM) contamination of the environment has increased to levels that are harmful for living organisms, mainly because of anthropogenic activities. HMs are non-degradable pollutants, and, thus, they persist indefinitely in diverse environmental matrices. Among HMs, zinc (Zn) can be included; it has an atomic number of 30 and atomic weight of 65.38, it is the 24rd most abundant

element on earth, and it is an essential trace element for all living beings, including plants. Zinc is a constituent of many proteins, it is also an enzyme cofactor and it is fundamental for optimum plant growth and development (Broadley et al., 2007). However, at high concentrations in the soil, Zn is phytotoxic, and plants that accumulate it through root absorption or deposition, pose health risks to consumers (Bolan et al., 2014). Therefore, remediation of HM polluted soils is imperative and necessary to reduce their impact on plants, ecosystems, landscape, soil microbial biodiversity and human health.

Phytoremediation is a green bio-technology, solar driven and cost-effective, associated with many additional benefits such as: conversion of plant biomass into bioenergy, sustaining of biodiversity, soil stabilization, and numerous other ecosystem services. Over the past decade, it has become clear that phytoremediation is assisted by soil or rhizosphere microorganisms often useful, and sometime necessary, to increase HM bioavailability and their subsequent uptake by plants not clear (Becerra-Castro et al., 2013; Ahemad and Kibret, 2014; Kamran et al., 2017). Recent evidence emphasizes that the success of phytoremediation of HM contaminated environments depends strongly on plant-microbiome interactions (Cicatelli et al., 2019). Plants and microbes coexist or compete for survival and their interactions play a vital role in plant adaption to HM pollution. Moreover, microorganisms, and their interaction with HMs in contaminated soils can contribute to their removal and, therefore, influence the efficiency and rate of the phytoremediation. Rhizosphere microorganisms, especially bacteria and fungi, colonize plant roots (Ahemad and Kibret, 2014), establishing an extensive microbial network that is mutually beneficial. The host plant constantly nourishes the microbiota through root exudates (up to 30% of photosynthesis by products) (Vives-Peris et al., 2020). The presence of the root apparatus, in fact, is spread throughout the microbial network, that perceives it and responses in turn, also affecting mobility and bioavailability of the HMs in the soil and in the rhizosphere, thereby protecting the plant from their toxicity (Shafigh et al., 2019). The amount and composition (simple molecules, sugars, organic acids, and secondary metabolites, as well as complex polymers, such as mucilage) of exudates vary in relation to the host genotype, developmental stage and, moreover, they are modulated by abiotic stresses (Rengel, 2015). In turn, rhizobacteria can promote plant growth by secreting beneficial chemical compounds, such as mineral phosphate solubilizers, indole acetic acid (IAA), siderophores, and ACC deaminase (Wang et al., 2018; Gupta and Pandey, 2019), affecting the absorption of pollutants by changing soil pH, excreting surfactants or chelating substances (Rajkumar et al., 2012) and altering redox potentials (Rengel, 2015). Rhizosphere fungi, such as mycorrhiza, are also able to enlarge soil exploration of plant roots, improving the uptake and translocation of nutrients and HMs from soils to the different plant organs. Currently, phytoremediation has not yet reached the level of highly efficient and fast clean-up technologies, and therefore many studies have focused their attention on strategies to improve soil phytoremediation efficiency, also using microbes able to assist plants in the processes increasing HM bio-availability and accessibility, plant growth, etc. A promising strategy for the managing of HM contaminated soils includes the use of metal tolerant poplar clones. In fact, it is widely recognized that the genus *Populus* has several features that are suitable for effective soil reclamation, such as rapid growth, a deep and spreading root system, marked adaptability to different pedoclimatic conditions and remarkable capability to vegetative reproduction, which makes its propagation quite easy. In addition, poplars are highly tolerant of different contaminants, and a large clonal variability in metal-resistance or accumulation traits (Kopponen et al., 2001; Laureysens et al., 2005; Dos Santos Utmazian and Wenzel, 2007; Cicatelli et al., 2014). Several poplar clones, belonging to the species *Populus alba* L. and *Populus nigra* L., have been screened for their HM tolerance during a field trial on a soil highly polluted by Cu and Zn. Among these, a black poplar clone named N12 was selected for its high survival, HM tolerance and accumulation (Castiglione et al., 2009).

In the present study, Zn phytoremediation, operated by the N12 black poplar clone, was assisted by compost (CMP) soil amendment. Compost may be easily obtained from artificially controlled microbial degradation of organic wastes [e.g., municipal solid and agricultural organic wastes (Singh and Agrawal, 2008)]. Compost can be used to improve the physico-chemical and biological properties of the soil, by modifying its porous structure (for an improved root penetration), water storage capacity and resistance to erosion, and also by introducing new organic matter, nutrients and microbes. All of these contribute to increased crop growth and yield, and provide additional genera and species of microorganisms potentially useful to plant health (Sudharsan Varma and Kalamdhad, 2015).

Several studies have clarified the structure and variation of root-associated microbiomes in different plants species employed in bio- phytoremediation (Jamir et al., 2019; Zadel et al., 2020). At present, as far as we know, very little information is available on how microbial communities assemble in the rhizosphere, defined as the top soil layer of 1–2 mm surrounding the plant roots, if it is polluted by a metal such as Zn and amended with compost (CMP). The main objectives of our study were to: (i) investigate the Zn phytoremediation performance of the N12 multi-metal tolerant black poplar clone, assisted by CMP amendment, and (ii) investigate the changes of its associated rhizosphere microbiome in relation to Zn addition to the soil and CMP amendment.

MATERIALS AND METHODS

Experimental Design

The experiment was conducted using N12 poplar cuttings (12) sprouting separately in single pots and grown in a greenhouse to minimize the impact of other environmental stress factors. Pots were filled using an agricultural soil (**Supplementary Table S1**, T0 soil). One portion of this soil was amended with certified high-quality compost (CMP, 20% of the pot volume) obtained from the organic fraction of municipal solid waste. The N12 poplar clone, belonging to a collection of *Populus nigra* L. that originated from an Italian natural population, was selected for this experimentation because during a field

trial on a multi-metal polluted site (Castiglione et al., 2009) it was shown to be tolerant to high concentrations of Cu and Zn. Cuttings (20 cm long) were placed overnight under running tap water and then singularly put into plastic pots of 5 liters of volume (3 cuttings per treatment group), containing soil amended or not with CMP. After 2 months of growth, Zn was added to the soil as a nitrate salt, avoiding the solution leaching, in three successive doses every week, up to a final concentration of 450 mg kg^{-1} soil dry weight (DW). The pot trial included the following four experimental groups: CNT (3 plants on unpolluted soil); Zn450 (3 plants on Zn polluted soil); CMP (3 plants on unpolluted and CMP amended soil); CMP + Zn450 (3 plants on Zn polluted and CMP amended soil).

Leaf Morphometric Measurement

At the end of experimentation, leaf area and length, average and maximum width were recorded on five completely expanded leaves of each poplar plant, using a portable leaf area meter (LI-3000C; LI-COR, Nebraska, United States). The total number of measured leaves is 60.

Plant and Soil Collection

At the end of the experimentation (about 3 months after planting), whole intact saplings were harvested from each pot. Roots, carefully washed with distilled water to eliminate soil sediments, stems and leaves were collected and separately dried at 75°C to constant weight, for measurements of their DW and for estimation of Zn content. At the start of the experimentation, soils, CMP amended or not, were analyzed to evaluate the main physical and chemical features. For pH determination, 10 g of soil were placed in 25 mL of deionized water and shaken for 2 h. Soil organic carbon and Organic Matter Content (OMC) were determined using the Walkley and Black (1934) protocol. For determination of available phosphate, the method described by Olsen et al. (1954) was used. Total C, total N, and the C/N ratio were determined through the combustion method of elemental analysis. The barium chloride triethanolamine method (Youden and Mehlich, 1938) was employed to estimate soil cation exchange capacity (CEC). Furthermore, three soil cores were collected at the start and at the end of experimentation from pots of each experimental group to determine the total and available Zn metal content. Soil cores were pooled, mixed, sieved (2 mm) and dried at 75°C to constant weight and processed for metal content analysis as described below.

Plant and Soil Metal Content

Dried soils were pulverized in a planetary ball mill (PM4, Retsch, Germany), while plant organs were pulverized in a mortar (leaves, roots) using liquid nitrogen, or reduced to ash (stems) by baking at 550°C for 2 h. For each plant organ, three biological replicates were analyzed. All the matrices (soils and plant organs) were digested with an acid mixture (HNO_3 65%; HF, 50% = 2:1v/v) in a microwave oven (Milestone Srl – 24010, Sorisole BG, Italy) using the following digestion program: 1 min at 250 W, 1 min at 0 W, 5 min at 250 W, 4 min at 400 W, 4 min at 600 W, 5 min at 250 W. The method of Lindsay and Norvell (1978) was used to estimate available Zn concentrations in soil extracts obtained from the dried granulometric fraction. Element concentrations were determined by ICP-OES (Optima 7000DV. PerkinElmer Italia Spa – 20126, Milan, Italy). Standard reference material (Mackey et al., 2004) was analyzed in order to verify the accuracy of the obtained results. Standard solutions of Zn were used to generate the calibration curve to convert emission readings into the analyte concentrations.

Rhizosphere Soil Collection and DNA Extraction

At the end of experimentation, soil particles adhering to the fine roots, with a diameter of about 2 mm or less, were sampled and considered as rhizosphere soil, while the fine roots were collected from poplar plants of each experimental group, pooled and placed in a tube containing 25 mL of sterile physiological solution (0.9% NaCl). Tubes were vortexed at maximum speed for 15 s and then shaken for 1 h at room temperature to release the majority of the microorganisms adhering to the roots. Roots were recovered and transferred to a new sterile 50-mL tube with 25 mL of physiological saline (0.9% NaCl), sonicated at low frequency for 5 min to further disrupt tiny soil aggregates and recover the attached microbes. The roots were then removed from this solution. The solutions containing fine sediment and microorganisms were pooled and centrifuged for 20 min at 5000 rpm to pellet the microorganisms. Pelleted microorganisms were washed twice with physiological saline, and finally resuspended in 20 mL of the same solution. Aliquots of 1 ml were flash-frozen in liquid nitrogen and stored at −80°C until processing. Aliquots of rhizosphere solutions (100 μL) were mixed in the lysis buffer of the DNA EXTRACT-N-AMP kit (Sigma-Aldrich, Milan, Italy), following the supplier instructions, for a rapid DNA extraction.

Amplicon Library Preparation and NGS Sequencing

Microbial DNAs were extracted in triplicate from rhizosphere solution of each experimental group and PCR amplified. The V3-V4 region of the bacterial 16S rRNA genes was amplified with 341F (5′-CCTACGGGRSGCAGCAG-3′) and 909R (5′-TTTCAGYCTTGCGRCCGTAC-3′) specific primers, the following PCR thermal profile was used: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min and elongation at 72°C for 1 min. with additional final elongation step at 72°C for 5 min. The fungi ITS2 region was amplified using the ITS3 (5′-GCATCGATGAAGAACGCAGC) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) primers and the following PCR thermal profile was employed: initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and elongation at 72°C for 1 min, with additional final elongation step at 72°C for 5 min. The amplicon libraries were processed following the manufacturer's instructions (BMR

Genomics, Padua, Italy) and sequenced by BMR Genomics (Padua, Italy).

Sequences Analysis

Illumina sequence data were sorted based on unique barcodes and quality-controlled using the Quantitative Insights Into Microbial Ecology (Qiime2, version 2017.8)[1] with plugins-demux[2] dada2 (Callahan et al., 2015) and feature-table (McDonald et al., 2012). α - and β-diversity analyses were performed by using plugins alignment (Katoh and Standley, 2013) diversity[3]. For taxonomic analysis, a pre-trained Naive Bayes classifier based on the SILVA 138 (Operational Taxonomic Units) OTUs database, in the case of 16S rDNA[4], which has been trimmed to include the V3-V4 region of 16S rRNA gene, bound by the 341F/909R primer pair, was used. While, the classifier, for fungi ITS2 DNA sequences, was pre-trained on UNITE database version 7–99%, and applied to paired-end sequence reads to generate taxonomy tables. Taxonomic and compositional analyses were conducted by using plugins feature-classifier[5] (Bokulich et al., 2018), taxa[6] and composition (Mandal et al., 2015).

Microbiome Diversity Indices

The raw data were used to prior α-diversity analyses using Observed OTUs Shannon Simpson and Chao1 metrics in Qiime2 α-diversity plugins (Faith, 1992). The total frequency that each sample was be rarefied to prior to computing diversity metrics was 6820 in the case of 16S rDNA and 59788 in the case of ITS. The differences among the experimental groups were assessed with the Kruskal-Wallis test. β-diversity was estimated by calculating the Dice non-phylogenetic β-diversity distance (Lozupone and Knight, 2005). All values were expressed as means ± standard deviations of triplicate analyses. Analysis of variance (PERANOVA) was performed and *P*-values were then obtained using 999 permutations. Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to detect the bacterial taxonomic biomarkers across the different treatments (Segata et al., 2011).

Statistical Analysis

A preliminary test to assay the Gaussian distribution, homogeneity variance and homoscedasticity were performed on data in R environment (R Development Core Team, 2011) through Shapiro-Wilk test, Levene test and Bartlett test, respectively (Shapiro and Wilk, 1965). After that, biomass production, morphometric data, metal concentration and accumulation in different plant organs and soil were tested in R environment by Kruskal and Wallis One-Way Analysis of Variance by ranks (Wallis, 2008), followed by *post hoc* Nemenyi test (Graves et al., 2015).

[1]https://docs.qiime2.org/2017.8/

[2]https://github.com/qiime2/q2-demux

[3]https://github.com/qiime2/q2-diversity

[4]https://www.arb-silva.de/documentation/release-138/

[5]https://github.com/qiime2/q2-feature-classifier

[6]https://github.com/qiime2/q2-taxa

RESULTS

N12 Sapling Growth

At the end of experimentation, N12 poplar saplings, grown on Zn spiked soils, did not show any symptoms of toxicity or stress. Zinc addition had no effect on biomass production of roots, stems and leaves (**Figure 1**). In contrast, the 20% CMP soil amendment improved growth and exerted a positive effect on the poplar saplings, mainly on their leaves (**Table 1**). Specifically, leaf biomass was significantly greater in saplings grown on CMP amended soil (without Zn) if compared with those grown on not-amended soils (CNT and Zn450). Moreover, poplar saplings belonging to CMP group had very large and dark green leaves. The morphometric analysis revealed that the leaves of saplings grown on CMP amended soils were significantly more expanded than those of saplings grown on unamended ones (**Table 1**).

Soil Characterization

Physico-chemical analyses were performed on unplanted soils with and without CMP amended, and both soils showed similar results (**Supplementary Table S1**). In unplanted soils, the total amount of Zn was below the guideline values established for a green area, and the bioavailable fractions of Zn were low. At the end of the experiment, Zn concentrations increased, as a consequence of the artificial contamination of both soils (data not shown). The available fractions of Zn, initially low, were not significantly modified in the presence of poplar saplings as well as by CMP soil amendment (data not shown).

Zn Content in N12 Saplings

Zinc reached the highest concentrations in the roots and stems (ca. 1,200 and 800 $\mu g\ g^{-1}$, respectively) and the lowest in leaves (ca. 190 $\mu g\ g^{-1}$; **Table 2**) in Zn450 and CMP + Zn450 treatments. The total amount of Zn accumulated by the total biomass of the saplings increased after Zn addition, but with no significative difference between the two experimental groups with CMP soil amendment (**Figure 2**).

The Rhizosphere Microbiome of N12 Saplings

The rhizosphere microbiome of N12 poplar saplings grown on a soil spiked with Zn with and without CMP amendment, was analyzed. In addition, phytoremediation capability of the saplings was investigated in relation to rhizosphere microbiome biodiversity. In order to evaluate the interaction between microorganisms living on the root surface and the saplings, and more specifically, to investigate a correlation between phytoremediation capacity of this clone and its rhizosphere microbiome, DNAs were isolated from microbial communities collected from fine roots surface and the 16S rDNA and ITS regions were PCR amplified and deep sequenced.

Biodiversity of the Root Bacterial Microbiome

For 16S rDNA, 160,000 gene from 12 samples were sequenced with an average read length of 600 bp (300 × 2). Quality filtering, denoising and the removal of chimeric sequences reduced this

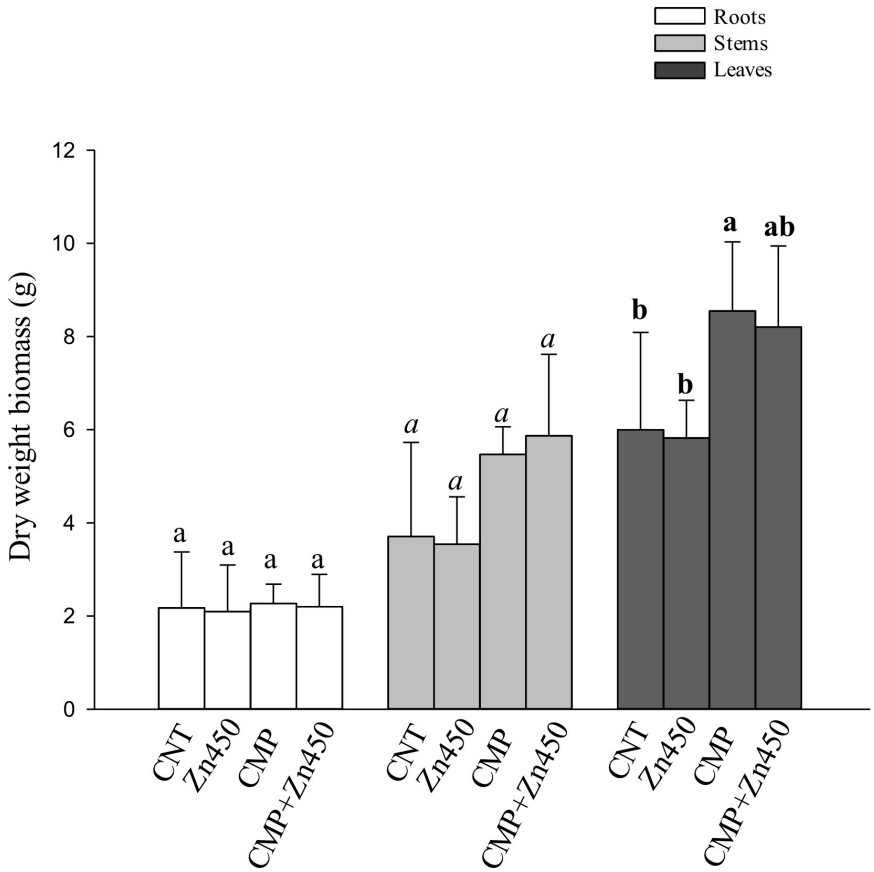

FIGURE 1 | Biomass (dry weight, g) of N12 leaves, roots and stems at harvest and for each treatment. Different letters indicate statistically significant differences among treatments compared to the leaves ($p < 0.05$). Bars indicate standard deviations.

TABLE 1 | Leaf area (cm^2), length, average width and maximum width (cm) of N12, grown under different conditions, were determined (mean value ± standard deviation five replicates for each sapling of each experimental thesis).

	Leaf area	Leaf length	Average width	Maximum width
CNT	27.05 ± 9.18 [a]	6.92 ± 1.20[a]	3.83 ± 0.87[a]	6.15 ± 1.07[a]
Zn450	19.59 ± 3.35[a]	5.91 ± 1.02[a]	3.23 ± 0.30[a]	5.29 ± 0.53[a]
CMP	42.81 ± 8.02[b]	9.16 ± 1.54[b]	4.61 ± 0.51[ab]	8.07 ± 0.70[b]
CMP + Zn450	48.22 ± 9.53[b]	9.77 ± 1.41[b]	4.90 ± 0.82[b]	8.62 ± 1.03[b]

Different letters indicate significantly different ($P < 0.05$) values for treatments with reference to the same leaf morphometric parameter.

TABLE 2 | Zinc concentrations ($\mu g\ g^{-1}$) in roots, stems and leaves of the N12 grown under different conditions (mean value ± standard deviation of three replicates for each experimental thesis).

	Roots	Stems	Leaves
CNT	346.82 ± 44.38[a]	596.98 ± 18.88	71.78 ± 2.41
Zn450	1247.77 ± 525.62[b]	800.60 ± 59.40	194.07 ± 5.47
CMP	261.58 ± 8.68[a]	626.82 ± 19.45	73.15 ± 25.49
CMP + Zn450	680.75 ± 84.20[c]	817.81 ± 24.34	87.91 ± 18.93

Different letters indicate significantly different ($P < 0.05$) values for treatments with reference to the same organ.

number to 108,387. The ASV was 436 and the rarefaction curves are reported in **Supplementary Material** (**Supplementary Figure S1**). The α biodiversity was estimated, the Observed OTUs and both Shannon and Simpson indices calculated. The Kruskal-Wallis pairwise comparison among the four treatments (CNT, Zn450, CMP, CMP + Zn450) were not statistically significant for

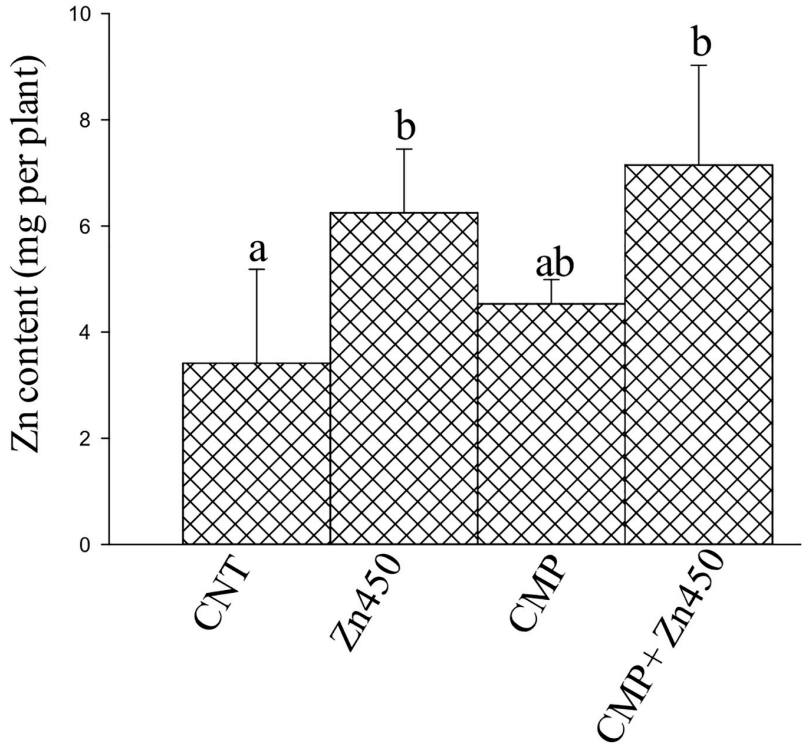

FIGURE 2 | Total Zn amount in the N12 saplings at the end of experiment and for each treatment. Different letters indicate significantly different values for treatments ($p < 0.05$). Bars indicate standard deviations.

TABLE 3 | Kruskal-Wallis pairwise comparison of two α-diversity indices **(A)** Shannon and **(B)** Simpson among the experimental groups.

Thesis 1	Thesis 2	H	*p*-value	*q*-value
(A) Shannon index				
Thesis 1	**Thesis 2**	**H**	***p*-value**	***q*-value**
CNT (*n* = 3)	**CMP (*n* = 3)**	**3.86**	**0.049**	**0.099**
CNT (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**3.86**	**0.049**	**0.099**
CNT (*n* = 3)	**Zn450 (*n* = 3)**	**3.86**	**0.049**	**0.099**
CMP (*n* = 3)	CMP + Zn450 (*n* = 3)	0.43	0.512	0.615
CMP (*n* = 3)	Zn450 (*n* = 3)	0.05	0.827	0.827
CMP + Zn450 (*n* = 3)	Zn450 (*n* = 3)	0.43	0.512	0.615
(B) Simpson index				
Thesis 1	**Thesis 2**	**H**	***p*-value**	***q*-value**
CNT (*n* = 3)	**CMP (*n* = 3)**	**3.86**	**0.049**	**0.099**
CNT (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**3.86**	**0.049**	**0.099**
CNT (*n* = 3)	**Zn450 (*n* = 3)**	**3.86**	**0.049**	**0.099**
CMP (*n* = 3)	CMP + Zn450 (*n* = 3)	1.19	0.275	0.275
CMP (*n* = 3)	Zn450 (*n* = 3)	1.19	0.275	0.275
CMP + Zn450 (*n* = 3)	Zn450 (*n* = 3)	1.19	0.275	0.275

Statistically significant values are indicated in bold ($p < 0.05$).

Observed OTUs, whilst all treatments affected both Shannon and Simpson indices ($p < 0.05$) when compared to CNT (**Table 3**).

The α diversity was greater in the case of CMP, CMP + Zn450 and Zn450 compared to CNT ($p < 0.05$). Correlation analyses revealed that Observed OTUs were negatively correlated with total sapling biomass ($r = -0.57$, $p < 0.05$) and total sapling Zn accumulation ($r = -0.58$, $p < 0.05$). In particular, the number of Observed OTUs was negatively correlated with root ($r = -0.58$,

$p < 0.05$) and stem biomass ($r = -0.60$, $p < 0.05$), and stem Zn accumulation ($r = -0.58$, $p < 0.05$).

The β diversity was calculated in order to highlight the differences among the experimental groups using the Dice metrics. The results (**Table 4**) showed that the Dice distance among the analyzed groups was statistically significative ($p \leq 0.1$).

Bacterial community composition

The taxa bar plot was carried out at three different hierarchic levels: phylum, class and family. At phylum level (**Figure 3A**), the results revealed the presence of 15 phyla. The most abundant were Proteobacteria and Firmicutes.

The Firmicutes phylum was predominant in CNT and its relative abundance was reduced in the other three experimental groups, whilst, Proteobacteria phylum increased its relative abundance as well as Actinobacteria and Acidobacteria phyla. All the other phyla showed a relative low frequency and were strongly influenced by Zn addition in the case of the Zn450 (e.g., Patescibacteria) or CMP + Zn450 (e.g., Chloroflexi). At the class level, Gammaproteobacteria (**Figure 3B**) dominated the CMP rhizosphere microbiome, and their relative abundance was not affected by Zn addition. On the contrary, the CNT thesis was characterized by a high relative frequency of Bacilli, which, in turn, was negatively affected by Zn addition (Zn450). In this case, the Alphaproteobacteria, Blastocatella, and Holophagae (belonging to the phylum of Acidobacteria) classes increased greatly compared to the CNT. When the CMP and CMP + Zn450 groups were considered, several phyla and classes (absent in the other groups) were present, such as Anaerolineae, Thermoleophilia and other classes belonging to the Acidobacteria phylum (Subgroups 6, 17, and 22).

At the family level (**Figure 4**), 62 OTUs were identified, and the most abundant were represented by Xanthomonadales, Pseudomonadales, and Betaproteobacteriales (belonging to the Phylum Proteobacteria, class Gammaproteobacteria), Bacillales (belonging to the Phylum Firmicutes, class Bacilli), and Rhizobiales (belonging to the Phylum Proteobacteria, class Alphaproteobacteria). The rhizosphere microbiome of CNT was characterized by the highest relative frequency of Bacillales (phylum Firmicutes, class Bacilli), and the lowest of Xanthomonadales, while other families were represented at low relative frequency. Moreover, some families were particularly enriched in some specific rhizosphere microbiome; even though CMP amendment and/or Zn addition deeply modified the rhizosphere community; the bacteria families, initially characterizing the CNT rhizosphere microbiome were still present in the other experimental group, but with different relative frequency (Rhizobiales, Verrucomicrobiales, Pseudomonadales, Betaproteobacteriales, Xanthomonadales, and Bacillales). In particular, Zn alone favored the presence of Micrococcales, Pyrinomonadales, and Dongiales classes.

In the case of CMP group, the most represented families were Xanthomonadales, Pseudomonadales and Betaproteobacteriales, and they were not affected by the Zn addition (CMP + Zn450 vs. CMP). However, CMP amendment favored the presence of Aeromonadales, Desulfuromonadales, Tistrellales, whilst Zn addition and CMP amendment (CMP + Zn450) modified the rhizosphere microbiome favoring the presence of Corynebacteriales, Gaiellales, Salinisphaerales, and Pseudonocardiales families.

Qualitative and quantitative analysis of rhizobacteria communities

A qualitative and quantitative analysis of the rhizosphere microbiome was carried out in order to evaluate the differences, in terms of the classes or families, that were attributable to CMP amendment and/or Zn addition. In particular, the Venn analysis (**Table 5**) revealed that the experimental group with the highest number of unique classes was that of CMP (7 bacteria classes), followed by the other three groups in decreasing order: CMP + Zn450 > Zn450 > CNT (4, 1 and 0 unique classes, respectively). A large part of the bacteria classes was shared among the different groups (6; **Table 5**).

In order to investigate whether some bacteria phylum, class or family was a potential marker of the diverse rhizosphere microbiome, a LEfSe analysis was performed (**Figure 5**). Seventy-five root Absolute Sequence Variants (ASVs) were identified with LEfSe ($p < 0.05$, log10 LDA score > 3.5) among the different experimental groups. In particular, members belonging to Bacillales family (belonging to Phylum Firmicutes, class Bacilli) may be considered a marker of CNT microbiome, whilst Actinobacteria (Streptomycetales) and Acidobacteria (Subgroup_6) were for Zn450. The CMP and CMP + Zn450 rhizosphere microbiome were characterized by the presence of Gemmatimonadales and Verrucomicrobiales, or Pseudomonadales and Cellvibrionales (Gammaproteobacteria) classes, respectively.

TABLE 4 | The pairwise PERMANOVA tests whether Dice distances between samples within the same experimental group are more similar to each other than they are to samples from the other groups.

Thesis 1	Thesis 2	Sample size	Permutations	pseudo-F	*p*-value	*q*-value
CNT	**CMP**	**6**	**999**	**6.19**	**0.090**	**0.110**
CNT	**CMP + Zn450**	**6**	**999**	**7.57**	**0.100**	**0.110**
CNT	**Zn450**	**6**	**999**	**5.71**	**0.100**	**0.110**
CMP	**CMP + Zn450**	**6**	**999**	**4.95**	**0.090**	**0.110**
CMP	**Zn450**	**6**	**999**	**7.20**	**0.100**	**0.110**
CMP + Zn450	**Zn450**	**6**	**999**	**8.52**	**0.100**	**0.110**

Statistically significant values are indicated in bold ($p \leq 0.1$).

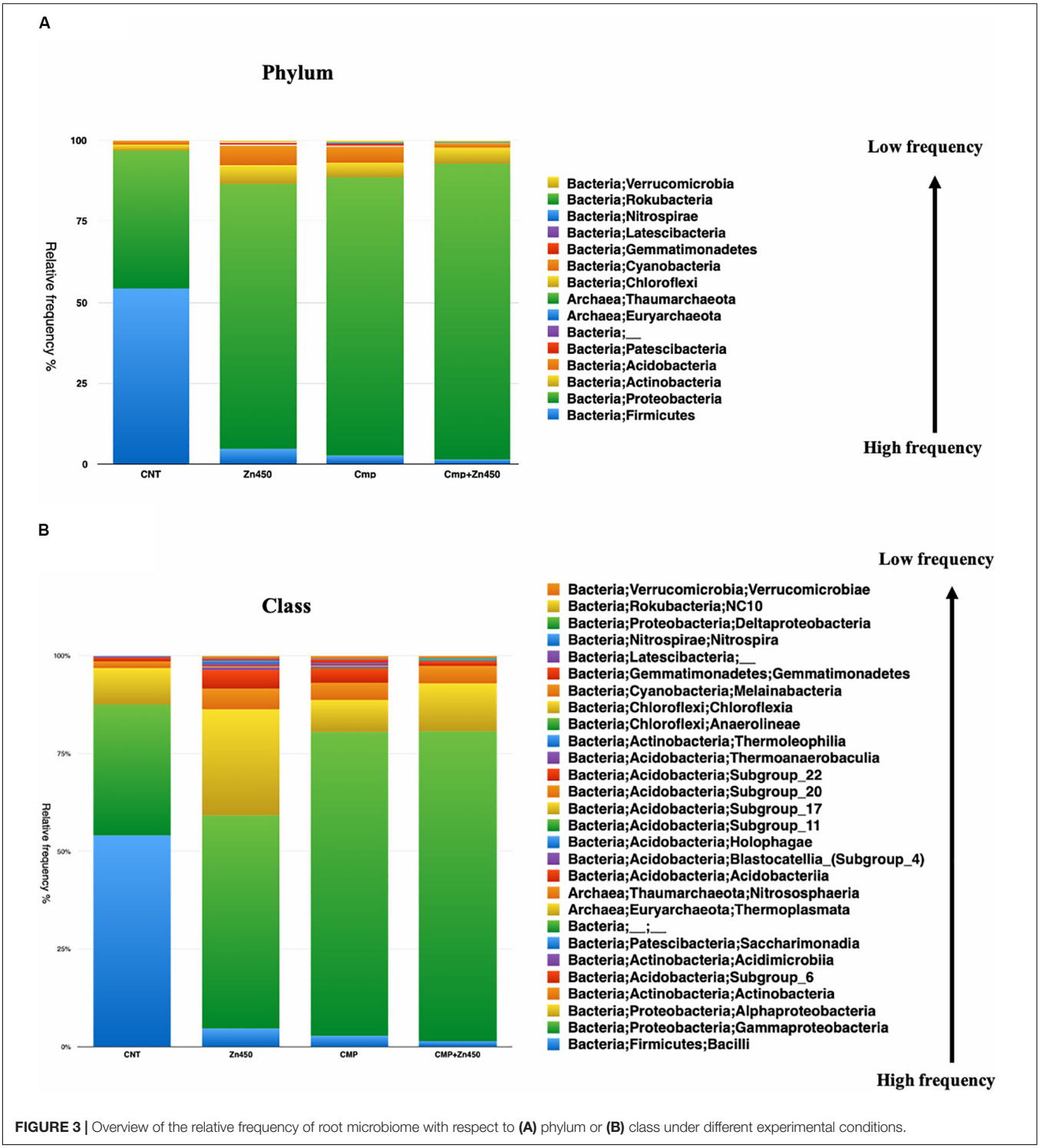

FIGURE 3 | Overview of the relative frequency of root microbiome with respect to **(A)** phylum or **(B)** class under different experimental conditions.

Biodiversity of Fungal Rhizosphere Microbiome

ITS sequencing generated 1,184,489 gene sequences and, after quality filtering, denoising and removal of chimeric sequences, 1,015,382 genes sequences remained. The ASV was 718 and the rarefaction curves are reported in **Supplementary Figure S2**. The α biodiversity was investigated for rhizosphere fungal communities and, in general, both CMP amendment and/or Zn addition altered them. In particular, the lowest α diversity was observed in the case of CMP + Zn450 (**Table 6**).

Furthermore, the comparison of Observed OTUs and Simpson indices revealed statistically significative differences among all treatments ($p < 0.05$), with the exception of

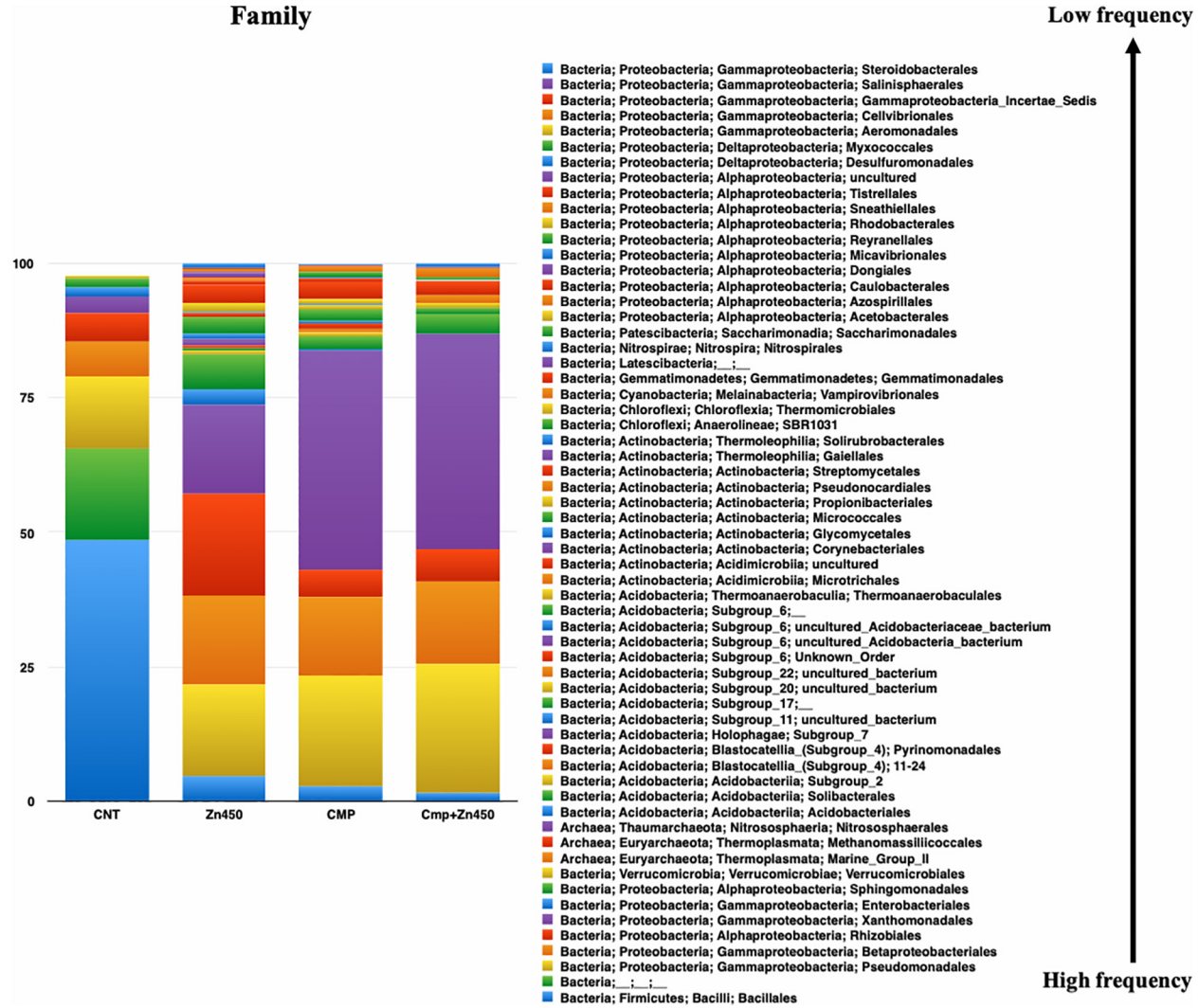

FIGURE 4 | Overview of the relative frequency of root microbiome with respect to family, under different experimental conditions.

the comparison between CMP and Zn450. In addition, each treatment significantly affected the Shannon indices ($p < 0.05$) when compared with CNT, with the exception of CMP. Although for this index, the comparison between CMP and Zn450 was not statistically significant (**Table 6**). Correlation analyses revealed that the Shannon index and N 12 sapling biomass were not statistically correlated ($r = 0.22$; $p < 0.05$). On the contrary, the Pearson coefficient highlighted that Shannon diversity and Zn addition were strongly negatively correlated ($r = -0.82$; $p < 0.005$). A similar result in terms of correlation was obtained estimating the correlation between Zn addition and Chao 1 ($r = -0.77$; $p < 0.005$), or Simpson index ($r = -0.77$; $p < 0.005$).

The b diversity was calculated in order to highlight the differences among the experimental groups using Dice metrics. The results (**Table 7**) highlighted that all the groups were well distinguished and the distance among them was statistically significant ($p \leq 0.1$).

Fungal community composition

The taxa bar plot was reported at three hierarchic levels: phylum, class and family. At phylum level (**Figure 6A**) Ascomycota, Basidiomycota, Mortierellomycota, Glomeromycota, and Chytridiomycota phyla characterized the rhizosphere fungal community. Ascomycota phylum was the dominant one, however, the CMP experimental group showed also a relative high frequencies of Basidiomycota and Mortierellomycota phyla respect to the other experimental groups.

At the class level (**Figure 6B**), the differences in relative frequency between CMP amended or unamended soil were highly evident. Zinc addition greater affected the fungal microbiome rhizosphere classes of CMP amended soils compared to those of unamended soil. In fact, only the relative frequency of the less abundant classes: Lecanoromycetes, Agaricomycetes and Leotiomycetes, belonging to the phylum of Ascomycota, were affected in the case of Zn450 and increased with respect

TABLE 5 | Venn table of the shared or unique OTUs among experimental groups.

Experimental theses	Number of shared OTUs	OTUs
CNT; Zn450; CMP;	6	Alphaproteobacteria
CMP + Zn450		Verrucomicrobiae
		Acidobacteria; Subgroup_6
		Actinobacteria
		Bacilli
		Gammaproteobacteria
CNT; Zn450; CMP + Zn450	1	Acidimicrobiia
Zn450; CMP;	2	Nitrospira
CMP + Zn450		Gemmatimonadetes
CNT; Zn450	1	Saccharimonadia
Zn450; CMP	4	Acidobacteriia
		Thermoplasmata
		Blastocatellia
		Thermoanaerobaculia
CMP; CMP + Zn450	1	Anaerolineae
Zn450	1	Holophagae
CMP	7	Acidobacteria; Subgroup_19
		Acidobacteria; Subgroup_18
		Deltaproteobacteria
		Acidobacteria; Subgroup_22
		Acidobacteria; Subgroup_24
		Acidobacteria; Subgroup_23
		Acidobacteria; Subgroup_17
CMP + Zn450	4	Acidobacteria; Subgroup_13
		Acidobacteria; Subgroup_12
		Thermoleophilia
		Acidobacteria; Subgroup_11

to the CNT. In CMP + Zn450, the Zn addition deeply modified the fungal rhizosphere community, mainly for the most abundant classes (e.g., Pezizomycetes, Sordariomycetes, and Dothideomycetes; CMP vs. CMP + Zn450), but also for the less represented ones (e.g., Agaricomycetes, Pezizomycetes, and Mortierellomycetes).

This trend was confirmed when the third hierarchic level was considered (**Figure 6C**). The most represented families in CNT were not affected by the addition of Zn (CNT vs. Zn450; e.g., Pezizales, Pleosporales, etc.) the less represented families slightly increased, as in the case of those belonging to Lecanoromycetes and Leotiomycetes (Helotiales). CMP amendment slightly augmented the frequency of some families less represented in the CNT group (CNT vs. CMP). On the contrary, when the fungal rhizosphere of CMP and CMP + Zn450 were considered a sensible modification of the class composition, due to Zn addition, was observed. In particular, the relative frequency of Mortierellales (Tremellomycetes) and Cantharellales (Agaricomycetes) was negatively affected.

Qualitative quantitative analysis of fungi rhizosphere community

The fungi community was investigated from qualitative and quantitative points of view, aiming to identify those fungal families that were specifically associated to the treatments of the different experimental groups. At the family level, the Venn analysis (**Table 8**) revealed that three, two, two, and five fungal families were specific to CNT, Zn450, CMP, and CMP + Zn450, respectively.

In particular, the CMP group was enriched by fungal families belonging to the Sordariomycetes and Rhizophydiales classes; whilst Agaricomycetes, Eurotiales, Atheliales, and Leotiomycetes were present when the CMP + Zn450 was considered. Interestingly, five families, Cantharellales, Mortierellales, Agaricales, Pezizales and Pleosporales, were present in all experimental theses.

A LEfSe analysis (**Figure 7**), as in the case of bacterial communities, was performed to identify hypothetic taxonomic markers (phyla, classes or families) of the specific analyzed experimental groups.

Only 47 root ASVs (LEfSe. $p < 0.05$, log10 LDA score > 3.5) were enriched among the different experimental groups. Dothideales and Pleosporales families (both belonging to the phylum of Ascomycota, class Dothideomycetes) can be considered as markers of the CNT; Rhizophydiales (phylum Chytridiomycota. class Rhizophydiomycetes), Mortierellales (phylum Mortierellomycota, class Mortierellomycetes), Cantharellales and Agaricales (both belonging to the phylum of Basidiomycota class Agaricomycetes) were markers of CMP, while Eurotiales (phylum Ascomycota, class Eurotiomycetes) was maker of CMP + Zn450. No one specific marker was identified for Zn450 group.

DISCUSSION

The N12 black poplar clone was previously selected as being highly tolerant of two heavy metals: Cu and Zn (Castiglione et al., 2009). Moreover, research conducted by us during the last 15 years has revealed its remarkable capacity for growth and phytostabilization in differentially contaminated soils (Cu, Fe, and Pb), and also for phytoextraction of Cd and Zn (Baldantoni et al., 2014).

The present study was conducted to explore the dynamics of the rhizosphere microbiome associated to N12 saplings in the context of HM phytoremediation. As expected, N12 poplar saplings grew normally under all experimental conditions. In fact, they had a normal biomass and showed no signs of toxicity in the presence of Zn. Moreover, they accumulated high amounts of the metal, confirming, once more, its particular suitability to cultivation on HM polluted soils. Based on the percentage and type of CMP added to the pot soil, the amendment exerted positive, albeit limited effects on the growth and biomass, mainly affecting the leaves, as well as on Zn removal from the soil, whilst they had no measurable effect on Zn soil bioavailability and its uptake into the plants.

Yields of different trees or crops, as well as plant biometric and morphometric parameters, can be improved as a result of soil amendments, as in the case of maize and barley (Hernández et al., 1991; Roca-Pérez et al., 2009; Lamari and Strelkov, 2010), sunflower (Moreno et al., 1997), wheat (Lamari and Strelkov,

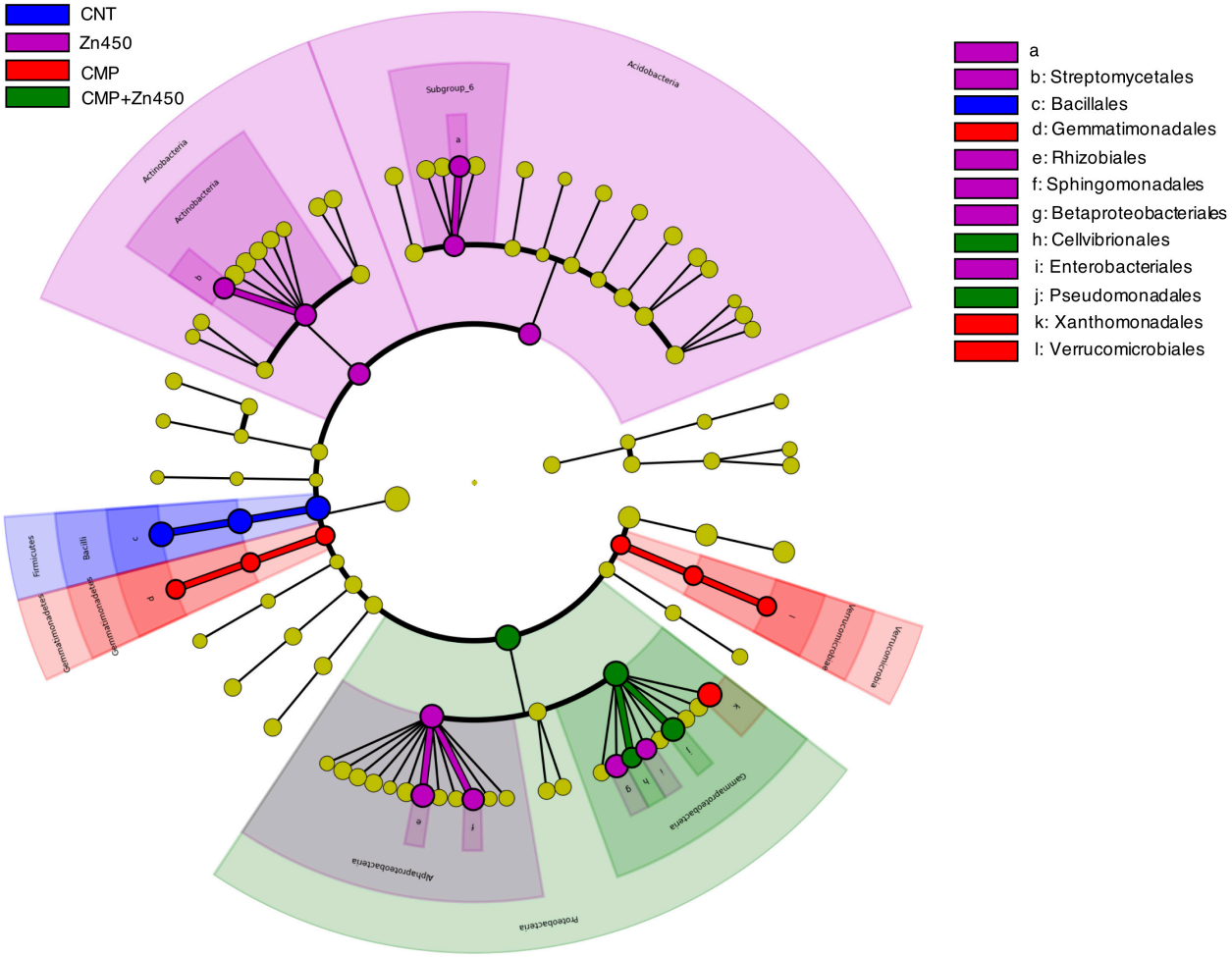

FIGURE 5 | Biomarkers of the rhizosphere microbiome of different experimental groups. LEfSe analysis was used to validate the statistical significance and the size effect of the differential abundances of the taxa of bacterial rhizosphere community of N12 poplar saplings (Kruskal–Wallis and Wilcoxon rank-sum $p < 0.05$ and LDA score > 3.5). In the cladogram, the taxonomic classification shows class, order, and family of the different experimental groups, while the genus is represented, with different letters, on the right side of the figure.

2010), fast growing trees (Madejón et al., 2016). It is known that CMPs of different origins, containing considerable amounts of nutrients, *humus* and microorganisms (Farrell et al., 2010), have beneficial effects on metabolism of soil biota, on dynamic of nutrient uptake and on physical soil properties, as well as on the enzymatic activity involved in phosphorus and nitrogen cycles, that enhance plant growth and productivity (Semida et al., 2014). Some studies (Sotta et al., 2019) suggest that Zn alone interferes with leaf morphology, and, in general, retardation in growth rate or biomass production, as well as the effects on plant morphology, might be due to interference of HMs with the processes of plant mineral uptake (Kidd et al., 2017; Kushwaha et al., 2018).

In our study, CMP alone, or in combination with Zn, had a positive and significant effect on the morphological parameters analyzed, especially on those of leaves. In fact, the increase of biomass and leaf expansion higher than CNT, is a clear indication that CMP may be protective with regard to Zn uptake and toxicity.

Both the CNT and CMP soils used in the experiment did not show evident differences in pH and nutrient content (e.g., Zn total or bioavailable fractions). The CMP supplied can significantly modify physical and chemical properties of the soil of CMP group, and in fact it slightly increased organic matter content and CEC respect to CNT. However, the amendment had no effect on Zn bioavailability and uptake by the poplar saplings. On the contrary, it favored Zn soil stabilization. The hazard of inorganic contaminants, such as metals and metalloids, arises from their absorption and accumulation in the cells. In fact, although Zn is an essential micronutrient for all living organisms, an excess of it can be toxic (Sharma et al., 2013).

Zn mobility in soil may be altered by the presence of some oxidized forms of Fe, Ca, Al, Mn, and P, and also by organic matter. Soil organic amendments can reduce solubility and mobility of metals, immobilizing them and/or decreasing their leaching (Huang and Salt, 2016). Various sorption processes, adsorption to mineral surfaces, formation of stable

TABLE 6 | Kruskal-Wallis pairwise comparison of two α diversity indices **(A)** 1256 Shannon, **(B)** Simpson, and **(C)** Observed Otus among the experimental groups.

(A) Shannon index

Thesis 1	Thesis 2	*p*-value
CNT (*n* = 3)	CMP (*n* = 3)	0.512
CNT (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CNT (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	Zn450 (*n* = 3)	0.512
CMP + Zn450 (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**

(B) Simpson index

Thesis 1	Thesis 2	*p*-value
CNT (*n* = 3)	CMP (*n* = 3)	0.275
CNT (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CNT (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	Zn450 (*n* = 3)	0.512
CMP + Zn450 (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**

(C) Observed OTUs index

Thesis 1	Thesis 2	*p*-value
CNT (*n* = 3)	CMP (*n* = 3)	0.275
CNT (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CNT (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	**CMP + Zn450 (*n* = 3)**	**0.049**
CMP (*n* = 3)	Zn450 (*n* = 3)	0.512
CMP + Zn450 (*n* = 3)	**Zn450 (*n* = 3)**	**0.049**

In bold are indicated the statistically significant differences ($p < 0.05$).

complexes with organic ligands, surface precipitation and ion exchange, co-precipitations can contribute to reduced mobility of the contaminants (Colugnati et al., 1995). Moreover, these sorption/dissolution processes are influenced by pH, CEC, redox potential, soil constituents, and in general, a single mechanism does not explain the immobilization of elements into the soil matrix (Kabata-Pendias, 2000).

However, the supply organic amendments to the soil can modify CEC, which, in turn, improves soil trace and oligo element (e.g., Zn) retention. Some studies have revealed that CMP, applied alone or in combination with others (e.g., biochar), increased the CEC of the soil due to the input of stabilized organic matter, which is rich in functional groups (such as carboxylic and phenolic acid groups) released into the soil exchange sites (Ouédraogo et al., 2001; Yang et al., 2019). Paradelo et al. (2011) analyzed in detail metallic chelates of the soil, observing that organic matter can play a key role in the immobilization of trace elements by forming stable compounds with them. Other studies have revealed that pH increased after the CMP soil addition, with a consequent reduction of trace element bioavailability (Pérez-De-Mora et al., 2006; Baker et al., 2011; Zhou et al., 2011; Strachel et al., 2017). However, the significance of the effects on metal bioavailability might be due to the type and amount of CMP added. Taiwo et al. (2016) observed that the higher the CMP dose added to the soil, lower was the Zn bioavailability.

In our study, we found that N12 saplings were able to accumulate Zn in all of the organs analyzed (roots, stems and leaves): the highest concentration of Zn was observed in the roots followed by the stems and leaves. The binding of contaminants at root cell walls, or their accumulation and storage into the vacuole, are recognized avoidance mechanisms for trace metals in plants (Krzesłowska, 2011). Moreover, in our study, Zn uptake decreased with the addition of CMP, mainly in leaves, which, in turn, increased their size and biomass. In general, we found that the addition of both Zn and CMP to the soil did not affect Zn accumulation, probably because CMP is able to decrease Zn availability throughout bio-accumulation and bio-adsorption processes.

Our results are consistent with previous findings where the application of CMP did not increase Zn concentrations in the plant organs or it decreased it slightly (Zhou et al., 2017). Soares et al. (2019) reported that broad bean plants accumulated increasing levels of Zn in the treatments with low or moderate CMP addition to the soils; in contrast, its content decreased significantly in the different organs of the plants with higher soil CMP additions. The authors postulated that this phenomenon might be caused by the lower available Zn content into the soil due to CMP sequestration.

Although it is known that the *P. nigra* N12 clone is suitable for Zn phytoextraction (Castiglione et al., 2009; Baldantoni et al., 2014), there is no information on the rhizosphere microbiome responses to Zn addition and/or CMP amendment of soil, or the role of microbiome in the phytoremediation processes of this black poplar clone. In order to shed light on these processes and to assess variations caused by Zn soil

TABLE 7 | The pairwise PERMANOVA tests whether Dice distances between samples within the same experimental group are more similar to each other than they are to samples from the other groups.

Thesis 1	Thesis 2	Sample size	Permutations	pseudo-F	*p*-value	*q*-value
CNT	**CMP**	**6**	**999**	**19.09**	**0.101**	**0.109**
CNT	**CMP + Zn450**	**6**	**999**	**27.55**	**0.102**	**0.109**
CNT	**Zn450**	**6**	**999**	**19.17**	**0.090**	**0.109**
CMP	**CMP + Zn450**	**6**	**999**	**42.28**	**0.097**	**0.109**
CMP	**Zn450**	**6**	**999**	**42.29**	**0.109**	**0.109**
CMP + Zn450	**Zn450**	**6**	**999**	**108.03**	**0.101**	**0.109**

Statistically significant values are indicated in bold ($p \leq 0.1$).

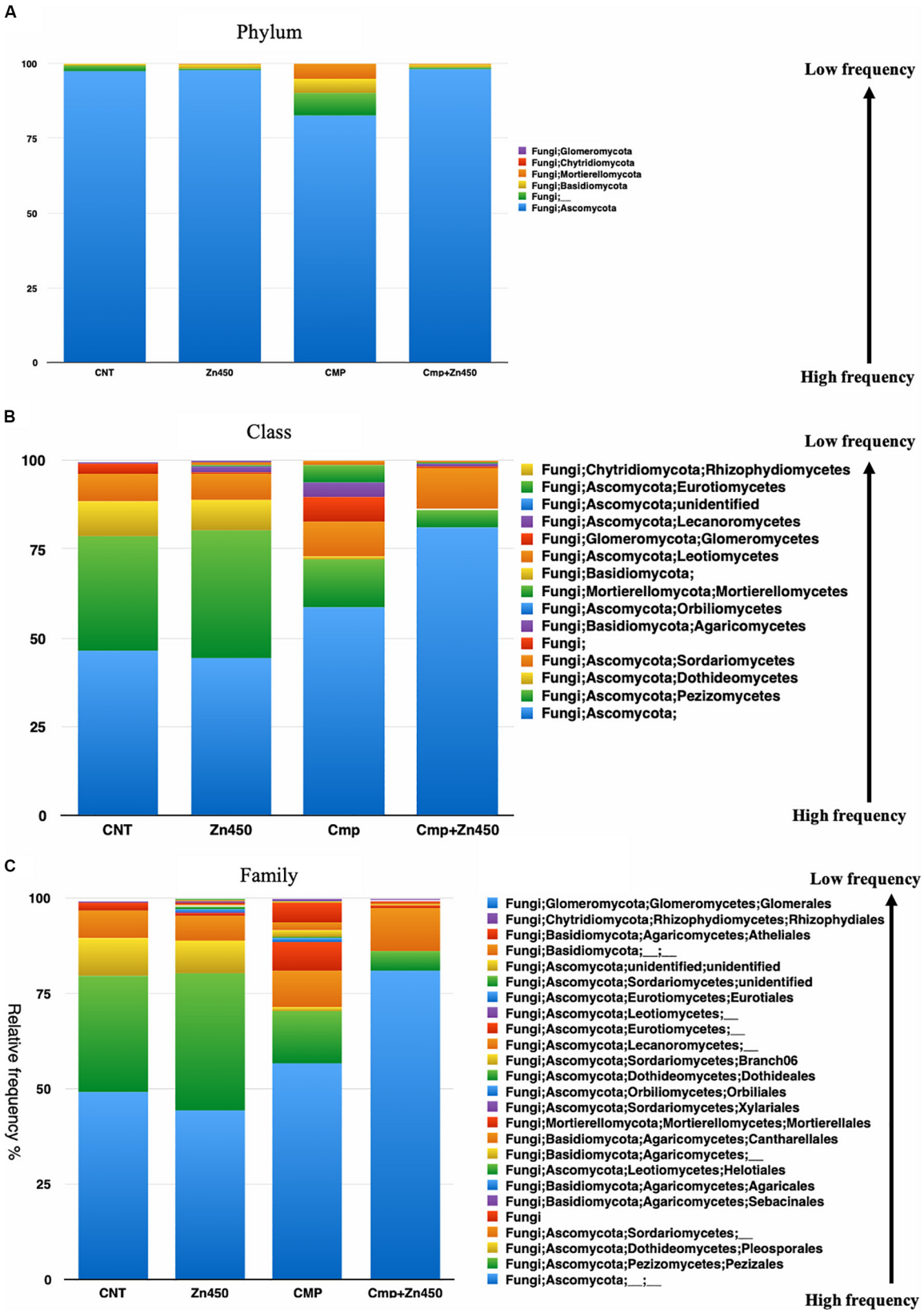

FIGURE 6 | Overview of the relative frequency of root surface fungi with respect to **(A)** phylum, **(B)** class or **(C)** family under different experimental groups.

TABLE 8 | Venn table of the shared or unique OTUs among groups.

Experimental groups	Number of shared OTUs	OTUs
CNT; ZN450; CMP; CMP + ZN450	5	Cantharellales Mortierellales Agaricales Pezizales Pleosporales
CNT; ZN450; CMP	5	Glomerales Leotiomycetes;__ Sebacinales Helotiales Agaricomycetes;__
CNT; ZN450; CMP + ZN450	1	Xylariales
CNT; ZN450	1	Sordariomycetes;__
CNT; CMP	2	Dothideales Orbiliales
ZN450; CMP	1	Lecanoromycetes;_
CMP CMP + ZN450	1	Sordariomycetes;_
CNT	3	Sordariomycetes; Branch07 Lecanoromycetes;__ Eurotiomycetes
ZN450	2	Sordariomycetes; Branch11 Sordariomycetes; unidentified
CMP	2	Sordariomycetes; Branch15 Rhizophydiales
CMP + ZN450	5	Agaricomycetes;_ Eurotiales Atheliales Leotiomycetes;_ Sordariomycetes; Branch19

addition and/or CMP amendment, we analyzed the rhizosphere microbiome of N12 saplings because it may contribute to plant growth, health (Richter-Heitmann et al., 2016) and metal tolerance. Our interest comes from several studies which have clearly established that the rhizosphere corresponds to the plant-soil compartment harboring the highest microbe richness diversity (Guo et al., 2019; Latini et al., 2019; Brereton et al., 2020). Soil rhizospheres are very complex matrices in relation to their bacterial and fungal communities, which can be readily analyzed in terms of diversity and composition, by deep sequencing (Burges et al., 2017; Fan et al., 2018). Furthermore, species richness diversity and their abundance levels may be inferred by the α-diversity indexes. Our experiment showed that the microbiome assembly during phytoremediation was positively influenced not only by Zn contamination, but also by CMP soil amendment. Furthermore, the CMP rhizosphere microbiome showed the highest α-diversity, followed by CMP + Zn450, Zn and CNT. We also detected a slight alteration of microbial diversity by Zn addition and an increase attributable to CMP amendment. Firmicutes, Proteobacteria, Acidobacteria and Actinobacteria were the dominant phyla of the rhizosphere communities of all N12 poplar saplings, although some differences were observed among the different groups.

Firmicutes and Proteobacteria and, to lesser extent, Actinobacteria and Acidobacteria dominated the CNT rhizobacterial microbiome. Firmicutes phylum was predominant in CNT, and the species, belonging to it, have been widely studied and recognized as "plant-beneficial" bacteria (Mendes et al., 2013). LEfSe analysis, which was mainly used to identify significantly enriched bacterial taxa among all analyzed microbiome rhizospheres, confirmed, once more, that the Bacillales family is potential biomarker of the CNT. Whereas the presence of Proteobacteria, which has been considered to be an indicator of nutrient-rich soils, indicated that a good quality and rich soil was used here.

Information about the effects of Zn pollution on the rhizobacteria communities of the poplar is very few. In general, HMs have a toxic impact on microbe communities when their content is high in the soil. In fact, it has been reported that a high soil metal contamination reduces the species number of microbes, and also bacterial and fungal diversity, and this it was particularly true for bulk soils (Rajapaksha et al., 2004; Stefanowicz et al., 2008; Azarbad et al., 2013).

Root secretions can significantly modulate bioavailability of metals in the soil, including their concentration and toxicity, and, consequently, affect the microbial communities of the rhizosphere (Chen et al., 2003; Zhou et al., 2011). In our study, a detrimental effect of Zn addition was not observed at the whole bacterial community level, however its addition to the soil resulted in a differential abundance of particular taxa. Compared to the CNT rhizosphere microbiome, Proteobacteria, Acidobacteria and Actinobacteria phyla increased and that of Patescibacteria became apparent. Several genomic and metagenomic studies have shown that members of the Patescibacteria "superphylum" showed reduced metabolic capabilities that likely limit their cultivation (Kantor et al., 2013; Rinke et al., 2013). This superphylum is also involved in hydrogen production, sulfur cycling (Wrighton et al., 2012; Kantor et al., 2013), and anaerobic methane oxidation (Peura et al., 2012), and it is responsible for the removal of conventional contaminants from the soil, including HMs and/or antibiotics (Wang et al., 2018; Yang et al., 2019).

Substantial changes were observed in the rhizobacterial community structure of N12 poplar saplings when grown on soils amended with CMP. Our results were consistent with other studies that have reported the effect of CMP on soil biodiversity (Hartmann et al., 2015; Ye and Tang, 2016), and its alteration during the phytoextraction process. Organic amendments can modify soil physico-chemical properties and provide nutrients and vast amounts of microorganisms, the composition of which largely depends on the source material (Sun et al., 2015; Yang et al., 2019). Thus they can create conditions that are favorable for the growth of microorganisms that are beneficial to plants, while simultaneously inhibiting others (Husson, 2013; Azarbad et al., 2015; Ye and Tang, 2016). The Proteobacteria, Acidobacteria and Actinobacteria phyla displayed greater abundance in the N12 rhizosphere microbiome of CMP amended soils compared to CNT. The abundance of these phyla might be explained by the

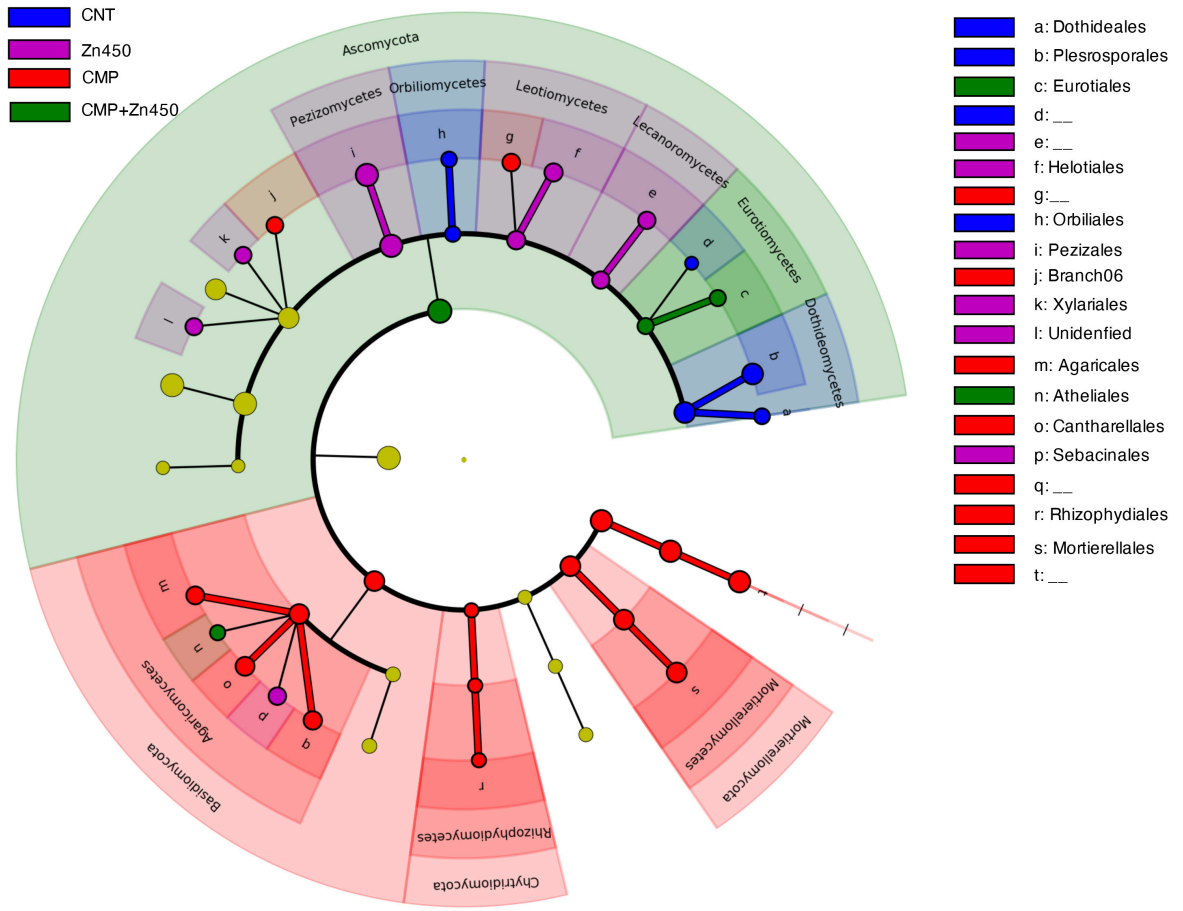

FIGURE 7 | Biomarkers of the rhizosphere microbiome of the different experimental groups. LEfSe analysis was used to validate the statistical significance and the size effect of the differential abundances of the taxa of fungal rhizosphere community of N12 poplar saplings (Kruskal–Wallis and Wilcoxon rank-sum $p < 0.05$ and LDA score > 3.5). In the cladogram, the taxonomic classification shows class, order, and family of the different experimental groups, while the genus is represented, with different letters, on the right side of the figure.

increase in soil carbon content due to the addition of organic matter. According to the trophic life histories of soil bacteria, these phyla include "copiotrophic" bacteria (r-strategists), which use labile carbon for their metabolism and growth, and this allows them to grow faster in nutrient-rich environments (Fierer et al., 2007; Trivedi et al., 2013; Deng et al., 2020).

Although the LEfSe analysis indicated that Gemmatimonadales and Verrucomicrobiales, or Pseudomonadales and Cellvibrionales are biomarkers of CMP and CMP + Zn450, respectively, all rhizobacterial communities of N12 poplar saplings grown on CMP amended soils were quite similar when Zn was added to the soil. Therefore, this suggests that Zn addition changes only weakly the structure of the microbial community when CMP is added to the soil. At phylum level, the CMP + Zn450 rhizosphere was characterized by the presence of Chloroflexi, previously identified as green non-sulfur bacteria, that includes a relatively understudied bacterial phylum with diversified metabolism and, in some cases, with a strong resistance to HMs (Gremion et al., 2003; Azarbad et al., 2015). Bacterial taxa belonging to this phylum were reported to be prevalent in nutrient poor soils (Will et al., 2010), in oligotrophic ecosystems, such as soils of high-elevation regions where vegetation is patchy, or decreases in the presence of high levels of nitrogen (Ding et al., 2013). In our study, the relative abundance of Chloroflexi increased in Zn450 + CMP soil, confirming, to some extent, the results reported for the rhizosphere of *Elsholtzia splendens* Nakai, a Cu-tolerant plant native to China, where a relationship between the abundance of Chloroflexi and a higher level of carbon source was detected in the rhizosphere soils, supporting its sensitivity to soil nutrient content (Jiang et al., 2016).

Correlation analysis was performed between microbial biodiversity indices and Zn content in the different organs of N12 poplar saplings, revealing that biodiversity was significantly correlated with the metal content in roots and stems, the two main accumulating organs, and even with the leaf area. This suggests that greater bacterial diversity stimulates Zn absorption by changing the physico-chemical characteristics of the rhizosphere, and, at the same time, impacting nutrient uptake and, consequently, plant growth.

Most of our current knowledge on rhizomicrobial diversity relates to bacterial communities, while information on fungal communities is quite scarce (Cicatelli et al., 2019). Studying and understanding fungal communities is of paramount importance since fungi comprise a major portion of the biomass and biodiversity of the Earth soil. In fact, fungi play crucial roles in maintaining soil processes which affect the functioning of the largest part of the ecosystems (Narendrula-Kotha and Nkongolo, 2017). For this reason, we also investigated the effects of Zn addition on the fungal rhizosphere communities of the N12 poplar saplings grown on CMP amended soil. Although the α-diversity of the rhizobacterial community increased in all experimental settings, compared to the CNT, in the case of the fungal community the trend was the opposite, with diversity being significantly reduced in CMP, Zn450 and CMP + Zn450 soils. The α-diversity of fungal rhizosphere was negatively correlated with the Zn content in the soils of N12 poplar saplings; in fact, this occurred in both Zn450 and Zn450 + CMP soils. This suggest that Zn soil addition selected only metal tolerant fungal communities (lower biodiversity), which, in turn, would improve the capacity of the plant to take up and accumulate Zn and counteract its negative effects. Contrary to what we observed, Kamal et al. (2010) found that species richness and diversity, represented by the Shannon–Wiener index, increased at moderate levels of soil pollution, while it was reduced at the higher ones, simulating in this way a homeostatic response. The increment of fungal biodiversity at HM moderate concentrations could also be a stress response, whereby fungal ecotypes better adapted to unpolluted soil, allow other fungi (probably less competitive in unstressed soils but better adapted to heavy metals) to colonize the roots and complete their life cycles. Few studies have reported the negative effects of metals on fungal growth and reproduction (Azevedo and Cássio, 2010; Goupil et al., 2015). These studies showed that metal toxicity varies on the basis of the fungal species, type of metal and its concentration, nutrient availability, and plant species diversity.

In our study the presence of Zn did not affect the more represented classes and families, however, Zn addition reduced negatively affected the number of the less represented OTUs represented in CNT and CMP. Several studies revealed that the fungal rhizosphere microbiome can sustain plants during their life cycles, especially in stressful conditions, through mechanisms such as chelation with organic ligands, transportation out of the cells, and biotransformation of the ions to less bioavailable or less toxic metal species. Vascular plants host a great variety of fungi in all of their organs. In addition, being susceptible to soil-borne pathogens, plant roots are also colonized by non-pathogenic or mutualistic fungi such as endomycorrhizal fungi (AMF), ectomycorrhizal fungi (EMF), and dark septate endophytes (DSE). The AM fungi comprise about 150 species of zygomycetous fungi, and EM fungi include about 6.000 species that are primarily Basidiomycetes, along with a few Ascomycetes and Zygomycetes (Saikkonen et al., 1998).

Differently to that observed in the case of rhizobacteria community, the CNT fungal rhizosphere community was not modified by soil Zn addition (Zn450). For all of the other experimental groups, Ascomycota and Basidiomycota phyla were abundantly represented (about 95%). Only the less represented classes of CNT, such as Lecanoromycetes, Agaricomycetes and Leotiomycetes, belonging to the phylum of Ascomycota, increased in the case of Zn450. Fungi, especially those belonging to Ascomycetes and Basidiomycetes, are able to degrade very complex organic compounds including cellulose and lignin, but many of them also live as root symbionts (mycorrhizas) and obtain simple sugars from their plant partners (van der Heijden et al., 2015). The range of Zn toxic concentrations among fungi is highly variable (generally from 10 up to 500 mg L^{-1}) depending on the species, strains, or even the type of growth media (de Oliveira and Tibbett, 2018). Høiland (1995) tested metal toxicity in Basidiomycota, and found Cd to be very toxic, whilst Zn affected only moderately this genus. The large part of the fungal taxa identified in our study was unaffected by Zn soil addition. A greater effect on the fungal rhizosphere microbiome was due to the CMP amendment. Wu et al. (2019), found that the same three fungal phyla (Basidiomycota, Ascomycota, and Mortierellomycota), which predominated in CNT, Zn450, CMP, and CMP + Zn450 groups, were not significantly affected by CMP, except for a slight increase in abundance of Ascomycota.

The fungal community composition of CNT and CMP differed in relation to four classes: Pezizomycetes, Dothideomycetes, Agaricomycetes, and Mortierellomycetes. The CMP reduced the relative frequencies of the first two classes (both belonging to Ascomycota phylum), whilst it increased in the case of Agaricomycetes (Basidiomycota phylum) and Mortierellomycetes (Mortierellomycota phylum). Furthermore, these four classes were also the ones that were more sensitive to Zn addition in the case of CMP + Zn450. In fact, when CMP + Zn450 was compared to CMP, the relative frequency of Agaricomycetes and Mortierellomycetes, which are characteristic of the CMP, was reduced by the Zn addition. These differences in the relative abundance of specific OTUs, due to the different ecologic driven force (CMP and/or Zn), were also investigate trough LEfSe analyses in order to identify specific markers in each experimental group. The CNT fungal rhizosphere microbiome was characterized by the presence of Dothideales and Pleosporales families (both belonging to the phylum of Ascomycota, class Dothideomycetes), whilst CMP was characterized by the presence of Rhizophydiales (phylum Chytridiomycota, class Rhizophydiomycetes), Mortierellales (phylum Mortierellomycota, class Mortierellomycetes), Cantharellales and Agaricales (both belonging to the phylum of Basidiomycota class Agaricomycetes). In particular, Eurotiales family (phylum Ascomycot, class Eurotiomycetes) was identified as maker for CMP + Zn450. This family was recognized as a taxon able to tolerate organic pollution; in fact, Harcourt and State (2014) observed that Eurotiales family (Ascomycota phylum) was able to thrive in extreme environments, such as those polluted by crude oil. It is noteworthy, that in a few studies of the ability of fungi to grow in polluted environments, Ascomycetes are highly represented compared to Basidiomycetes (Kozdrój and Van Elsas, 2000; Obire and Anyanwu, 2009; Thion et al., 2012). Hartikainen et al. (2012) also recognized that Basidiomycetes tolerated

better lower Zn concentrations than Ascomycetes and Zygomycetes, whilst the opposite was true at higher Zn concentrations. This finding indicates that changes in fungal communities occur when different amounts of Zn are added to the soil and they are also directly related to its increasing concentrations of the metal (Hartikainen et al., 2012). In our study, as expected, soil CMP addition modified the fungal communities and increased the biomass of the saplings especially of leaves, however, this improvement was not positively correlated with fungal diversity of the fungal rhizosphere microbiome, but, probably, related to the input of organic matter. In contrast, Zn addition negatively affected the fungal rhizosphere microbiome in terms of biodiversity; this was expected given the selective pressure exerted by high Zn doses added to the soils in our experimental groups.

CONCLUSION

Phytoremediation is one of the most widely studied green technologies for soil and water reclamation. In the last 20 years, many studies have focused on the identification of the best plant species to accumulate, extract, degrade or volatilize, inorganic or organic contaminants in specific pedoclimatic conditions. However, although these studies have improved knowledge and yielded interesting results, a thorough understanding of the interactions between plants and soil/rhizosphere microorganisms, and of roles that microbes play in phytoremediation processes, is still far away. In addition, soil amendment with CMP represents both a source of organic matter and of microorganisms, both of which are useful to improve the physico-chemical soil properties (often impoverished by metal pollution) and also increase the biodiversity. The resistance and resilience of soil microbiomes in turn can improve plant tolerance to contaminants and promote their growth. In this study, CMP amendment was able to improve biomass of N12 poplar saplings, in particular that of leaves, and, mostly, it counterbalanced the negative effects of Zn soil addition on their biomass. A greater biomass due to CMP amendment allowed a lower Zn accumulation in the different plant organs preserving plant health and, at the same time, improving the total Zn content in the N12 poplar saplings. Our experimental results demonstrated that CMP amendment induced different effects on bacterial and fungal rhizosphere microbiome; in fact, in the case of bacteria, CMP amendment deeply affected the rhizosphere microbiome, which in turn was more resilient to the presence of Zn. In contrast, in the case of fungal rhizosphere microbiome, CMP amendment strongly modified the community structure. Moreover, the Zn addition mainly affected the fungal rhizosphere microbiome of saplings grown on CMP amended soil. Our study confirmed that the rhizosphere microbiome is characterized by a complex network of relationships through which plants obtain several advantages. In our opinion, further studies on phytoremediation should be focused on these relationships with the goal of understand how these networks may improve phytoremediation effectiveness and opening the route to phytoremediation phase 2.0.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/metagenomics/, PRJEB39028 and https://www.ebi.ac.uk/metagenomics/, PRJEB39025.

AUTHOR CONTRIBUTIONS

FG, AC, and SC: conceptualization. FG and AC: formal analysis, investigation, methodology, and validation. AC, SC, and MT: funding acquisition. AC: supervision. All authors: writing – original draft, review, and editing.

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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.2020.01677/full#supplementary-material

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frontiers in Microbiology

ORIGINAL RESEARCH
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Response of Soil Fungal Community to Drought-Resistant *Ea-DREB2B* Transgenic Sugarcane

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Drought limits crop productivity, especially of sugarcane, which is predominantly grown in the subtropical parts of China. Soil microbes perform a wide range of functions that are important for plant productivity and responses to drought stress, and fungi play an important role in plant–soil interactions. The *Ea-DREB2B* gene of sugarcane, *Saccharum arundinaceum*, is involved in regulating the response to drought stress. In this study, fungal communities of the transgenic (TG) sugarcane variety GN18, harboring the drought-tolerant gene *Ea-DREB2B* and its corresponding non-TG wild-type (WT) variety, FN95-1702, were investigated in three soil compartments (rhizoplane, rhizosphere, and bulk soil) by assessing the internal transcribed spacer region using Illumina MiSeq. As the soil microbial community is also affected by various environmental factors, such as pH, carbon availability, and soil moisture, we determined the total carbon (TC), total nitrogen (TN), and total phosphorus (TP) contents in the rhizoplane, rhizosphere, and bulk soil compartments to explore the associations between soil fungal communities and host plant characteristics. The differences between the soil fungal communities of TG and WT plants were detected. The alpha diversity of TG fungal communities was more correlated to environmental factors than the beta diversity. The abundance of operational taxonomic units (OTUs) enriched in TG root-related area was far more than that in the root-related area of WT plants. Thereinto, more saprotrophs were enriched in the TG root-related area, indicating altered niches of fungal guilds around TG roots. These results revealed that host plant genotype did play a key role for strengthening plant–fungi interaction and enhancing beneficial fungal function in the root-related area (rhizoplane and rhizosphere) of TG sugarcane in order to respond to drought stress.

Keywords: drought resistance, *Ea-DREB2B*, transgenic sugarcane, fungal community, environmental factor

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INTRODUCTION

Drought is a major constraint for plant growth and agricultural productivity in many parts of the world. There are several aspects of the plant response to drought, such as sensing stress, activating systemic signaling pathways, and genetically regulating the responses (Zolla et al., 2013). Many genes involved in plant responses to drought have been identified, and some of these have been

effectively used to improve drought tolerance (Gosti et al., 1995; Iuchi et al., 2001; Li et al., 2017) by developing new drought-tolerant crop varieties through genetic modifications to increase crop productivity and reduce costs (Romão-Dumaresq et al., 2016).

Soil microbes have a wide range of functions that are important for plant productivity, such as cycling nutrients, inducing disease resistance, and responding to environmental stresses, including drought and salinity (Zolla et al., 2013). Soil fungi play important roles in ecosystem nutrient cycling and as mutualists and pathogens of host plants (Yang et al., 2017). The fungi colonized in endosphere, rhizoplane, and rhizosphere of plant have particularly important feedback effects on the responses of their host plants to climate change (Gehring et al., 2017). Several studies have documented variations in fungal communities among different plant genotypes (Dunfield and Germida, 2004; Hunter et al., 2015). Sugarcane, as a significant resource of sugar and ethanol, has high requirements for irrigation and is very sensitive to water shortage (Ferreira et al., 2017); therefore, genetic engineering has been applied to enhance its drought resistance (Ramiro et al., 2016).

The genes encoding dehydration-responsive element-binding (DREB) transcription factors recognized in *Arabidopsis thaliana* have been reported to enhance drought tolerance in genetically modified plants (Mizoi et al., 2012). *Ea-DREB2B* is a member of the DREB family and cloned from the hardy sugarcane *Saccharum arundinaceum*. It regulates the expression of a few stress-inducible genes and plays a crucial role in promoting plant resistance to drought and salinity (Lata and Prasad, 2011; Augustine et al., 2015). The transgenic (TG) sugarcane (GN18) harboring *Ea-DREB2B* was obtained by genetic transformation using a gene gun using inducible promoter RD29A and sugarcane variety FN95-1702 as the receptor material. Xu et al. (2018) reported that TG sugarcane (GN18) has significantly better drought resistance than non-TG sugarcane. Abscisic acid (ABA) is a stress-responsive hormone that plays important roles in drought sensing and responses (Vargas et al., 2014). The CBF/DREB regulon is activated by the ABA-independent pathway (Saibo et al., 2009), and SlDREB3 expression in tomatoes influences several ABA-related processes by reducing ABA levels and responses, hence increasing photosynthesis (Upadhyay et al., 2017). Worchel et al. (2013) reported that the plant photosynthetic pathway affects arbuscular mycorrhizal (AM) fungal–plant symbiosis. Further, fungal community structure and diversity are affected by many environmental variables (Yang et al., 2017). Although DREB2s are known to contribute greatly to enhance drought and salinity tolerance in plants (Chen et al., 2009; Matsukura et al., 2010; Li et al., 2017), few studies have focused on the fungal communities in the soil of TG plants harboring DREBs (Yu et al., 2013).

Therefore, in the present study, we examined the effects of TG sugarcane harboring *Ea-DREB2B* on fungal communities in three soil compartments (rhizoplane, rhizosphere, and bulk soil). The aims of this study were as follows: (1) to determine the alteration in the diversity and composition of fungal communities in the rhizoplane, rhizosphere, and bulk soil around TG and non-TG wild-type (WT) plants; (2) to explore the associations between fungal community diversity and environmental factors (C, N, and P levels); and (3) to elucidate the complex interactions among fungi, plants, and the soil environment. These results can provide insights into the potential effects of using genetic modification to improve plant stress resistance in the context of a broader ecosystem, thereby offering guidance for burgeoning new genetically modified varieties of sugarcane.

MATERIALS AND METHODS

Study Site

This study was conducted in the forage-growing area of Guangxi University, in Quli, Fusui, Chongzuo, China (between 107°31′ and 108°06′E, and 22°17′ and 22°57′N), in the summer of 2018. The average temperature during the study period was 21.3°C, and the total annual precipitation for the entire region was 1,050–1,300 mm (**Supplementary Figure S1**). The fields, which have been cultivated long term with sugarcane, had the following soil properties: lateritic red earth, pH of 5.15, 19.47 g/kg of organic matter, 0.84 g/kg of total N (TN), 2.98 g/kg of total P (TP), 7.11 g/kg of total K, 136 mg/kg of alkaline-hydrolyzed N, 83 mg/kg of available P, and 77.1 mg/kg of available K. We compared the properties and the fungal community diversity of the soil around the roots of the TG sugarcane variety GN18 with those of the WT variety FN95-1702. Xu et al. (2018) derived GN18 using FN95-1702 as the acceptor parent with the inducible promoter RD29A and a gene gun to achieve overexpression of *Ea-DREB2B* to improve drought resistance. Our experiment consisted of a random block design with six blocks containing both sugarcane varieties. In this experiment, random block design was adopted, with a total of six blocks, each with an area of 30 × 4.2 m. Each block contained two sugarcane varieties (three rows per plant). The distance between the two varieties was 2.1 m, and the distance between any two sugarcanes was 30 cm, with 46 sugarcane planted in each row (Zhao et al., 2020).

Soil Sample Collection and Physicochemical Analysis

Sampling was conducted at the late jointing stage on November 18, 2018. Bulk soil was collected from five sampling sites between two lines of sugarcane with a standard soil corer each block using five-point sampling method. We excavated 12 sugarcane plants (six plants each variety) showing similar characteristics each block for extracting soil samples of rhizoplane and rhizosphere, and each replicate was mixed with soil collected from 12 sugarcane roots, which were excavated from two blocks through random selection in six blocks (Zhao et al., 2020). Rhizoplane soil sampled from the plant root surface was removed by sonication for 5 min (Edwards et al., 2015). Rhizosphere soil samples were separated by vortexing the roots for 20 s. Each composite soil sample was homogenized and stored at −80°C for less than 24 h before DNA extraction. We divided each soil sample into three technical replicates of 0.5 g each for physicochemical analysis. Total carbon (TC) (Batjes, 1996) and

TP (Sommers and Nelson, 1972) contents were measured as previously described, and TN content was measured using the Kjeldahl method (Bremner and Tabatabai, 1972).

DNA Extraction, Polymerase Chain Reaction, and Illumina Sequencing

Microbial DNA was extracted for each soil sample (3 replicates × 1 g) using the E.Z.N.A Soil DNA kit (Omega Bio-tek, Inc., Norcross, GA, United States) following the manufacturer's instructions. The fungal internal transcribed spacer-2 (ITS-2) region was amplified from each sample using the primers ITS3F (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4R (5′-TCCTCCGCTTATTGATATGC-3′) (Tj et al., 1990). Primers were synthesized by Invitrogen (Invitrogen, Carlsbad, CA, United States). PCRs, containing 25 μl of 2 × Premix Taq (Takara Biotechnology, Dalian Co., Ltd., China), 1 μl of each primer (10 mM), and 3 μl of DNA (20 ng/μl) template in a volume of 50 μl, were amplified by thermocycling: 5 min at 94°C for initialization; 30 cycles of 30-s denaturation at 94°C, 30 s annealing at 52°C, and 30- s extension at 72°C; followed by 10 min of final elongation at 72°C. The PCR instrument was BioRad S1000 (Bio-Rad Laboratory, CA, United States). The PCR was conducted using the S1000 Thermal Cycler (Bio-Rad) with initial denaturation for 5 min at 94°C, followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, and 72°C for 10 min. The PCR products were sequenced by Magigene Technology (Guangzhou, China) using an Illumina HiSeq 2500 platform. Sequences analyses were performed by usearch software (V10[1]). Sequences with ≥ 97% similarity were assigned to the same OUT. Sequences were assigned to each sample based on their unique barcode and primer using Mothur software (V1.35.1[2]), after which the barcodes and primers were removed and got the effective Clean Tags. The ITS-2 sequences obtained in this study have been deposited in the National Center for Biotechnology Information Sequence Read Archive (SRA) database with accession number SRP257722.

Statistical and Bioinformatics Analysis

Alpha diversity was estimated from the richness, Shannon diversity, and evenness of the fungal operational taxonomic units (fOTUs). Correlations between alpha diversity and soil properties were determined using the "corrplot" package (Wei et al., 2017) in R v. 3.6.3. Changes in the relative abundance of fungal communities in each compartment were evaluated using the "alluvial" and "ggplot" packages in R v. 3.6.3 (Wickham and Wickham, 2007). Distance-based redundancy analysis (dbRDA) was used to evaluate the relationships between soil characteristics and soil fOTUs. Mantel test was used to study the relationship between alpha diversity and environmental factors and between the enriched fOTUs and environmental factors. Mantel tests, principal coordinate analysis (PCoA), and dbRDA were performed using the "vegan" package in R v. 3.6.3 (Oksanen et al., 2013). All statistical analyses (analysis of variance and Tukey's *post hoc* test) and Spearman's rank correlations between phylum abundance and soil properties were performed using SPSS v. 22.0 (SPSS Inc., Chicago, IL, United States). The effect of soil compartments on fOTU abundance was investigated using edgeR (Robinson et al., 2010) on trimmed mean of M-values (TMM)-normalized data (Robinson and Oshlack, 2010) and visualized using ternary plots. TMM-normalized data were then used to calculate the Spearman rank correlations between OTUs to construct co-occurrence networks.

[1]http://www.drive5.com/usearch/

[2]http://www.mothur.org

RESULTS

Fungal Alpha Diversity and Beta Diversity

The indices of fOTU richness, Shannon diversity, and evenness were used to represent fungal alpha diversity. These indices of TG sugarcane in all three soil compartments were lower than those of WT plants (**Figures 1A–C**). Additionally, the indices of fOTU Shannon diversity and evenness were different between rhizoplane and bulk soil in TG sugarcane, which was not observed in WT sugarcane. Pearson correlation analysis indicated that fungal alpha-diversity indices in two sugarcanes were both positively correlated with the C:N ratio but were negatively correlated with other environmental factors (**Figures 1D,E**). Besides, the TC and TN contents increased in TG root-associated areas (rhizoplane and rhizosphere) but decreased in the TG bulk soil area compared with those of WT (**Supplementary Table S1**). Phylogenetic analysis of fungal membership and composition was performed to evaluate fungal beta diversity using unweighted and weighted UniFrac distances, respectively (**Figures 2A,B**). The PCoA plots indicated good separation of weighted UniFrac distances for the soil fungal communities in different soil compartments (**Figure 2B**). Additionally, the fungal composition of the TG plant soil was remarkably different from that of WT soil based on weighted UniFrac metric. The mantel text indicated that the phylogenetic composition based on weighted UniFrac metric significantly contributed to the separation of fungal communities in both varieties. Further, a different relationship was observed between fungal structure based on weighted UniFrac metric and environmental factors in two sugarcanes. Fungal structure based on weighted UniFrac metric in TG showed no relationship with TC and C:N, while in WT sugarcane, fungal structure based on weighted UniFrac metric was significantly related with TC and C:N (**Figures 2C,D**).

Fungal Community Composition and Abundance

There was a marked difference in fungal relative abundance at the class level between the TG and WT varieties. Compared with that with the WT plant, the relative abundance of Agaricomycetes was significantly higher, whereas the abundances of Dothideomycetes, Pezizomycotina, Eurotiomycetes, and Sordariomycetes were lower in root-related area of TG plants. In the bulk soil, the relative abundances of Eurotiomycetes and Sordariomycetes were higher for TG than for WT plants. And, the relative abundance of Tremellomycetes was significantly

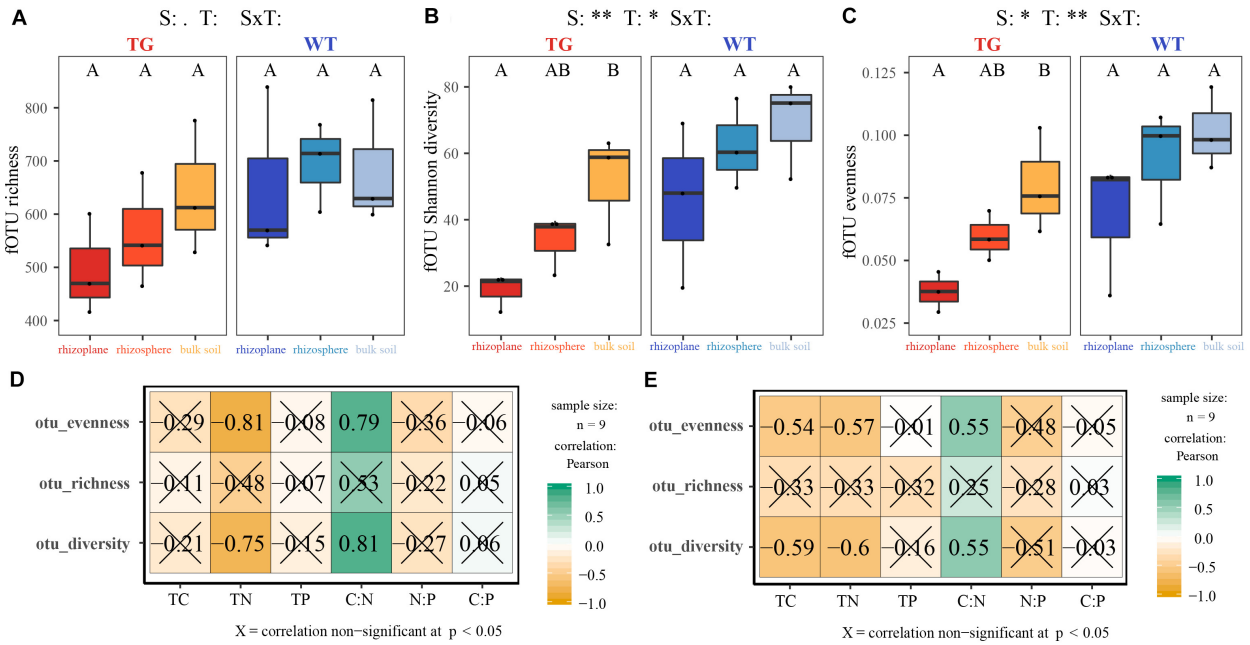

FIGURE 1 | (A–C) Fungal alpha diversity indices represented by richness, Shannon diversity and evenness in each soil compartment and variety. **(D,E)** The correlation between indices of fungal alpha diversity in TG **(D)** and WT **(E)** and environmental factors using Pearson analysis. *0.01 < *P* value < 0.05; **P* value < 0.01.

higher in the bulk soil of WT plants compared with that with of TG plants (**Figure 3A**). Based on dbRDA, the soil compartment and sugarcane variety explained 46.0 and 3.5% of the variation in fungal composition, respectively. The dominant fungal phyla across all soil samples were Agaricomycetes, Sordariomycetes, Eurotiomycetes, Pezizomycotina, Dothideomycetes, and Tremellomycetes. Apart from Agaricomycetes, which was found in the rhizoplane and rhizosphere of TG plants, Sordariomycetes, Eurotiomycetes, Pezizomycotina, Dothideomycetes, and Tremellomycetes were all present in the bulk soil of TG plants. Among the soil properties, C (r^2 = 0.329, P = 0.046), N (r^2 = 0.562, P = 0.005), and C:N (r^2 = 0.731, P = 0.001) were significantly correlated with the distribution of fungal species (**Figure 3B**).

Fungal Abundance Patterns in Transgenic and Wild-Type Plants

We identified fOTUs that were differentially abundant among the soil compartments. Three fOTUs (fOTU8, fOTU116, and fOTU33) exhibited the largest differences in abundance for TG plants. For the WT plants, fOTU43, fOTU142, and fOTU48 were the most differentially abundant OTUs. Agaricomycetes (fOTU8, fOTU43, and fOTU48) were enriched in the root-related area of both TG and WT plants. Eurotiomycetes (fOTU116 and fOTU33) was enriched in the bulk soil of TG plants, whereas the abundant fungal class Leotiomycetes (fOTU142) was found to be enriched in the bulk soil of WT plants (**Figure 4**).

We employed co-occurrence network analysis to identify groups of fungi with similar abundances according to the soil compartment. The groups were separated into six and seven modules (for TG and WT plants, respectively) based on similarities in abundance. There was marked separation of fungal groups between the root-related area (rhizoplane and rhizosphere) and bulk soil of TG plants (**Figure 5A**). Modules M6 (representing Sordariomycetes) and M4 (representing Agaricomycetes) comprised a high proportion of the fOTUs (**Figure 5C**) and were specifically abundant in the root-enriched area of TG plants (**Figure 5B**). Modules M1, M12, M2, and M3, which comprised a relatively high proportion of fOTUs (**Figure 5C**), were enriched in the bulk soil of TG plants (**Figure 5B**). There was similar separation of fOTUs between the root-related area and bulk soil of WT plants (**Figure 5D**). Modules TM1, TM11, and TM26 were mainly present in the root-enriched areas of WT plants (**Figure 5E**), whereas modules TM18, TM16, TM3, and TM12, which comprised fOTUs that were more abundant (**Figure 5F**), were enriched in the bulk soil of WT plants. Further, a set of fOTUs in Chytridiomycetes and Ustilaginomycetes were detected in the root-enriched area of TG plants, with no presence in the WT-enriched area. The disputed class Zygomycota (Zygomycota_cls_Incertae_sedis) was found only in module M3 in the bulk soil-enriched area of TG plants, whereas Tremellomycetes were found only in module TM18 in the bulk soil-enriched area of WT plants (**Supplementary Figure S2**). Apart from these species, other fungal (Eurotiomycetes, Sordariomycetes, Pezizomycotina_cls_Incertae_sedis, Dothideomycetes Agaricomycetes, and unassigned) groups were present in both TG and WT sugarcanes (**Supplementary Figure S2**). The relative abundances of Agaricomycetes, Sordariomycetes,

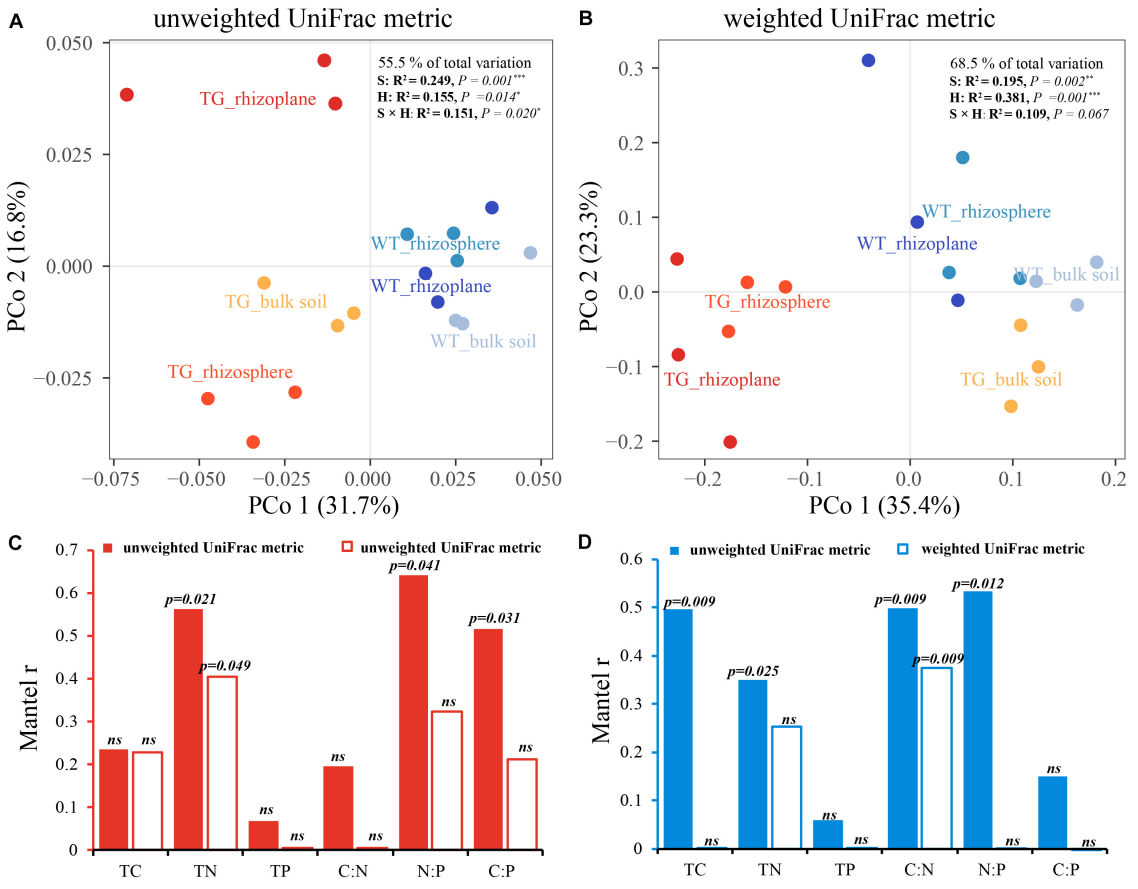

FIGURE 2 | (A,B) Principal coordinate analyses (PCoAs) using unweighted UniFrac metric **(A)** and weighted UniFrac metric **(B)** indicate that the largest separation between fungal communities is spatial distribution of three areas (PCo1) and the second largest source of variation is cultivar (PCo2). **(C,D)** Correlation between environmental factors and phylogenetic membership and composition in two sugarcanes by using Mantel test.

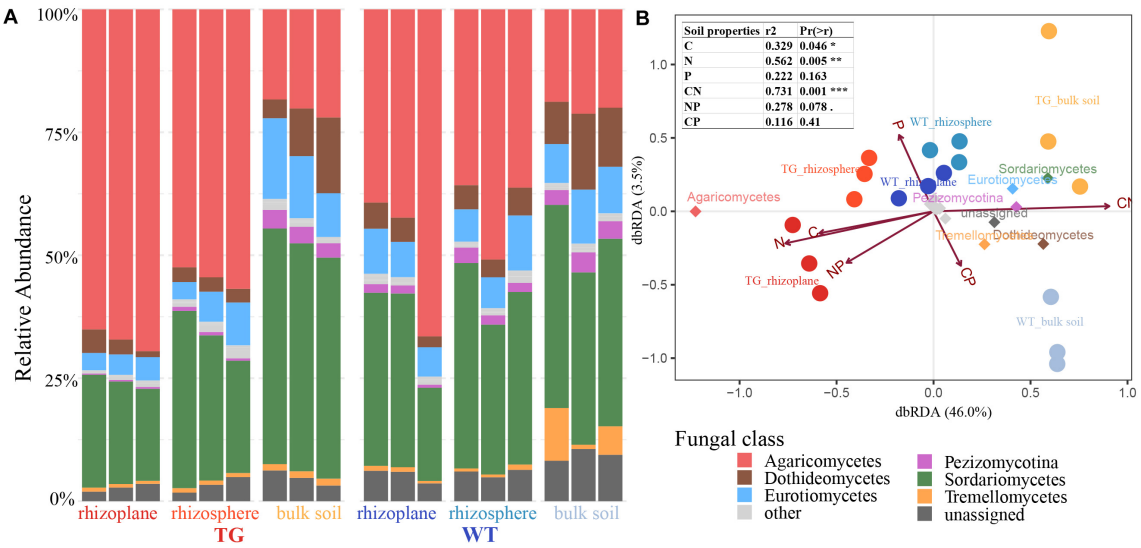

FIGURE 3 | (A) Fungal relative abundance with class of each compartment of two sugarcanes, with three repetitions in each compartment. **(B)** Distance-based redundancy analysis of different zones, abundant classes, and six environmental factors (arrows) indicates the dominant communities and influential environmental factors.

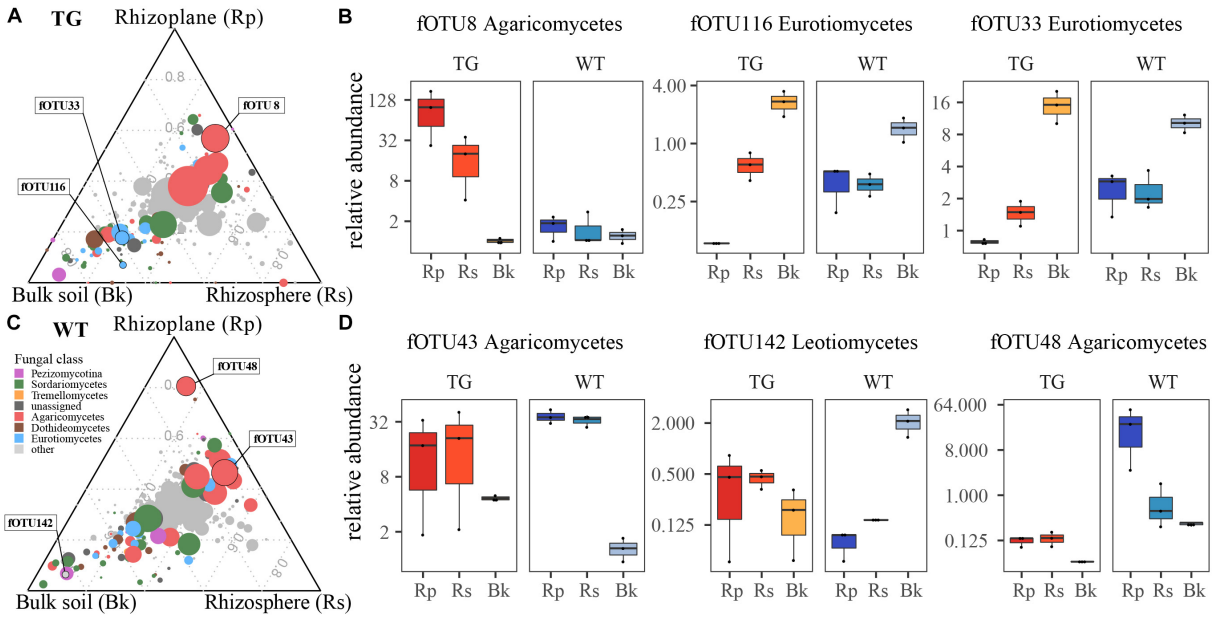

FIGURE 4 | (A,C) Identifying fungal operational taxonomic units (fOTUs) that vary in abundance in response to different soil compartment in two sugarcanes. **(B,D)** The relative abundances of a few representatively responsive fOTUs in rhizoplane, rhizosphere, and bulk soil compartments, which are also indicated in the ternary plots.

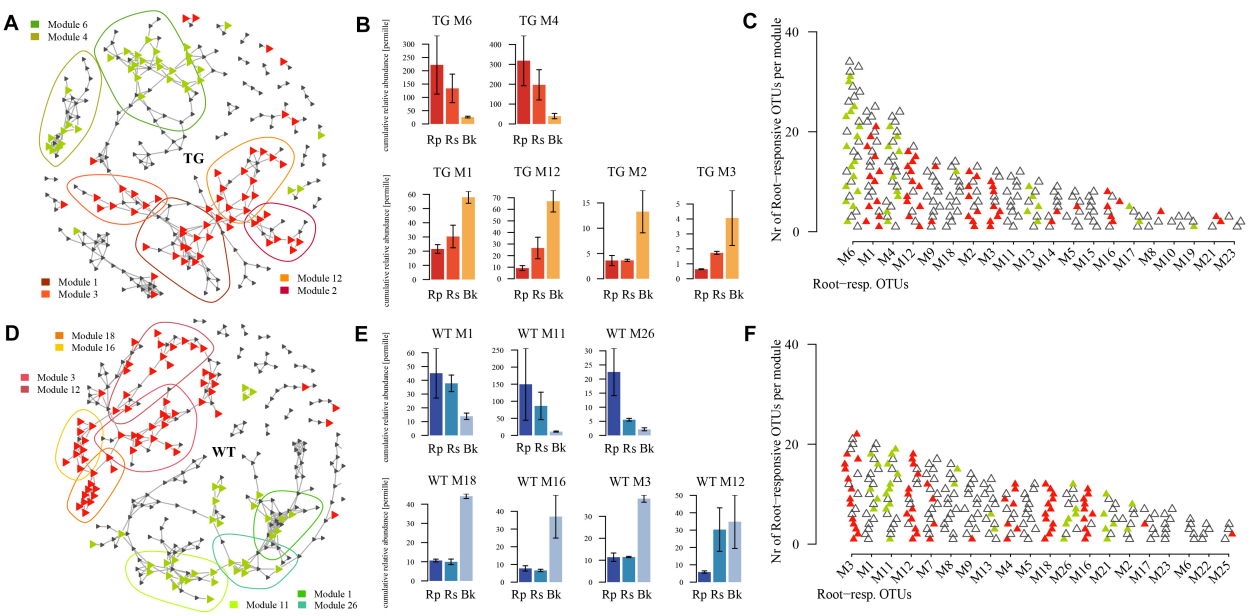

FIGURE 5 | (A,D) Co-occurrence networks visualize the significant positive pairwise correlations [$r > 0.7$, $P < 0.001$; indicated by links between operational taxonomic units (OTUs)] between fOTUs (triangles) in transgenic (TG) **(A)** and wild type (WT) **(D)**. The green triangles within the green circle indicate the root-enriched (rhizoplane-enriched and rhizosphere-enriched) fOTUs, and the red triangles in the red circle indicate the bulk soil-enriched fOTUs. **(B,E)** Cumulative relative abundance of all fOTUs in the responsive modules in root-enriched and bulk soil-enriched areas. The cumulative relative abundance indicates the overall response of the fungi in the responsive modules. **(C,F)** Top 20 most populated modules, ranked by decreasing numbers of fOTUs with root-enriched and bulk soil-enriched fOTUs being colored in green and red, respectively.

and Dothideomycetes all declined in the root-enriched area but increased in the bulk soil-enriched area of TG sugarcane compared with those of WT plants. The relative abundance of Eurotiomycetes and Pezizomycotina_cls_Incertae_sedis declined in root- and bulk soil-enriched areas of TG sugarcane compared with that of WT plants (**Supplementary Figure S3**).

Correlations Between Fungal Operational Taxonomic Units and Soil Properties and Their Functional Roles

We used Mantel testing to analyze the relationship between fOTUs in root-enriched and bulk soil-enriched areas and environmental factors. Root-enriched fOTUs indicate the OTUs, as shown in **Figure 4** (M6 and M4 in TG; and M1, M11, and M26 in WT), that were relatively enriched in the rhizoplane and rhizosphere. Similarly, bulk soil-enriched fOTUs indicate the OTUs (M1, M12, M2, and M3 in TG, and M18, M16, M3, and M12 in WT) that were relatively enriched in bulk soil. The relative abundance of TG root-enriched fOTUs of 940 was far more than that of WT root-enriched fOTUs of 411. The internal correlations among the environmental factors were stronger in the soil of the TG plants than in that of WT plants. The fOTUs in the TG plant soil compartments were significantly correlated with C, N, and C:N and N:P ratios. However, the bulk soil-enriched area fOTUs were more highly correlated with environmental factors than were root-enriched fOTUs in TG plants (**Figure 6A**). The correlation between fOTUs and environmental factors was weaker in the soil of WT plants than in that of TG plants. The fOTUs in the WT root-enriched area were related only to TN, and those in the WT root-enriched area were related only to the C:N ratio (**Figure 6B**). The internal relationships among environmental factors were significantly stronger in TG than in WT plants. Functional annotation of the OTUs predicted seven trophic modes, including saprotroph–symbiotroph, saprotroph, pathotroph–saprotroph, symbiotroph, pathotroph–symbiotroph, pathotroph–saprotroph–symbiotroph, and pathotroph. Among the fOTUs in both the TG root-enriched area and WT root-enriched area, most fOTUs were assigned to the saprotroph and pathotroph–saprotroph function (**Figures 6A,B**). However, the quantity of TG root-enriched fOTUs was larger than that of WT. Most fOTUs in the TG plant bulk soil-enriched area were categorized as pathotroph–saprotroph–symbiotroph (**Figure 6A**).

DISCUSSION

Variation in Fungal Diversity in the Soil of Transgenic Plants

In the present study, differences of the fungal diversity have been observed in all three compartments of TG and WT sugarcanes under the same planting conditions. However, Romão-Dumaresq et al. (2016) have indicated that the plant genotype (TG and non-TG) does not significantly change the fungal diversity of sugarcane in the rhizosphere, which is the opposite of our results. As previous studies showed, fungal diversity was affected in lettuce and *Arabidopsis thaliana* due to plant genotype (Hunter et al., 2015; Urbina et al., 2018), while no significant differences of fungal diversity between the WT and TG lines were found in potato and rice (Milling et al., 2005; Sohn et al., 2016). These conflicting results arise from different functional genes in plants. *Ea-DREB2B* belongs to the DREB subfamily, the members of which are capable of regulating drought response via ABA-dependent as well as ABA-independent pathways (Vargas et al., 2014). Thus, altered ABA pathways in TG plants affect the pattern of TG sugarcane exudates to some extent due to the overexpression of *Ea-DREB2B*. Accumulating evidence suggests that plant-produced metabolites can significantly shape the microbiome composition and activity (Dunfield and Germida, 2004). Root exudation was previously shown to enhance sugarcane rhizosphere fungal

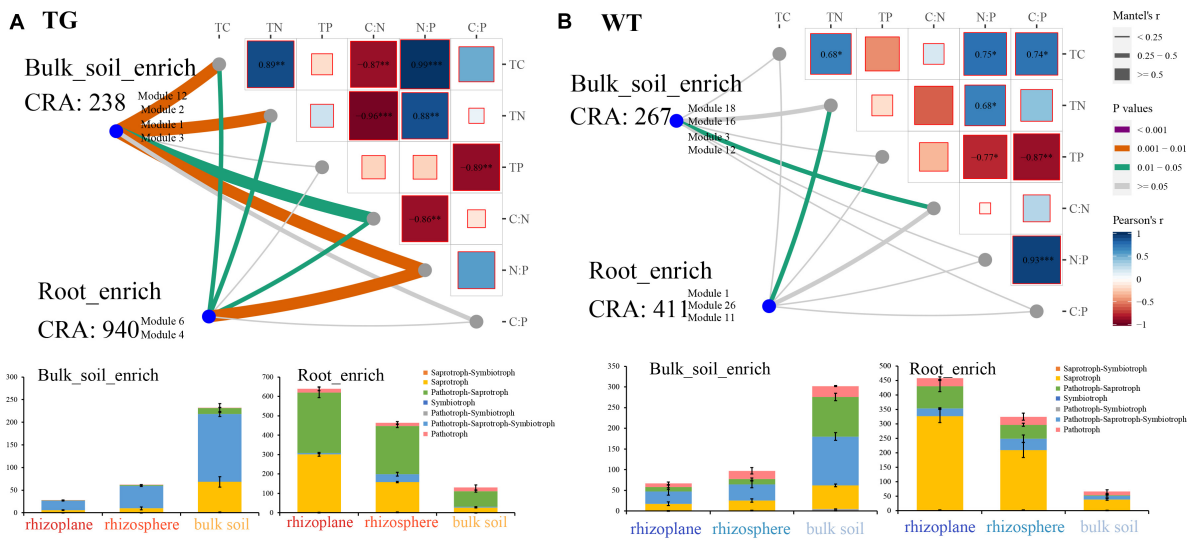

FIGURE 6 | (A) Correlation between fungal operational taxonomic units (fOTUs) represented by cumulative relative abundance (CRA) in root-enriched modules (M6 and M4) and bulk soil-enriched modules (M1, M12, M2, and M3) and environmental factors in transgenic (TG) plant using Mantel test; fungal trophic modes of fOTUs enriched in TG root and bulk soil areas. **(B)** Correlation between fOTUs in root-enriched modules (M1, M11, and M26) and bulk soil-enriched modules (M16, M3, and M12) and environmental factors in WT plant using Mantel test; fungal trophic modes of fOTUs enriched in WT root and bulk soil areas.

activity, which can in turn affect plant growth and fitness via hormone production or the mineralization of available nutrients (Lian et al., 2018). In our results, the roots structure of TG sugarcane was significantly more developed than that of WT (**Supplementary Figure S4**), and C and N contents in TG root-associated compartments increased compared with those of WT (**Supplementary Table S1**), suggesting a more nutritious living environment for TG fungal species and plant roots. Interestingly, unlike the relationship trend observed between indices representing alpha diversity in both TG and WT and environmental factors, the phylogenetic composition based on weighted UniFrac metric representing beta diversity of TG fungal species was not related to C:N, which differed from that of species with WT. It has been reported that fungi can endure environmental changes that are less well tolerated by other organisms, indicating a more stable structure of fungal species than that of other microorganisms (de Vries et al., 2018). Indeed, comparing the present results with the results of our previous study (Zhao et al., 2020), the structure of fungal species around plant roots was less affected by changes in soil environment compared with that of bacteria in TG plants. Overall, our results reveal that fungal diversity is affected by changes in soil living environment that primarily result from plant genotype.

Variation in Fungal Community Composition Between Sugarcane Varieties

There was greater variation in fungal composition between the root-associated areas and bulk soil with TG plants than with WT plants. Fungal communities are recruited from the soil in a manner consistent with the two-step selection of bacterial communities: first from the bulk soil to the rhizosphere and then from the rhizosphere to the endosphere (Bulgarelli et al., 2013). We found clear alterations in all three soil compartments with TG sugarcane compared with those with WT plants (**Figure 3A**), suggesting that the soil fungal communities migrated under the influence of genotypes. Taking the two-step recruitment of fungal communities into consideration, we speculate that the soil fungal community of TG sugarcane migrated from outside to the inside, from the bulk soil to the rhizosphere, from the rhizosphere to the rhizoplane, and finally into the root. The host plant greatly affects the fungal diversity and composition in the soil around the roots, and certain fungal species are particularly affected by the host plants (Urbina et al., 2018). We found a markedly higher relative abundance of Agaricomycetes in the rhizoplane and rhizosphere of TG sugarcane compared with that of WT plants, but a higher relative abundance of Sordariomycetes and Eurotiomycetes was observed in the bulk soil of TG plants compared with that of WT plants (**Figure 3A**), suggesting fungal colonization, particularly by certain fungal species. Agaricomycetes accounts for the major mycorrhizal taxa, which can form a mutualistic symbiotic relationship with the roots of plants to enhance the uptake of immobile nutrients from the soil (Douds and Millner, 1999). Inoculation with *Funneliformis mosseae* (an AM fungus) on the roots of trifoliate orange enhances the relative abundance of Sordariomycetes and has been widely reported to improve soil health (He et al., 2019). Sordariomycetes comprises one of the major responders to drought stress (Meisner et al., 2018). Members of Ascomycota, the phylum containing Sordariomycetes and Eurotiomycetes, can tolerate stressful conditions such as low nutrient availability, enabling them to achieve more efficient resource use in challenging environments (Li et al., 2020). Notably, TC, TN, and the C:N ratio were the most important factors explaining fungal community structure (**Figure 3B**), indicating that changes in fungal composition are caused by the effects of fungi altering the physiochemical environment. Fungal community colonization depends on environmental conditions when the resource becomes available for colonization; that is, when the habitat is altered, most fungal communities are usually replaced by other species that are more combative or better able to tolerate conditions within the resource by virtue of certain modifications in their structure or physiology (Boddy and Hiscox, 2017).

Enhanced Relationship Between Transgenic-Enriched Fungal Operational Taxonomic Units and Environmental Factors

Analysis of the network co-occurrence patterns of two sugarcane types showed the same trend in our study: certain fOTUs of both varieties were enriched in the root and bulk soil areas (**Figure 5**). However, a significantly closer relationship was observed between isolated fOTUs in two enriched areas and environmental factors in TG plants than in WT plants (**Figure 6**). These phenomena indicate that, to a certain degree, the structure or function of fungal species around TG roots changed compared with that around WT. As a multitudinous class of soil microorganisms, fungi comprise multiple functional groups, including decomposers, mutualists, and pathogens (Bardgett and Wardle, 2010). Saprotrophs, a group of fungi making up 50–80% of the overall fungal community (Schmidt et al., 2019), are principal decomposers in terrestrial habitats. In our study, more saprotrophs were enriched in TG root areas than in WT root areas, indicating the increased proportion of saprotrophs and the altered fungal-rhizosphere niche. As a previous study showed, soil environmental changes may shift the original niches of the three fungal guilds (pathotroph, saprotroph, and symbiotroph) in root-associated areas, potentially influencing decomposition within that ecosystem (Bödeker et al., 2016). Furthermore, it has been reported that the rate of decomposition is intimately related to the C:N ratio (Dighton, 2007), and a reduced decomposition rate has been shown with higher C:N than with lower ratios, which is consistent with our results; we observed lower C:N in TG root-related areas than in that of WT with increased saprotrophs (**Supplementary Table S1**). It has also been reported that, compared with ectomycorrhizal fungi (symbiotrophic fungi), saprotrophs even play a more important role in the production of labile C-targeting hydrolase enzymes (Talbot et al., 2013). Additionally, fungal species that colonize bulk soil and that have no interaction with plant roots seem to depend on nutrients inherent in the soil to acquire nutrition, which may explain

the close relationship between bulk soil-enriched fOTUs and environmental factors. Thus, enhanced relationship between TG root-related fOTUs and environmental factors and altered fungal niches with more saprotrophs around the TG roots develop the beneficial function of fungi and have a positive impact on the environment by facilitating plant growth and development, thereby further enhancing plant resistance to drought stress.

CONCLUSION

Genetic regulation is considered a crucial mechanism by which plants respond to drought stress. In this study, we applied several analytical approaches to investigate the impacts of TG *Ea-DREB2B*-carrying sugarcane plants on the fungal communities in the soil. Our study revealed that alterations in fungal communities by TG sugarcane are closely related to environmental factors. Our study also revealed that the apparent migration of fungal communities in the soil compartments of TG sugarcane corresponds to changes in their living environment. Additionally, an enhanced relationship between fOTUs and environmental factors and more abundant saprotrophs were observed in TG root-enriched areas, suggesting strengthened plant–fungal interactions in TG-enriched areas. Furthermore, judging from the results of microbial diversity and network analyses in the present study and our previous study, the structure of fungal species is more stable than that of bacterial communities with changing living environment due to different plant genotype (Zhao et al., 2020). Although we did not find a direct relationship between the enhanced drought response of TG sugarcane and altered fungal communities, the increase in specific beneficial fungi in TG soil compartments and the enhanced relationship between TG-enriched fOTUs and environmental factors contribute substantially to improving the drought response capability of TG sugarcane. However, it should be recognized that soil fungal communities are affected by many other natural sources of variation. Therefore, more comprehensive studies on the effects of TG plants on fungal communities in the soil, considering other potentially important factors, are required.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, SRP257722.

AUTHOR CONTRIBUTIONS

XZ, ZW, QL, and SX contributed to design of the experiments, data analysis, and manuscript writing. XZ, QL, SX, and HY contributed to experimentation. HY and MZ contributed to data interpretation. 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.2020.562775/full#supplementary-material

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Understanding the Impact of Cultivar, Seed Origin, and Substrate on Bacterial Diversity of the Sugar Beet Rhizosphere and Suppression of Soil-Borne Pathogens

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The rhizosphere microbiome is crucial for plant health, especially for preventing roots from being infected by soil-borne pathogens. Microbiota-mediated pathogen response in the soil-root interface may hold the key for microbiome-based control strategies of phytopathogens. We studied the pathosystem sugar beet—late sugar beet root rot caused by *Rhizoctonia solani* in an integrative design of combining *in vitro* and *in vivo* (greenhouse and field) trials. We used five different cultivars originating from two propagation sites (France, Italy) with different degrees of susceptibility towards *R. solani* (two susceptible, one moderately tolerant and two cultivars with partial resistance). Analyzing bacterial communities in seeds and roots grown under different conditions by 16S rRNA amplicon sequencing, we found site-, cultivar-, and microhabitat-specific amplicon sequences variants (ASV) as well as a seed core microbiome shared between all sugar beet cultivars (121 ASVs representing 80%–91% relative abundance). In general, cultivar-specific differences in the bacterial communities were more pronounced in seeds than in roots. Seeds of *Rhizoctonia*-tolerant cultivars contain a higher relative abundance of the genera *Paenibacillus*, *Kosakonia*, and *Enterobacter*, while *Gaiellales, Rhizobiales*, and *Kosakonia* were enhanced in responsive rhizospheres. These results indicate a correlation between bacterial seed endophytes and *Rhizoctonia*-tolerant cultivars. Root communities are mainly substrate-derived but also comprise taxa exclusively derived from seeds. Interestingly, the signature of *Pseudomonas poae* Re*1-1-14, a well-studied sugar-beet specific biocontrol agent, was frequently found and in higher relative abundances in *Rhizoctonia*-tolerant than in susceptible cultivars. For microbiome management, we introduced microbial inoculants (consortia) and microbiome transplants (vermicompost) in greenhouse and field trials; both can modulate the rhizosphere and mediate tolerance towards late sugar beet root rot. Both, seeds and soil, provide specific beneficial bacteria for rhizosphere assembly and microbiota-

mediated pathogen tolerance. This can be translated into microbiome management strategies for plant and ecosystem health.

Keywords: sugar beet cultivars, seed microbiome, root microbiome, vermicompost, biocontrol, breeding, *Pseudomonas poae* RE*1-1-14

INTRODUCTION

Developing concepts for microbiome-based crop management strategies is challenging due to the multi-fold interactions in these complex systems. It further requires a deep scientific understanding of microbial community dynamics. Microbiome network structure and microbial diversity in the rhizosphere of plants are linked with tolerance towards pathogen invasion (van Elsas et al., 2012; Berg et al., 2017). The rhizosphere is the critical soil-plant interface for resource exchange and interaction between the plant and soil microbiota (Weller et al., 2002; Philippot et al., 2013). The crucial involvement of rhizosphere-associated microbiota for growth promotion and stress tolerance in crops is known for more than a century (Hiltner, 1904), but was impressively accelerated by the progresses in omics-technologies (Mendes et al., 2012; Raaijmakers and Mazzola, 2016). Due to the importance of microbial rhizosphere assembly for plant health, it was intensively studied in the last decades (Berendsen et al., 2012; Bakker et al., 2013). Diverse factors shaping the microbial rhizosphere community have been identified, with plant genotype and soil traits as the most important determining factors (Berg and Smalla, 2009). However, both plant genotype as well as soil quality, were strongly changed in the period of increasingly intensified agriculture. This resulted in changes in plant-associated microbial communities and reduced indigenous antagonistic potential towards plant pathogens, especially towards soil-borne pathogens (Cardinale et al., 2015; Perez-Jaramillo et al., 2017; Banerjee et al., 2019). Crops often lack clear genetic resistance to soil-borne pathogens. Cook and colleagues postulated already in 1995 that plants could compensate this by recruiting antagonists of pathogens from the soil microbiome. This was now evidenced by omics-technologies (Mendes et al., 2012; Carrión et al., 2019). Although, there are first studies indicating that breeding for resistance towards soil-borne pathogens unintentionally shaped the structure and function of the rhizosphere microbiome, e.g. in the pathosystem common bean (*Phaseolus vulgaris*)—*Fusarium oxysporum* (Mendes et al., 2018a; Mendes et al., 2018b), a generalized relationship between the rhizosphere microbiome structure and plant immunity/tolerance could not be established yet.

Seeds were identified as carrier and ideal target for rhizosphere's microbiome management (Berg and Raaijmakers, 2018). During the last years, the vertical transmission *via* seed or propagule endophytes has been described for many plants (e.g. Johnston-Monje et al., 2016; Klaedtke et al., 2016; Bergna et al., 2018). However, the role of seed endophytes and their interplay with soil microorganisms in rhizosphere assembly and microbiota-mediated pathogen response is not yet understood (Berg and Raaijmakers, 2018). Furthermore, many basic insights f.e. about the impact of propagation site on seed endophyte assemply are still missing (Bergna et al., 2018). Seed endophytes play an important role in the respective plant-pathogen interactions (Shade et al., 2017). To what extent microbial seed communities are conserved or otherwise shaped by the propagation sites' soil communities may be important for pathogen susceptibility of the next plant generation. Furthermore, only seed endophytes that survive the dynamic process of germination will be represented in the endophytic community of the seedling and its rhizosphere (Shade et al., 2017).

Sugar beet is an interesting model crop for microbiome studies (Zachow et al., 2008; Mendes et al., 2012; Kusstascher et al., 2019a; Kusstascher et al., 2019b) known for their genome and breeding history (Würschum et al., 2013; Dohm et al., 2014). Sugar beet (*Beta vulgaris* L.) is an important root crop and the main source of sucrose in moderate climates. It is grown on approximately $4.8*10^6$ hectares, resulting in $2.7*10^8$ tonnes yield in 2018, with Russia and France being the main producers worldwide (FAOSTAT, 2019). One of the major pathogens in sugar beet is the fungus *Rhizoctonia solani* J.G.KÜHN [teleomorph: *Thanatephorus cucumeris* (A.B.Frank) Donk], which causes a variety of different plant diseases and has a broad host range (Ogoshi, 1996). Especially the late root rot, a disease caused by *R. solani* of the anastomosis group AG2-2IIIB leads locally to high yield losses over 50%, and is estimated to affect 5%–10% of the acreage in Europe and over 24% in the United States (Büttner et al., 2004; Jacobsen, 2006). Although partially *Rhizoctonia*-resistant or -tolerant sugar beet cultivars are commercially available, they usually are less productive or lack resistance/tolerance towards other diseases (Jacobsen, 2006). Therefore, microbiome-based disease management may be an interesting alternative in the future. Microbiomes can be managed either directly by applying microbiome transplants; single or mixed microbes with bioactive properties; or microbiota-active metabolites, or indirectly by changing environmental conditions in a way that microbiomes also shift their structure and function from dysbiosis into a healthy state (Berg, 2009). One possible approach to directly shape rhizosphere communities is using vermicompost as microbiome transplant. Vermicompost is a biofertilizing substrate produced by earthworms, which are one of the key taxa for soil functionality

Abbreviations: ASV, amplicon sequence variants; BEL, sugar beet cultivar BELLADONNA KWS, *Rhizoctonia*-susceptible; BER, sugar beet cultivar BERETTA KWS, *Rhizoctonia*-susceptible; cv, Cultivar; FR, France; ISA, sugar beet cultivar ISABELLA KWS, *Rhizoctonia*-effective (intermediate); IT, Italy; LAE, sugar beet cultivar LAEITIA KWS, *Rhizoctonia*-tolerant; MAT, sugar beet cultivar MATTEA KWS, *Rhizoctonia*-tolerant; PCoA, Principal coordinate analysis; r^S, mean relative abundance, romanized version of ancient Egyptian: Ra, deity of the sun.

(Drake and Horn, 2007; Singh et al., 2020). Properties of vermicompost as disease-suppressing microbiome transplant are promising, since it already showed suppression of *Rhizoctonia* in cucumber (Simsek Ersahin et al., 2009).

This study focuses on the origin of root microbiota in general and for *Rhizoctonia* tolerance in particular as well as potential microbiome-based biocontrol options. Microbiome modulation approaches consisted of either bacterial inoculant (three strains belonging to *Pseudomonas* and *Serratia*) or microbiome transplant (vermicompost) application. We investigated the following hypotheses: I) Most seed endophytes of sugar beet survive until the process of germination, II) The bacterial seed endophyte communities differ within seeds of the same cultivar depending on the origin of the mother plants, III) *Rhizoctonia*-tolerant and –susceptible cultivars enrich different bacterial taxa in their rhizosphere, IV) *Rhizoctonia*-tolerant and –susceptible cultivars enrich similar taxa from different substrates, and V) *Rhizoctonia* tolerance can be mediated in susceptible cultivars by seed treatment with bacterial biocontrol agents. Our aim was to investigate bacterial communities in sugar beet and correlate them to the variables cultivar, seed origin, *Rhizoctonia* susceptibility and growth substrate.

MATERIAL AND METHODS

Experimental Setup

We analysed the naturally composed seed microbiome ("seed"), the roots of soilless germinated seedlings ("*in vitro*") and roots of seedlings grown in different substrates ("*in vivo*"). Five sugar beet cultivars were chosen based on their phenotypic characteristics regarding the susceptibility towards the fungal phytopathogen *Rhizoctonia solani* (J.G.Kühn), the causative agent of late root rot in sugar beet; two susceptible cultivars [BELLADONNA KWS (BEL), BERETTA KWS (BER)], one moderately tolerant [ISABELLA KWS (ISA)] and two cultivars with partial resistance and tolerance [LAETITIA KWS (LAE)] MATTEA KWS (MAT); *Rhizoctonia*-tolerant cultivars]. Seeds of every cultivar were provided by KWS SAAT SE & Co. KGaA (Einbeck, Germany) from two different seed production sites, one in situated in France (Departement Lot-et-Garonne), one in Italy (Region Emilia Romagna). All samples were repeated 4 times.

For seed samples, 20 seeds per replicate were washed three times with sterile distilled water and activated for 4 h in 2 ml water. Sugar beet seeds were not surface-sterilized to simulate field-like conditions. For soilless cultivated sugar beet seedlings ("*in vitro*"), eight activated seeds were placed in germination pouches (Mega International, Newport, MN, USA) with two pouches per replicate. To avoid decoupling the rhizosphere microbiome from plant selection and to avoid training effects from agricultural field management including chemical compounds like fertilizer, pesticides, insecticides, and disseminated gene pools, we used potting soil instead of field soil for rhizosphere sampling. Nine activated seeds were placed in 7 cm × 7 cm × 13 cm pots with a soil: sand:vermiculite 3:1:1 (v/v) mixture, further denoted as "potting soil" (for details see **Supplementary Table 1**). To investigate the effects of natural-based biofertilizer on rhizosphere diversity, the setup was repeated for BEL, BER, LAE, and MAT using a natural product based on earthworm casts (**Supplementary Table 1**), further denoted as "vermicompost". Vermicompost was used either as sole substrate or as amendment to potting soil. For the latter approach, ca. 15 g vermicompost was folded in the upper few centimetres of the potting soil before seeds were planted. Growing conditions were 23°C, 60% humidity and 16:8 h light/dark conditions for 2 weeks in all approaches.

DNA Extraction

Activated seeds and *"in vitro"* roots weighed and grounded with 2 to 5 ml sterile 0.85% NaCl with mortar and pestle under sterile conditions. Suspensions were centrifuged at 16,500 × g for 20 min at 4°C and resulting pellets were stored at -70°C for further DNA extractions. DNA was extracted by mechanical disruption and homogenization of the pellet using and a FastPrep Instrument for 30 s at 5.0 ms^{-1} and FastDNA Spin Kit for Soil (MP Biomedicals, Illkirch, France). DNA was purified using GeneClean Turbo Kit (MP Biomedicals, Illkirch, France) to remove humic acids. Extracted DNA was treated with RNase (0.02 ng μl^{-1}) for 5 min at 65°C to obtain the template for PCR amplification of 16S rRNA genes from total community DNA.

Isolates From Sugar Beet and Vermicompost

Bacterial strains were isolated from vermicompost and sugar beet rhizospheres to compare culture-dependent and culture-independent results for the bacterial communities. For vermicompost and potting soil, 1 g substrate was diluted in 9 ml sterile 0.85% NaCl, vortexed, centrifuged at 16,500 g and 4°C for 20 min. For rhizosphere, supernatants of the suspensions used for amplicon sequencing were used. Bacteria were grown on R2A (Roth, Karlsruhe, Germany) at 30°C for 48 h. Clean CFUs were randomly chosen, isolated and grown on NA (Sifin GmbH, Berlin, Germany). Bacterial DNA was extracted using a "quick and dirty" protocol using a microwave for cell disruption; bacterial material was transferred to a 1.5 ml Eppendorf and rayed by a microwave for 3 min with closed lid, 30 µl TE buffer was added, vortexed and centrifuged at 16,500 x g for 2 min. Supernatant was used as template for a PCR using the bacterial universal primer pair 27F/1492R according to Lane et al. (1991). Amplifications were confirmed by gel electrophoresis in 1x TAE (0.8% Agarose). PCR-products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). Sequencing was conducted by LGC Genomics (Berlin, Germany). Resulting sequences were quality-checked using Seq Scanner 2 (Applied Biosystems) and identified with BLAST (https://blast.ncbi.nlm.nih.gov/) using refseq_rna and/or nr/nt database.

Amplicon Sequencing

The hypervariable V4 region of the 16S rRNA gene was amplified according to the protocol of Caporaso et al. (2011) using the primer pair 515f and 806r including Illumina cell flow adapters and sample-specific barcodes. Peptide nucleic acid (PNA) PCR

clamps (PNA Bio, Newbury Park, USA) were used to reduce plastid and mitochondrial 16S contamination (Lundberg et al., 2013). The PCR mix (30 µl) contained 1 × Taq&Go (MP Biomedicals, Illkirch, France), 0.2 mM of each primer, 1.5 µM activated (55°C for 5min) PNA mix (1:1 mPNA:pPNA) and 1 µl template DNA. PCR conditions were 96°C for 5 min; 30 cycles of 96°C for 1 min, 78°C for 5 s, 54°C for 1 min, 74°C for 1 min; 74°C for 10 min). Amplifications of the resulting 168 samples were confirmed by gel electrophoresis in 1x TAE (0.8% Agarose). PCR-products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). DNA concentrations were measured using Nanodrop 2000 (Thermo Scientific, Wilmington, USA) and equimolar aliquots of all samples were pooled for amplicon sequencing using an Illumina MiSeq v2 (250 bp paired end) platform conducted by LGC Genomics (Berlin, Germany).

Bioinformatics

Pre-processing and analysis of the sequencing data was performed in QIIME2 v. 2019.10 (Bolyen et al., 2019) and QIIME v. 1.9.1 (Caporaso et al., 2010). Paired sample reads were demultiplexed and primers were trimmed from sequences using cutadapt (Martin, 2011). Since a considerable amount of forward and reverse sequences were flipped, reverse reads were also trimmed to forward primers and *vice versa*, and the resulting data from both trimming steps was combined. The sequences were denoised using DADA2 (Callahan et al., 2016), aligned with MAFFT (Katoh, 2002) and their phylogeny was constructed with fasttree2 (Price et al., 2010). Taxonomy was assigned with VSEARCH algorithm (Rognes et al., 2016) and Silva 132 99% consensus database (Quast et al., 2013). Mitochondrial, plastid DNA, and taxonomically unassignable sequences were removed from table and representative sequences. Thus, the datasets contained 32,140 amplicon sequences variants (ASV) and read numbers ranging from 254 to 545,913 reads per sample. Seeds and *in vitro* roots were separately analyzed from rhizosphere and soil. Alpha (Shannon, observed OTUs and evenness) and beta diversity indices (Bray-Curtis dissimilarity, weighted UniFrac) were calculated in QIIME2 and visualized using the q2_emperor plugin (Vázquez-Baeza et al., 2013). Differences in alpha and beta diversity indices were calculated using Kruskal-Wallis and pairwise PERMANOVA implemented in QIIME2. To evaluate taxa that differ significantly regarding cultivar and *Rhizoctonia* tolerance, samples of seed, *in vitro*, and *in vivo* roots were rarefied according to their minimum read number [1,000, 2,400 (losing 2 samples) and 15,000, respectively]. Samples for ISA were removed in *Rhizoctonia* tolerance testing to perform pairwise comparisons. The remaining samples were collapsed to genus level and compared using the QIIME1 plugin group_significance.py with Kruskal-Wallis test (cultivar, substrate) and nonparametric t-test (*Rhizoctonia* tolerance, seed origin). Differences between Bray-Curtis distances and weighted UniFrac distances of seeds and roots were tested using the adonis (Anderson, 2001) command of the R script vegan 2.5 (Oksanen et al., 2018) implemented in QIIME2.

To evaluate number proportion of surviving seed endophytes, seed and *in vitro* root samples were compared on ASV level. Intersection of communities of seed, soil, and rhizosphere were compared on genus-level with mean values of relative sequences.

Field Design

Field trials were conducted in the course of efficacy tests in different years for the sugar beet cultivars BERETTA KWS (*Rhizoctonia*-susceptible, growing season 2009 and 2010), ISABELLA KWS (moderately tolerant, growing season 2016), and MATTEA KWS (*Rhizoctonia*-tolerant, growing season 2013). However, all field trials were performed under usual production conditions by local farmers. All sugar beet (*Beta vulgaris* L.) seed materials were generated and evaluated by KWS SAAT SE & Co. KGaA (Einbeck, Germany). Bacterial treatment was performed with a consortium of *Pseudomonas poae* RE*1-1-14 obtained from sugar beet (Zachow et al., 2010), *Pseudomonas brassicacearum* L13-6-12 (Grosch et al., 2005), and *Serratia plymuthica* 3Re4-18 (Berg et al., 2005) isolated from potato, which all show antagonistic activity towards *Rhizoctonia solani*. Formulations were prepared by Biotenzz GmbH (Graz, Austria) for integration in the commercial seed coating. Sugar beet seeds of *R. solani*-susceptible cultivars were routinely coated with the fungicides Thiram® (Cheminova Deutschland GmbH, Germany), Hymexazol® (Mitsui Chemicals, Tokyo, Japan) and the insecticides Imidacloprid (Gaucho®, Bayer CropScience, Leverkusen, Germany), Chlothianidin (Poncho®, Bayer CropScience, Leverkusen, Germany), Thiamethoxam (Syngenta Crop Protection, Basel, Switzerland), and Tefluthrin (Syngenta Crop Protection, Basel, Switzerland) respectively. The two test fields ("Kasten": 48°42'48.6"N 13°04'34.5"E and "Tabertshausen": 48°44'24.5"N 12°52'55.2"E) for the trials were located in the main growing area in Lower Bavaria, Germany. *R. solani*-infested barley kernels were used for artificial inoculation of the soil (60 kg/ha). Control plants were commercially prepared without microbial inoculants. Field trials were conducted in a randomized block design with four replicates per variant, each with six rows containing 30 plants per row. The *Rhizoctonia* disease index (RI) described by Büttner and coworkers (2004) with 1 to 9 (1—no disease, 9—plant dead, root completely rotted) and the number of beets were evaluated at harvest. Beets of the categories RI = 1 or 2 were defined as healthy beets. Statistical analysis was performed using SPSS version 25.0 (IBM Corporation, NY, USA). Correlation of number of beets at harvest, RI and percentage of healthy beets with bacterial treatment were tested pairwise (treated:untreated) for each cultivar and field separately using Mann-Whitney-U Test.

The 16S rRNA sequence of *Pseudomonas poae* RE*1-1-14 (NCBI Reference Sequence: NC_020209.1), *Pseudomonas brassicacearum* L13-6-12 (CP014693.1), and *Serratia plymuthica* 3Re4-18 (CP012097.1) was extracted from their reference genome and cross-checked with the amplicon dataset to evaluate presence or absence in our amplicon data samples.

RESULTS

Seeds Harbor Cultivar-Specific Bacterial Communities Strongly Influenced by Propagation Sites

Sugar beet seeds of five different cultivars originating from two different seed production sites contained a total of 1001 amplicon

sequences variants (ASVs). For seed samples, read numbers ranged from 1,062 to 152,738 reads per sample (for a summary, see **Supplementary Table 2**). Seeds of all cultivars share a core of 121 ASVs, which accounts for 80%–91% r^{ζ} (relative abundance) in the communities, while partially shared ASVs account for 4%–19% (**Figure 1A**); ASVs that are unique to a cultivar account for less than 4% r^{ζ} (See also **Supplementary Table 3**). The seed endophyte community is dominated by *Pseudomonas*, *Pantoea*, and *Paenibacillus* (**Figure 1B**). Significant differences in alpha diversity indices (Shannon, Pielou's evenness, observed OTUs) were not observed in seeds, except BER shows higher values for evenness than BEL and ISA (both $p = 0.027$). On the contrary, PERMANOVA results for differences in beta diversity indices (Bray-Curtis dissimilarity, weighted UniFrac distances) revealed to be largely significant (except: weighted UniFrac of BER:LAE; ISA:LAE). Main drivers of cultivar-dependent differences ($r^{\zeta} > 1\%$) in seeds were *Pseudomonas* spp., *Paenibacillus* spp. and *Massilia* spp. ($p<0.05$; Bonferroni-corrected).

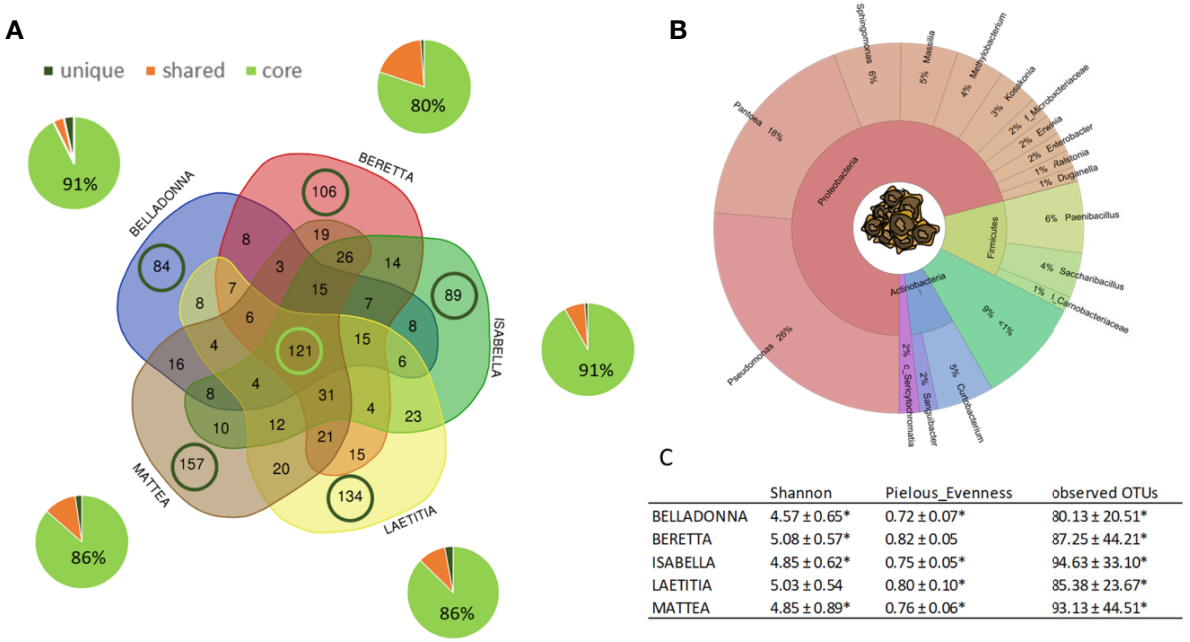
Seed origin was an important variable for alpha diversity (**Figures 1C**, **2A–C**), pairwise comparison within the same cultivars revealed significant differences (pairwise Kruskal-Wallis) for observed OTUs (all) and Shannon index (except LAE). Additionally, all cultivars differed significantly ($p < 0.05$) due to seed origin in Bray-Curtis and weighted UniFrac distances. Adonis test of Bray-Curtis and weighted UniFrac distances revealed the factors "Cultivar" and "Seed origin" explain more than 50% of the variance (**Supplementary Table 4**), with "Cultivar" being the more important one ($R^2 = 0.33$ and $R^2 = 0.32$, respectively; $Pr(>F) = 0.001$). Key genera that significantly differ due to seed origin are not the same for the different cultivars (**Supplementary Table 5**). When merging all seed data, *Sphingomonas*, *Methylobacterium* and unidentified *Sericytochromatia* show higher r^{ζ}-values in Italian seeds, while *Saccharibacillus*, *Kosakonia*, and *Erwinia* have higher proportion in French seeds; this is also indicated by PCoA-biplot of Bray-Curtis distances (**Figure 2B**).

Dominating Seed Endophytes Survive Host Germination

To assess the proportion of alive bacterial communities within seeds, 20 seeds each cultivar were cultivated under *in vitro* conditions in soilless germination pouches. The *in vitro* rhizosphere contained 397 ASVs, represented by 2,533,778 reads, with read numbers ranging from 254 to 137,909 reads per sample. Depending on cultivar, ASVs accounting for 63%–83% r^{ζ} found in the seeds could be confirmed to be present in soilless roots ("*in vitro*", **Figure 3A**).

Dominant phyla in soilless roots are *Proteobacteria* (range 75%–91% r^{ζ}, depending on cultivar), *Actinobacteria* (1%–18% r^{ζ}), and *Firmicutes* (2%–22% r^{ζ}). *Enterobacteriaceae*, mainly represented by *Kosakonia* (19% r^{ζ}) and *Pantoea* (18% r^{ζ}) account for 40% r^{ζ} in soilless roots, although *Methylobacterium* (28% r^{ζ}) is the most frequent genus.

The number and proportion of ASVs of successfully colonizing bacteria differ between cultivars (n = 45–70, ≙ 14%–22% of corresponding seed ASVs), but represent dominating taxa of seed endophytes. Tendentially, a higher proportion of seed



	Shannon	Pielous_Evenness	observed OTUs
BELLADONNA	4.57 ± 0.65*	0.72 ± 0.07*	80.13 ± 20.51*
BERETTA	5.08 ± 0.57*	0.82 ± 0.05	87.25 ± 44.21*
ISABELLA	4.85 ± 0.62*	0.75 ± 0.05*	94.63 ± 33.10*
LAETITIA	5.03 ± 0.54	0.80 ± 0.10*	85.38 ± 23.67*
MATTEA	4.85 ± 0.89*	0.76 ± 0.06*	93.13 ± 44.51*

FIGURE 1 | Overview on microbial community of sugar beet seeds. **(A)** number (VENN diagram) and corresponding abundance (pie charts) of amplicon sequence variants (ASVs) found in sugar beet seeds. Light green: seed core, found in all cultivars; dark green: cultivar-specific ASVs; orange: partially shared ASVs. **(B)** OTUs accounting for >1% relative abundance in the sugar beet seed microbial community on phylum and genus level. C: Alpha diversity indices of seed community; * = significantly differing according to Kruskal-Wallis test depending on seed origin (see also **Figure 2**).

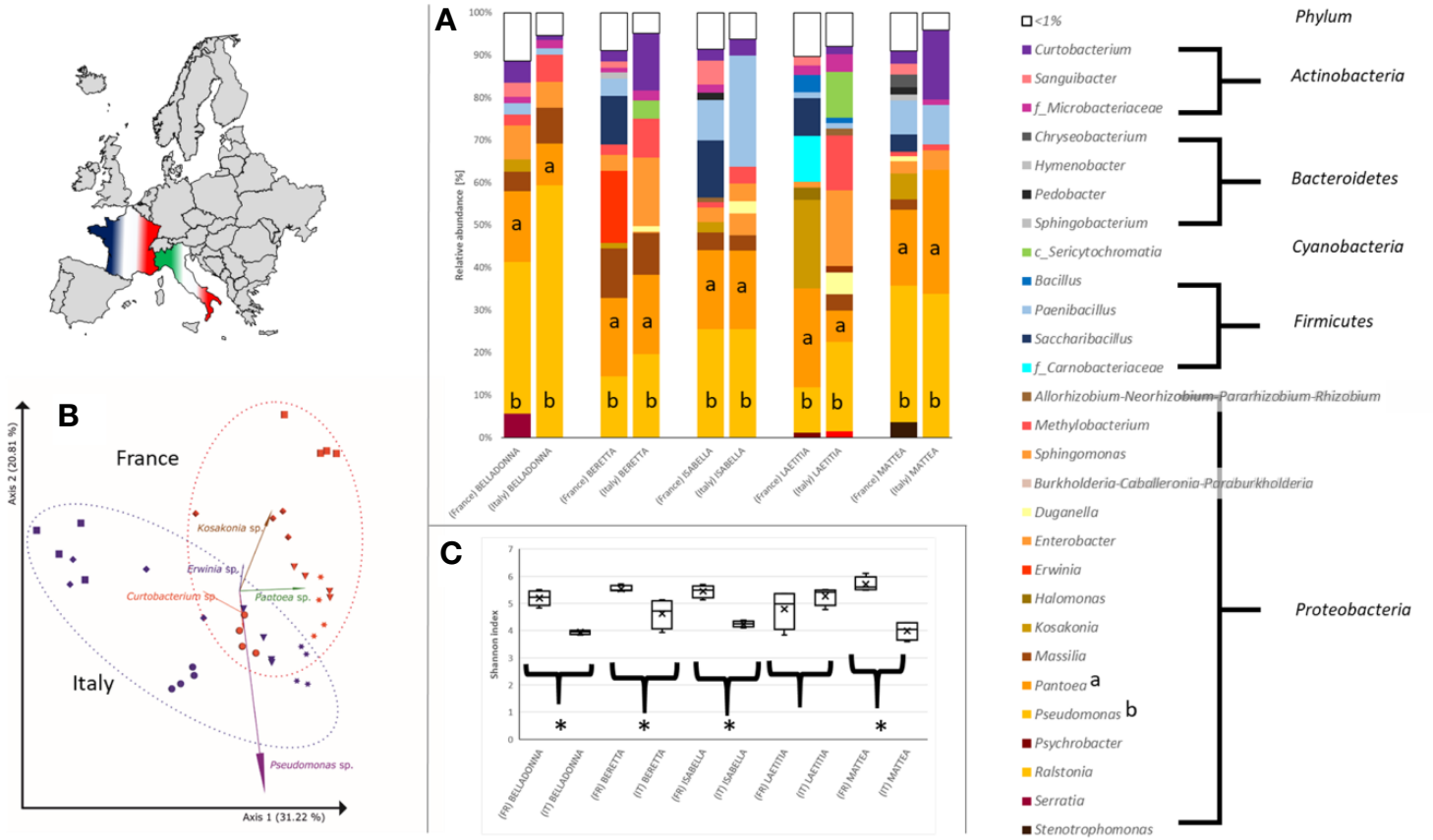


FIGURE 2 | Differences in the bacterial community in sugar beet cultivars' seeds due to origin. **(A)** Seed microbial community on genus level. All seeds are dominated to a different extent by *Proteobacteria*, especially *Pseudomonas* (a) and *Pantoea* (b). **(B)** PCoA-biplot of Bray-Curtis distance on genus level. Seed communities clearly cluster according to seed origin; spheres: cv. BELLADONNA; diamonds: cv. BERETTA; triangles: cv. ISABELLA; squares: cv. LAETITIA; stars: cv. MATTEA. **(C)** comparing Shannon diversity in seeds within the same cultivar due to seed origin; all seeds originating from France show a higher alpha diversity except cv. LAETITIA; * = significantly differing according to Kruskal-Wallis test depending on seed origin.

endophytes survive in soilless roots of *Rhizoctonia*-tolerant cultivars (14%–15% vs. 16%–21% of corresponding seed ASVs; **Figure 3A**). In all cultivars investigated, *in vitro* roots display a lower alpha diversity and a lower evenness (**Figure 3B**) than the corresponding seeds. ASVs representing up to 14% r^S (for LAE) in soilless roots were found to be unique and thus of unknown origin. In total, 230 ASVs unique to *in vitro* roots were found across all cultivars with 12 ASVs accounting for r^S >1% per sample. These ASVs belong to the genera *Curtobacterium*, *Bacillus*, *Pullulanibacillus* and *Methylobacterium*, taxa that are frequently present in both seeds and *in vitro* roots. Cultivar-dependent differences for alpha diversity (Shannon) were not found, and beta diversity indices (Bray-Curtis, weighted UniFrac) were only significantly different between BEL and MAT.

The Ingredients of the Rhizosphere: Soil and Seed Bacteria

The *in vivo* root community clearly differs from both soil and seed communities and is dominated by *Proteobacteria* (26% r^S), *Acidobacteria* (16% r^S), *Actinobacteria* (13% r^S), *Chloroflexi* (10% r^S), and *Planctomycetes* (7% r^S; **Figure 4A**). When the dataset was collapsed to the highest taxonomic annotation, the dominant taxa were *Acidobacteria* Subgroup 6 (7.0% r^S), the archaeal family *Nitrososphaeraceae* (3.9% r^S), and the genus *Pyrinomonadaceae* RB41 (3.9% r^S). Differences in root beta diversity due to seed origin were not significant. The bacterial core community of *in vivo* roots comprised of 3,228 ASVs, representing 82%–88% r^S depending on cultivar. Cultivar-specific ASVs account for 2.2%–3.1% of the abundance (**Supplementary Table 3**).

The bigger part of the root community is originating from soil. Genera accounting for 53.6% are exclusively assembled from soil and another 26.8% of the root community comprises taxa that are found in both seeds and soil (**Figure 4B**). Only a small proportion of the seed and soil microbiome (5.3% and 7.5%, respectively) is unique to their corresponding habitats, while we find 417 genera accounting for 15.25% of the rhizosphere microbiome to be unique. The key taxa found in seeds appear in lower relative abundances in rhizosphere. From the genera that exclusively originate from seeds, four are found in >50-fold r^S-values in root samples; 41 of soil-derived taxa are >50-fold increased (**Supplementary Table 6**).

Interestingly, *Archaea* were found in high abundances (up to 14.1% r^S) in the root and vermicompost-associated communities, with *Nitrososphaeraceae* (ad *Thaumarchaeota*) as dominant taxa (in average representing 91% of all archaeal taxa).

Indicator Bacteria for *Rhizoctonia*-Tolerant Cultivars

ASVs, that are exclusively found in seeds of *Rhizoctonia*-tolerant cultivars (n = 20) account for 0.83% r^S (LAE) and 0.88% r^S (MAT). When combining seeds, potting soil and roots samples, adonis test revealed the factor "habitat" to be the most important variable explaining the variance for Bray-Curtis and weighted UniFrac distances (R^2 = 0.421 and R^2 = 0.725, respectively). The factors

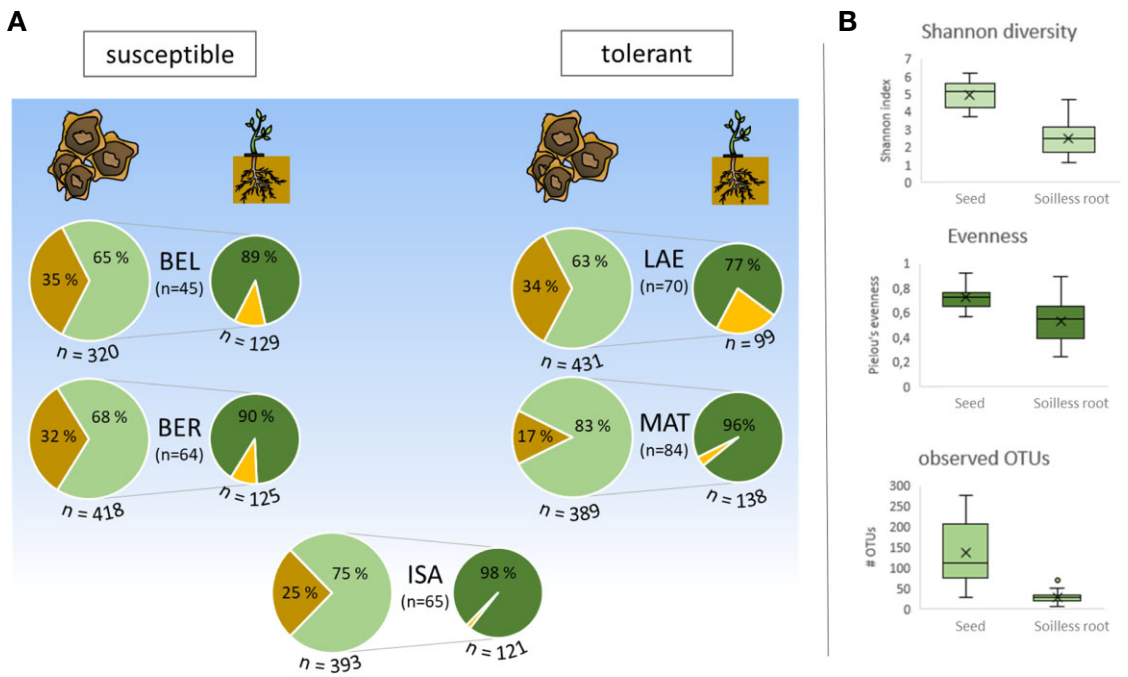


FIGURE 3 | Comparing seed and *in vitro* root microbial community on relative abundance of sequence variants (ASV). **(A)** fraction of surviving bacteria originating from seeds (left pie chart) in soilless seedlings (right pie chart). Percent values refer to mean relative abundance, while n stands for the number of involved ASVs (below pie charts: total number in microhabitat; between pie charts: number of shared ASVs); brown: unique in seeds; green: shared ASVs; yellow: unique *in vitro* root ASVs. **(B)** Differences in alpha diversity indices between seed and *in vitro* root communities.

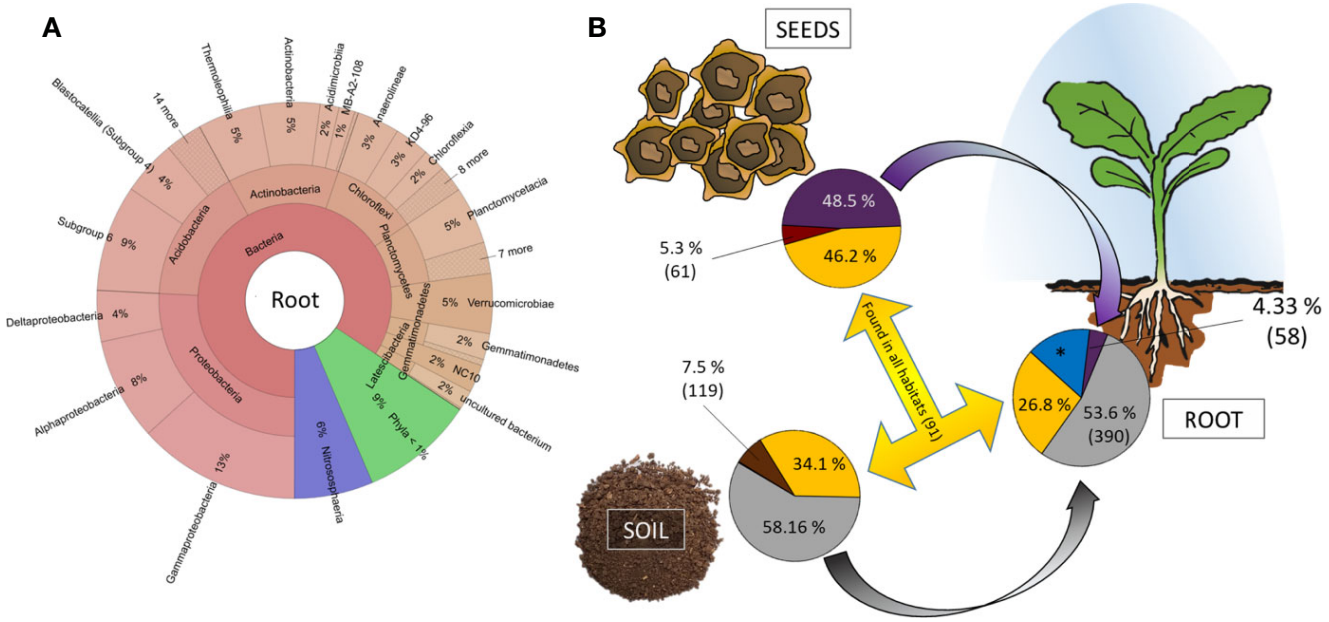


FIGURE 4 | Tracking the origin of bacterial genera found in the rhizosphere. **(A)** Krona chart of root communities on phylum level. Abundances are mean values across al cultivars. **(B)** Relative abundance and number of genera unique (soil: brown; red: seed; blue: root) and shared (yellow) between habitats. *: 417 genera accounting for 15.25% relative abundance exclusively found in rhizosphere and with thus unknown origin.

"seed origin" and "*Rhizoctonia*-tolerance" explain approximately the same amount of variance in both Bray-Curtis (R^2 = 0.029 and R^2 = 0.027) and weighted UniFrac distances [R^2 = 0.014 and R^2 = 0.015, respectively (**Supplementary Table 7**]. When exclusively testing root samples (seed origin = Italy) grown in the three different substrates, the factor "*Rhizoctonia*-tolerance" explains a higher percentage of variance than the factor "Cultivar" for both beta diversity indices (**Supplementary Table 8**).

Taxa significantly and positively correlated with *Rhizoctonia*-tolerance in seeds are *Halomonas* spp., *Paenibacillus* spp, *Enterobacter* spp., and *Kosakonia* spp. (**Figure 5A**), while *Massilia* spp. is higher in seeds of susceptible cultivars. In soilless roots, *Firmicutes*, mainly represented by *Paenibacillus* (except for LAE: *Pullulanibacillus* and *Bacillus*), are higher abundant in *Rhizoctonia*-tolerant cultivars than in susceptible ones, and *vice versa* for *Actinobacteria* (**Supplementary Table 9**), mainly represented by *Curtobacterium*. For the root samples, clustering of samples in PCoA plots due to *Rhizoctonia*-tolerance is less pronounced than in seeds (**Figure 5B**). A general trend across seeds, *in vitro*, and *in vivo* root samples is the higher abundance of *Firmicutes* in *Rhizoctonia*-tolerant cultivars.

Root communities of *Rhizoctonia*-tolerant cultivars show a trend (non-significant) towards higher r^S-values of *Gaiellales* (ad *Actinobacteria*) and *Rhizobiales* (ad *Proteobacteria*). r^S-values of *Bacteroidetes* and *Verrucomicrobia* are significantly (p = 0.02) higher in roots of susceptible.

Genera significantly higher in *Rhizoctonia*-tolerant cultivars differ according to used substrates (**Figure 5C**). However, the genus *Kosakonia*, which is one of the taxa correlated with *Rhizoctonia* tolerance in seeds, tends to be higher abundant in *Rhizoctonia*-tolerant root samples across all substrates.

The Impact of Substrates on Root Microbiota

Root samples of BEL, BER, LAE, and MAT grown in the three different substrates (potting soil, vermicompost and mixtures) were merged in a data subset. Differences in the overall composition of root microbiomes are already apparent in high taxonomic ranks. Vermicompost addition to potting soil highly increases r^S of *Proteobacteria* across all cultivars. *Firmicutes* and *Bacillus* spp. in particular are found in high abundance in pure vermicompost (**Figure 6A**). Results for pairwise Kruskal-Wallis test between "substrate" groups were filtered for genera that a) have r^S >1% in at least one of the substrate groups and b) show high r^S-values in one of the pure substrates and intermediate in mix of vermicompost-treated potting soil. Six genera matching these prerequisites were thus shown to be gradually enriched with increased use of vermicompost (**Figure 6A**).

The factor "substrate" explains around 68% of the variance in both Bray-Curtis and weighted UniFrac distances (**Supplementary Table 8**). In PCoA visualization plots, samples clearly cluster due to substrate (**Figure 6B**).

Cultivation-Dependent vs. -Independent Microbiome Assessment

Bacteria were isolated to find bioactive strains and compare cultivation-dependent to -independent approaches. We successfully isolated and identified 339 bacterial strains belonging to at

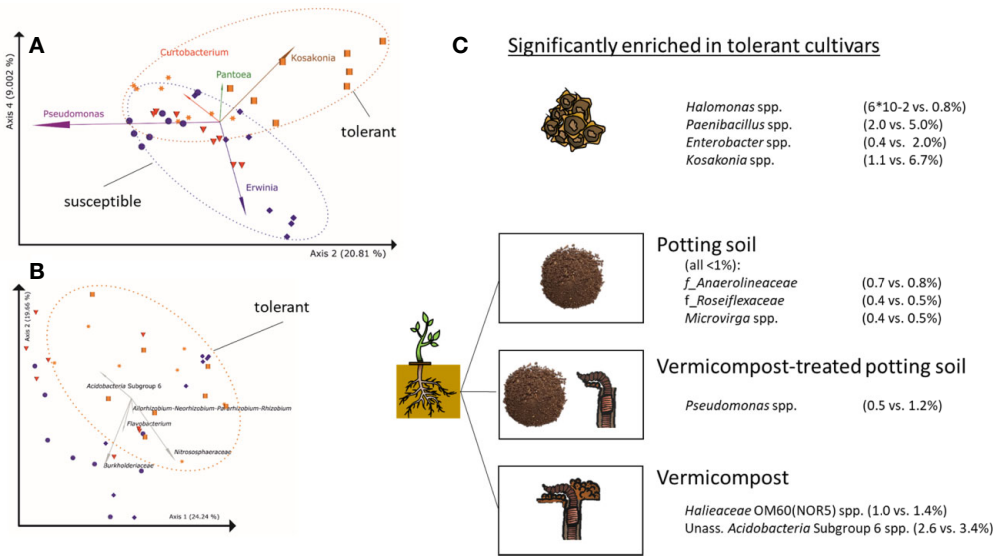


FIGURE 5 | Differences in bacterial root communities between *Rhizoctonia solani*-susceptible (violet), effective (red) and –tolerant (orange) cultivars **(A)** PCoA-biplot of Bray-Curtis dissimilarities in seeds on genus level; spheres: cv. BELLADONNA; diamonds: cv. BERETTA; triangles: cv. ISABELLA; squares: cv. LAETITIA; stars: cv. MATTEA. Note, that PCoA axes 2 and 4 separate the communities based on *Rhizoctonia* tolerance, while axes 1 and 2 separate them due to their origin (**Figure 2B**). For the visualization of the other combinations of PCoA axes, see **Supplementary Figure 1**. **(B)** PCoA-biplot of Bray-Curtis dissimilarities in roots on genus level; **(C)** Taxa significantly enriched in *Rhizoctonia* tolerance cultivars (genus level) in seeds and roots grown in potting soil, vermicompost-treated potting soil and vermicompost.

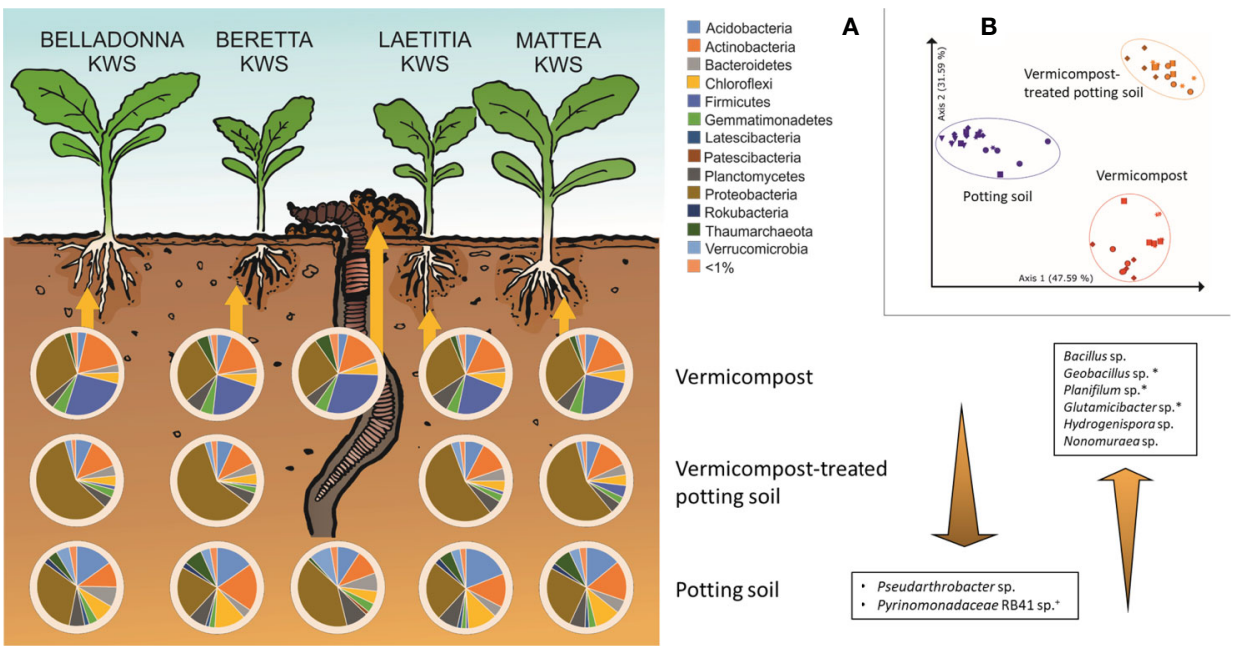


FIGURE 6 | Influence of different substrates on bacterial root communities in sugar beet. **(A)** Composition of bacterial root communities in different substrates on phylum level. Bacterial genera displayed that are significantly different (Bonferroni-corrected $p < 0.05$) between substrates, have >1% mean relative abundance and have intermediate abundance in VC-PS samples. $^{+}$: unique for potting soil; *: unique for vermicompost; PS: potting soil; VC-PS: vermicompost-treated potting soil; VC: vermicompost. **(B)** PCoA-plot of Bray-Curtis dissimilarities of root samples grown in different substrates, only samples with seed origin from Italy; spheres: cv. BELLADONNA; diamonds: cv. BERETTA; squares: cv. LAETITIA; stars: cv. MATTEA.

least 137 different species from pure vermicompost (n =192; 73 species), roots grown in vermicompost-treated potting soil (n = 95; 41 species) and roots grown in pure vermicompost (n = 112; 56 species, **Supplementary Table 10**). The most frequently isolated species associated with vermicompost was *Agromyces flavus*. Additionally, we found 62 strains of at least 20 different *Bacillus* species, with *B. firmus* (n = 15) being the most frequently isolated species. Furthermore, the diversity of *Microbacterium* spp. (n = 19; six species) and *Streptomyces* spp. (n = 12; 9 species) is worth mentioning.

Results of cultivation-dependent assessment clearly differ from amplicon data. On order level, a third up to half of all orders present in the amplicon data could be cultivated. When comparing r^{ζ}-values between cultivated strains and amplicon data, the most apparent difference is the high proportion of *Micrococcales* in the isolate dataset across all three groups. In contrast, sequences of *Micrococcales* account for r^{ζ} <3% to the amplicon dataset. (**Figure 7**). The dominance of *Bacillales* in vermicompost and *Betaproteobacteriales* in roots grown in soil-vermicompost mix is confirmed by both amplicon and cultivation data. *Rhizobiales* reached higher r^{ζ}-values in the amplicon dataset across all groups.

Inducing *Rhizoctonia* Tolerance Using a Consortium Containing *Ps. poae* Re*1-1-14

Sugar beet seeds with standard fungicide and were compared to a combined treatment with both bacterial consortium and fungicide treatment. The percental change in RI, total number of beets and number of healthy beets due to bacterial treatment were compared. Statistical pairwise comparisons did not result in significant differences ($p > 0.05$), most likely due to the patchy infestations of *Rhizoctonia* observed in the field and subsequently high standard deviation in the dataset. Nevertheless, trends towards different responses due to cultivar were observed: the application of the bacterial consortia showed strongest effects in the *Rhizoctonia*-susceptible cultivar, especially at the field with higher soil humidity and thus higher pathogen pressure ("Kasten"). Under these conditions a mean RI decrease of 26% as well as an increase in the number of healthy sugar beets of 65.4% relative to standard treatment could be achieved (**Supplementary Figure 2**) in susceptible cultivars. For tolerant or moderately tolerant cultivars, positive effects regarding RI and number of healthy beets were less pronounced or not observed. In average, the total number of sugar beets at the time of harvest increased by 5% using bacterial treatment.

DISCUSSION

We analyzed microbial communities in seed, roots and corresponding soil to investigate sugar beet microbiota assembly and composition. We found novel aspects, which can be translated to manage the plant microbiome. The bacterial root community of sugar beet derives from both seed and soil communities. Seeds of all sugar beet cultivars were highly colonized by bacteria and carried a selective core of the sugar beet microbiome, which contributed significantly to the rhizosphere assembly. We found relatively high alpha diversity values for the sugar beet seeds, with results comparable to pumpkin seeds (Adam et al., 2018) but significantly lower than in sugar beets at harvest (Kusstatscher et al., 2019). The high proportion of *Proteobacteria, Actinobacteria, Firmicutes* and *Bacteroidetes* found in sugar beet seeds is quite typical for seed microbiota (reviewed by Nelson, 2018). However, especially *Enterobacteriaceae* were identified as important component in seeds. For instance, *Enterobacteriaceae* and in particular *Klebsiella*, dominate in pumpkin seeds (Adam et al., 2018). *Pantoea*, one of the key taxa in sugar beet seeds, was also found to be dominant in *Brassica* seeds (Barret et al., 2015a)

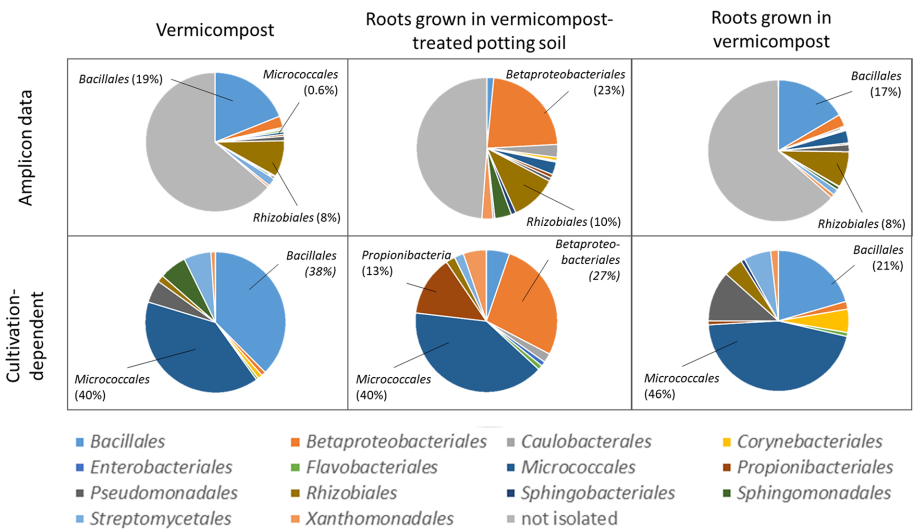


FIGURE 7 | Comparison of microbiome assessment using cultivation-dependent (isolation on R2A plates) and –independent (16SrRNA amplicon sequencing) approaches on order level.

except for oilseed rape (Rybakova et al., 2017). *Pantoea* comprises versatile lifestyles, including plant pathogens, plant growth promoters as well as strains commercially produced for biocontrol of phytopathogens. Thus, *Pantoea* is a model group for niche-specific adaptations (Walterson and Stavrinides, 2015). Furthermore, abundance of some *Enterobacteriaceae* (*Kosakonia* and *Enterobacter*) together with *Paenibacillus* are correlated with *Rhizoctonia* tolerance (**Figures 5A, C**) in sugar beet seeds. *Paenibacillus* is known to contain species with plant growth-promoting and pathogen-antagonistic properties (reviewed by Rybakova et al., 2016). Similarly, some *Enterobacter* species show antagonistic properties against *Rhizoctonia* (e.g. Abdeljalil et al., 2016). *Kosakonia radicinitrans* is known for both plant growth promotion and indirectly reducing pest pressure in *Arabidopsis thaliana* (Brock et al., 2018). Nevertheless, bacterial seed communities rather cluster due to origin (**Figure 2B**, displaying PCoA axes 1 and 2) than to *Rhizoctonia* tolerance (**Figure 5B**, displaying PCoA axes 2 and 4) of the cultivars.

One interesting fact is the higher abundance of different groups of *Firmicutes* (*Pullulanibacillus* in LAE, *Paenibacillus* in MAT, see **Supplementary Table 9**) in seeds of *Rhizoctonia*-tolerant cultivars. This indicates that the function provided by several different groups of bacteria is more important for the plant-microbe interaction than their exact taxonomic position; taxa significantly solely enriched in *Rhizoctonia*-tolerant cultivars account for less than 1% r^{ζ}. Although our study suggests which taxa could be correlated with *Rhizoctonia* tolerance, we are aware that five genotypes are not sufficient to suggest a general trend for microbial shifts due to breeding. This also applies for our results for *in vitro* roots and *in vivo* roots. However, other authors found different taxa associated with response upon fungal invasion with *R. solani* than we found in higher abundances in tolerant cultivars (Chapelle et al., 2016; Carrión et al., 2019).

This study is the first one to track seed-associated bacteria during germination at sequence level and correlate microbial communities to differences in *Rhizoctonia* tolerance. The majority of seed endophytes is able to survive the process of germination, although speaking of species number they only represent a subset of seed-associated microbiota (**Figure 3A**). These species are thus available for the next sugar beet generation, confirming vertical transmission of seed endophytes in sugar beet. Apparently, the proportion of surviving endophytes may still be underestimated due to our experimental setup, including higher exposure of the roots to oxygen and light compared to conditions in soil. This may apply for seed endophytic *Archaea*, which are found in 50% of seed samples but only in 35% of *in vitro* root samples. However, the bacterial community is shifted in the course of germination, which was also observed in *Brassica* plants (Barret et al., 2015b), wheat (Huang et al., 2016) and maize (Johnston-Monje et al., 2016). When focusing on general aspects of the *Rhizoctonia*-tolerant cultivars' microbial seed communities during germination (**Figure 3A**), the most striking facts are, that a) a higher proportion (absolute and relative) of seed-associated bacteria in cv. ISABELLA and cv. MATTEA seeds are still represented in *in vitro* root samples, and b) cv. LAETITIA shows the highest seed alpha diversity across all tested cultivars. Although we know that bacterial alpha diversity is usually correlated with pathogen tolerance (van Elsas et al., 2012), a high diversity may not be necessary if members of the microbial community provide all important functions to the plant-microbe-pathogen interaction. We hypothesize this to be the case in cv. ISABELLA and cv. MATTEA since the majority of seed endo- and epiphytes survive in the germinated seedlings.

The drop in microbial communities' diversity during germination (**Figure 3B**) is usually interpreted as selective force exerted by the seedling, favoring fast-growing r-strategists like *Pseudomonas*, *Bacillus* or *Trichoderma* (Barret et al., 2015a). In our dataset, *Methylobacterium* dominated soilless roots and they are regarded to be typical K-strategists due to their ability to metabolize C1-compounds. Therefore, we suggest the indirect selective force of the germinating host plants rather to favor seed endophytes that are adapted to their hosts' specific genotype (Berg and Raaijmakers, 2018) or the present cultivation conditions (Bergna et al., 2018). Given that this turnover of the seed core is proportionally more or less stable over generations, there are niches in the seed microbiome that can be colonized with bacteria enriched from soil, shaping the seed microbiome of the next generations' seeds (Bergna et al., 2018).

The soil microbiome can influence the rhizosphere microbiome composition of the seedlings and thereof influences the host (Nelson, 2018). We confirm soil as the main source of diversity in sugar beet rhizosphere. Nevertheless, a considerable amount of rhizosphere-inhabiting bacteria exclusively originates from seeds, and 26.8% of all bacterial genera could be provided by either bacterial soil or seed endophyte communities. Interestingly, 417 genera accounting for 15.3% of the rhizosphere community could not be traced back to either seed or soil (**Figure 4B**). The majority of these genera have low r^{ζ}-values (<0.5%). We regard these members of the root microbiome to be enriched in rhizosphere but under the detection threshold in soil and seed samples. This explains why alpha diversity in roots (Shannon H = 9.61 ± 0.56) is significantly ($p = 0.02$) higher than in soil samples (Shannon H = 8.94 ± 0.05). The differences in microbial communities due to seed origin, cultivar and *Rhizoctonia* tolerance are more pronounced in seeds than in roots. *Roseiflexaceae* and *Anaerolineaeceae*, which are significantly enriched in *Rhizoctonia*-tolerant cultivars, are worldwide distributed taxa (GBIF.org, 2020), found in water as well as soil habitats and mainly comprise yet monotypic genera. The genus *Microvirga* on the other hand is frequently found in root nodules of legumes in temperate climates (e.g. Msaddak et al., 2017). Seed origin was an important variable for bacterial seed communities. One should consider that crops are usually planted across large geographical areas and location-dependent bacterial communities were already revealed in maize (Johnston-Monje and Raizada, 2013) and common beans (Klaedtke et al., 2016). Although community differences due to the origin of seeds in both alpha and beta seed diversity indices are obvious and apply to several different bacterial taxa (**Figures 2A–C**), the cultivar—and thus the genotype as well as the phenotype (e.g. root architecture) of the host plant—is the more important factor explaining the seed communities' variance.

Considering earthworms, their ecosystem services and their microbes in agricultural practices holds a big potential for the agriculture of the future (Singh et al., 2020). The microbial community of vermicompost, as a product obtained mainly from organic litter earthworm casts, clearly differs from potting soil, with especially higher proportions of *Bacillus* (5.22 vs. 0.02%). High abundance of *Bacillus* spp. relates to the processing of organic matter in the gut of earthworms. In the course of digestion, the microbial community is exposed to several changes in conditions, including pH neutralization, higher water content, complete anoxia, secretion of digestive enzymes and enrichment of organic compounds including fermentation products from other microbes (reviewed by Drake and Horn, 2007). These conditions on the one hand favor facultative anaerobic or aerobic bacteria that are able to form endospores (Drake and Horn, 2007), and on the other hand activate endospores of *Bacillus* (Fischer et al., 1997). The genus *Bacillus* contains several species that are known for direct as well as indirect phytopathogen antagonism and plant growth-promoting effects (e.g. Pérez-García et al., 2011). We observed higher abundance of *Firmicutes*—in particular the genus *Bacillus*—in roots of *Rhizoctonia*-tolerant cultivars grown in untreated and vermicompost-treated potting soil. When using pure vermicompost as substrate, abundance of Bacillus was comparably high (4.1%–6.2% r^S) in all cultivars. We interpret this as specific enrichment of pathogen-antagonistic bacteria by *Rhizoctonia*-tolerant sugar beet cultivars from the substrate. Since the abundance of *Firmicutes* is generally higher in vermicompost than in potting soil, enrichment in the rhizosphere may not be necessary to achieve pathogen suppression. Some basic information on the microbial community when using vermicompost as substrate are concordant in both cultivation-dependent and –independent approaches. This includes the dominance of *Bacillales* in vermicompost-associated samples and the dominance of *Betaproteobacteriales* in vermicompost-treated potting soil samples (**Figure 7**).

The effect of bacterial control agents in field trials was strongest for the *Rhizoctonia*-susceptible cultivar BERETTA, indicating a strong influence of the microbial community in the early stage of rhizosphere establishment. Applying bacterial control agents to *Rhizoctonia*-tolerant cultivars did not result in further increased, but partially showed slightly decreased health parameters. This may indicate interference of the applied control agents with native root bacteria. The bacterial treatment consisted of three strains, of which *Pseudomonas poae* RE*1-1-14 was originally isolated from sugar beet. This strain shows an interesting pattern across our dataset (**Supplementary Table 11**), since it is more frequently and in higher abundances in *Rhizoctonia*-tolerant cultivars. The genus *Pseudomonas* itself is frequently found across all life stages of sugar beet and several strains with promising biocontrol potential were found in sugar beet endosphere before (Zachow et al., 2008; Zachow et al., 2010). RE*1-1-14 was initially selected in a screening for antagonistic strains against several different sugar beet pathogens (*Phoma betae*, *R. solani* AG2-2IIIB, *R. solani* AG4 and *Sclerotium rolfsii*; Zachow et al., 2010). Despite the specific ASV was not exclusively found in *Rhizoctonia*-tolerant cultivars, *Ps. poae* seems to be an integral part of *Rhizoctonia*-tolerant cultivars and provide important plant-protecting functions to the seedling. Interestingly, *Ps. poae* is more frequently found in sugar beets originated from France, where sugar beets are grown on much larger geographical areas (EUROSTAT, 2019) and historically for a longer time span compared to Italian acreages. These factors are known to favor the development of soil communities suppressive to certain pathogens (Mazzola, 2002; Eberlein et al., 2020). Continuous cropping of sugar beet is known to influence the field soil community, accelerating the abundance of different taxa in the microbial soil community over time (Huang et al., 2020). We found partially cultivar-specific differences between root communities. Thus, continuous cropping of self-reproduced genotypes may shape both bacterial soil and seed endophyte communities, potentially leading to a cultivar-dependent enrichment of taxa that are beneficial and/or usually uncommon in seeds.

CONCLUSIONS

Overall, found differences in the microbiomes of *Rhizoctonia*-susceptible and -tolerant sugar beet cultivars across various life stages. In seeds, the genus *Kosakonia* may play a role in pathogen tolerance. Seed communities in sugar beet differ due to seed origin and cultivar; differences are more pronounced than in seedling roots and rhizospheres, although substrate heavily determine root community structure. Further investigation with a higher number of different *Rhizoctonia*-susceptible and tolerant cultivars are needed for confirming general trends in sugar beet-associated bacterial communities with *Rhizoctonia*-tolerance. This investigation can be explored in more detail by implementing crop breeding strategies that include the trait "plant microbiome". Future strategies for sustainable agriculture might be able to include microbiome management.: 1) Selection of seed production sites could include soil microbiome analysis as seeds take up sets of microbes differently involved in stress response in the next generation. 2) The crop/plant microbiome can be adjusted *via* seed treatments that add further microorganisms to the soil 3). The entire soil microbiome can be managed by microbiome transplants.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ebi.ac.uk/ena, PRJNA299370 https://www.ebi.ac.uk/ena, PRJEB37140.

AUTHOR CONTRIBUTIONS

AW, GB, CZ, and HM drafted the manuscript. CZ, AW, HM, AG, NT, RT, and GB planned the experimental setup. AW, CZ,

and HM performed molecular, microbial, and *ad planta* work. AW conducted bioinformatic analyses. AW, CZ, HM, GB, NT, and RT either planned and/or performed the field trials. 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.2020.560869/full#supplementary-material

SUPPLEMENTARY FIGURE 1 | PCoA-biplots of sugar beet seed endophyte communities on genus level displaying Bray Curtis dissimilarities using PCoA axes 1 and 3 **(A)**, 1 and 4 **(B)** and 2 and 3 **(C)**.

SUPPLEMENTARY FIGURE 2 | Results of field trials for relative in-/decrease of Rhizoctonia disease index (RI, according to Büttner et al., 2004), number of sugar beets and number of healthy sugar beets for the cultivars BER, ISA and MAT using a consortium of three bacterial antagonists additionally to standard fungicide treatment. Results are relative to sugar beet seeds with standard fungicide treatment. Comparisons did not result in significant differences but trends towards stronger positive effects in the Rhizoctonia-susceptible cultivar.

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Exploring the Microbiota of East African Indigenous Leafy Greens for Plant Growth, Health, and Resilience

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Indigenous leafy green vegetable crops provide a promising nutritious alternative for East African agriculture under a changing climate; they are better able to cope with biotic and abiotic stresses than cosmopolitan vegetable crops. To verify our hypothesis that the associated microbiome is involved, we studied archaeal and bacterial communities of four locally popular leafy green crops in Uganda (*Bidens pilosa*, *Solanum scabrum*, *Abelmoschus esculentus*, and *Gynandropsis gynandra*) and of four plant microhabitats (phyllosphere, root endosphere, rhizosphere, and soil) by complementary analyses of amplicon and isolate libraries. All plants shared an unusually large core microbiome, comprising 18 procaryotic families but primarily consisting of *Bacillus*, *Sphingobium*, *Comamonadaceae*, *Pseudomonas*, and one archaeon from the soil crenarchaeotic group. Microbiome composition did not differ significantly for plant species but differed for microhabitats. The diversity was, in general, higher for bacteria (27,697 ASVs/H = 6.91) than for archaea (2,995 ASVs/H = 4.91); both groups form a robust network of copiotrophic bacteria and oligotrophic archaea. Screening of selected isolates for stress and plant health protecting traits showed that strains of *Bacillus* and *Sphingomonas* spp. div. constituted a substantial portion (15–31%) of the prokaryotic plant-associated communities. Across plant species, microbiota were characterized by a high proportion of potential copiotrophic and plant-beneficial species, which was not specific by plant species. The use of identified plant-beneficial isolates could provide the basis for the development of consortia of isolates for both abiotic and biotic stress protection to improve plant and ecosystem health, ensuring food security in East Africa.

Keywords: plant microbiome, archaea, leafy green vegetables, PGPR, amplicon analysis

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INTRODUCTION

The adoption of sustainable agriculture practices is necessary in order to feed growing populations, especially in Sub-Saharan Africa, where food insecurity and malnutrition indices are the most alarming globally (Sikora et al., 2019). Sustainable agricultural practices are additionally important for biodiversity and ecosystem services, the maintenance of which is a global challenge and which is attracting increasing attention (Mariotte et al., 2018). Eastern African farming systems

comprise a tapestry of crops and livestock, which is dominated by smallholders and characterized by low productivity (Vanlauwe et al., 2014). A key factor explaining this low productivity is the high pest and pathogen pressure, and consequent production losses due to bioconstraints (Venkateswarlu et al., 2012; Dean et al., 2012; CIAT; BFS/USAID, 2017). The application of synthetic, chemical pesticides is a common coping strategy by farmers, but often using inappropriate, adulterated, out of date products, or generic compounds against which resistance has long since built up (Coyne et al., 2019). Consequently, in these smallholder systems, excessive misuse of pesticides may prevail, especially on crops which are prone to diseases and pests, such as vegetables (James et al., 2010). The detrimental effects of pesticide misuse are mostly reported in relation to human and animal health, but there are important considerations for ecosystem health too (Rosenstock et al., 1991; al-Saleh, 1994; Lemaire et al., 2006). The challenge therefore, is how to reduce the use of, and reliance on, pesticides and simultaneously improve yields while maintaining, facilitating, or enhancing biodiversity in farming systems.

A range of options is available to reduce dependency on synthetic pesticides, while their suitability depends on prevailing circumstances (Bender et al., 2016; Sikora et al., 2019). With the domestication of crops, their commercial exploitation, and the focus on breeding for ever-higher yielding cultivars, there has been a concomitant loss of resistance to stress factors as well as a decrease in microbial diversity (Mariotte et al., 2018; Cordovez et al., 2019). In contrast, indigenous plants that are less commercially exploited and less highly bred, but locally produced or gathered from natural habitats even, tend to be more robust and resilient (Venkateswarlu et al., 2012). In addition to their nutritional value, such properties have led to an increased interest in indigenous leafy green vegetables in Africa, where there is a need to raise the daily nutritional intake (Cernansky, 2015). Preferred traits include high levels of protein, iron, and other valuable nutrients, as well as their ability to better withstand biotic and abiotic stresses, compared to popular non-native vegetables, e.g., kale and cabbage (Kumar et al., 2010; Bartolome et al., 2013; Onyango et al., 2013; Cernansky, 2015; Ronoh et al., 2019). Further, these plants have a short and adapted life cycle, resulting in a lower vulnerability to irregular rainfall due to climate change. Speculation remains, however, why these plants are so robust and whether this robustness can be transferred between crops.

Plant fitness is a phenotypic expression and genotypically determined but can be modified by external factors, such as their associated microbiota. Plants and their associated microbiota combined represent a functional unity, the plant holobiont (Vandenkoornhuyse et al., 2015). Plant species are host to a high diversity and complexity of microbial communities, which vary depending on various external influences (Yeoh et al., 2017). These microbial communities can influence plant growth, productivity, adaptation, and health (Bulgarelli et al., 2013; Berg et al., 2016). Modes of action include nutrient supply, plant hormone production, and antagonism toward pathogens (Berg, 2009; Lugtenberg and Kamilova, 2009). Plant-microbe interactions have largely focused on bacteria and fungi, although archaea are widespread and stable components of plant microbiomes (Hardoim et al., 2015; Moissl-Eichinger et al., 2018). They have the potential to directly interact with the host plant by supporting nutrient supply and growth promotion *via* auxin biosynthesis (Taffner et al., 2018, 2019), while antagonistic properties are not yet known (Moissl-Eichinger and Huber, 2011). Our hypothesis is that the microbiome strongly contributes to the fitness and health of the indigenous leafy green vegetables and that the archaeal community is an important component. Identification of key species within these communities may be crucial to develop suitable biologically based options toward increased robustness and health in crops, and consequently toward the sustainable improvement of smallholder crop production systems in rural areas of Africa.

This study was aimed at characterizing the microbial communities of four leafy green crops grown in rural, smallholder conditions in Uganda, including blackjack (*Bidens pilosa* L.), nightshade (*Solanum scabrum* Mill.), okra (*Abelmoschus esculentus* Moench), and spiderwisp [*Gynandropsis gynandra* (L.) Briq.] and to assess the role of the bacterial and archaeal community on plant health. To achieve this, we combined next-generation sequencing and characterization of bacterial isolates as well as screening for antagonism toward five phytopathogenic fungi, including species of the top 10 economically important crop pathogens worldwide (*Botrytis cinerea*, *Fusarium oxysporum*, *Fusarium verticillioides*, *Sclerotium rolfsii*, and *Verticillium dahliae*; Dean et al., 2012).

MATERIALS AND METHODS

Experimental Design and Sampling Procedure

The leafy green vegetables blackjack, okra, nightshade, and spiderwisp were sampled in Kasangati, Uganda (0° 26' 33"N, 32° 36' 19"E) in April 2017. Four samples, each consisting of a single plant (blackjack, spiderwisp, and okra) or three individual plants (nightshade), were gently removed with the aid of a spade, placed in sealed air-tight plastic bags, stored in a cool box, and transferred to the laboratory; four bulked soil samples were also collected and stored in separate plastic bags. Soil parameters were analyzed by "AGROLAB Agrar und Umwelt GmbH" (Sarstedt, Germany). The soil texture was sandy loam with pH = 5.9, organic matter content of 3.7%, and nutrient contents of K = 413 mg kg^{-1}, P = 86 mg kg^{-1}, and Mg = 214 mg kg^{-1}. In order to homogenize the samples, 3 g of the phyllosphere (plant leaves and stalks), 5–10 g root material with adhering soil, and 5 g of soil per replicate were physically mixed in a BagMixer (Interscience, St. Nom, France) with 15 ml of 0.85% NaCl. Samples of root-adhering soil are further called rhizosphere. To obtain root endosphere samples, root samples were further surface sterilized with a 4% sodium hypochloride solution (NaClO) for 3 min, washed four times with 0.85% NaCl, resuspended in 15 ml NaCl, and then physically crushed with a sterile mortar and pestle. Samples were centrifuged at 16,500 *g* for 20 min at 4°C, and DNA extracts were then stored at −70°C for further processing.

Isolation and CFU Determination of Bacterial Strains

Bacterial strains were isolated according to the protocol of Bragina et al. (2012). Briefly, 100 µl of the 15 ml 0.85% NaCl suspensions of each microhabitat-sample were plated onto NBII agar (Sifin, Berlin, Germany) plates in dilutions ranging from 10^{-2} to 10^{-5}, incubated for 5 days at 20°C, and number of colony forming units (CFUs) determined and equated to fresh weight of the samples. A total of 512 randomly selected CFUs were isolated and stored in 20% glycerol at −70°C for further characterization.

Screening of Antagonistic Bacteria and Antifungal VOCs Production

The 512 bacterial isolates were each streaked onto a Waksman Agar (WA)-plate and exposed to a fungal pathogen, following the protocols of Berg et al. (2006). The fungal phytopathogens *B. cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii*, and *V. dahliae* were obtained from the strain collection of the Institute of Environmental Biotechnology (Graz University of Technology, Austria). Screenings were performed in triplicate and evaluated according to their antagonistic activity against pathogens according to Wolfgang et al. (2019). Isolates with strong antagonistic activity were further tested for volatile organic compound (VOC) production, using a two-clamp VOC assay (Cernava et al., 2015).

BOX-PCR Fingerprinting and Sequencing of Antagonistic Bacteria

BOX-PCR was performed to resolve bacterial genetic diversity, according to the protocol of Rademaker and de Bruijn (1997). Shortly, colonies of 20 bacterial isolates with strong antagonism against all tested pathogens were solubilized, transferred into glass-bead filled tubes, ribolyzed, and centrifuged. PCR amplification was conducted using the BOXA1R primer 5'-CTA CGG CAA GGC GAC GCT GAC G-3'. After separation by gel electrophoresis, resulting band pattern was compared with "Gel Compar II" V.5.1 (Applied Maths, Kortrijk, Belgium). Different isolates were further sequenced based on the 16S rRNA gene fragment and taxonomically identified by manual BLAST search.[1]

Abiotic Stress Assays and Phosphate-Solubilization Tests

Bacterial isolates with antagonistic activity toward the tested phytopathogens were additionally screened for resistance to abiotic stress, including drought, salinity, and reactive oxygen, as well as their potential to solubilize phosphate, as described by Zachow et al. (2013). In reactive oxygen species stress assays, bacterial isolates were cultivated overnight in LB (Lennox) medium (Carl Roth, Karlsruhe, Germany). Overnight cultures (5 µl) were added to 96-well plates filled with 195 µl LB in 10 different concentrations of tellurite (1, 3, 5, 7, 9, 10, 13, 15, 18, and 20 µg/ml), and hydrogen peroxide (from 100 to 1,500 µmol in 200 µmol steps, 1,750–4,000 µmol in 250 µmol steps), respectively. Growth of each isolate was measured after 24 h incubation at 30°C under agitation in four replicates using a plate reader (Infinite 200, Tecan Trading AG, Switzerland) at a wavelength of 600 nm (OD_{600}). For evaluation of the tolerated osmolarity level, bacterial isolates were cultivated in LB media with various NaCl concentrations (from 0 to 15% in steps of 1%). Growth was measured in four replicates after 24, 48, 72, and 144 h using the plate reader at 600 nm. In order to test for drought resistance, 20 µl of an overnight culture were dried under sterile conditions in a 96-well plate and were resuspended in 20 µl 0.9% NaCl after 1, 2, 5, 7, 14, 60, and 80 days. Further, 10 µl of the resuspended cells was dropped onto LB-agar plates in a dilution series, incubated and number of CFUs determined.

Screening for Plant-Growth Promoting Activities

Growth-promoting activities of bacterial isolates were tested on tomato (*Solanum lycopersicum* L. cv. Moneymaker, Austrosaat AG, Austria) plants according to Zachow et al. (2013). Tomato seeds were primed with bacterial cultures derived from three NBII plates grown overnight at 30°C suspended in 20 ml sterile water and were incubated for 4 h under agitation. Number of CFU per ml and OD_{600} of the suspensions were determined before seed priming (**Table 1**). Two germination pouches per strain were prepared with 8–9 seeds each. After 15 days, plants were harvested, leaf and root fresh weight were recorded. Roots were further pestled, and suspensions were plated on NBII plates in a dilution series for CFU determination. Plant growth was analyzed using Mann–Whitney U test.

Isolation of Total-Community DNA and Illumina Sequencing

Community DNA pellets from each microhabitat of soil, rhizosphere, root-endosphere, and phyllosphere of the four leafy green crops were subjected to PCR-based barcoding. First, extraction of DNA pellets was conducted using "FastDNA Spin Kit for soil" (MP Biomedical, Eschwege, Germany). PCR-products were purified with GENECLEAN TurboTM Kit (MP Biomedicals, Eschwege, Germany), following the manufacturer's instructions for genomic DNA. The bacterial PCR approach was carried out with the Illumina barcode universal bacterial primer set 515f-806r (Caporaso et al., 2011) and PNA Mix (Lundberg et al., 2013) to remove host plastid and mitochondrial DNA. In order to amplify the archaeal 16S rRNA gene, a nested PCR was performed using the archaea-specific primers 344f and 915r in the first PCR. In a second PCR approach, the modified primer pair S-D-Arch-0349-a-S-17/S-D-Arch-0519-a-A-16 (here 349f/519r; Klindworth et al., 2013) with an additional 10 bp primer-pad (TATGGTAATT/AGTCAGCCAG) and linker (GT/GG) was used, according to the protocols of the Earth Microbiome Project (Walters et al., 2016). In a third PCR, Golay barcodes were annealed (Hamady et al., 2008). All PCR reactions were conducted as previously described (Taffner et al., 2019).

[1]https://blast.ncbi.nlm.nih.gov/

TABLE 1 | Abiotic stress confrontation assays.

Strain-ID	Species	Origin	Drought	H_2O_2 (µmol/ml)	NaCl (24 h)	NaCl (48 h)	NaCl (72 h)	NaCl (6 days)	OD_{600} (ONC)	CFU/ml priming suspension	ΔRFW (%)	ΔSFW (%)	ΔGR
Soil-I-11	*Bacillus* sp.	Soil	Tolerant	0	0	0	8%	6%	1.8	10^{13}	67	46	+
Soil-I-14	*Bacillus* sp.	Soil	Tolerant	2,000	5%	7%	7%	7%	1.6	$4.2^{*}10^{14}$	83	6	+
Soil-I-45	*Sphingomonas* sp.	Soil	Tolerant	0	0	0	8%	11%	2.3	$3.2^{*}10^{14}$	−11	11	+
ORE-30	*Sphingomonas* sp.	Okra root	Tolerant	100	0	0	8%	10%	2.6	$>3^{*}10^{14}$	0	−24	+
ORE-44	*Sphingomonas* sp.	Okra root	Tolerant	0	0	0	8%	11%	2.8	$>3^{*}10^{14}$	21	−10	0
NSRE-37	*Bacillus* sp.	Nightshade root	Tolerant	900	0	0	0	0	1.7	$5^{*}10^{12}$	88	56	+

Growth after desiccation was measured by CFU/ml: tolerant: CFU above 10^5 after drought for 88 days. Reactive oxygen species test performed with hydrogen peroxide (H_2O_2): highest concentration of H_2O_2, the isolate could still tolerate. NaCl(x): maximum concentration (w/v) of NaCl in growth medium resulting in increased OD_{600} relative to the control after time x. Increase in root fresh weight (ΔRFW), shoot fresh weight (ΔSFW), and germination rate (ΔGR; +, increase; −, decrease; 0, no effect) relative to the corresponding control group.

Bacterial and archaeal PCR reactions were conducted in triplicate, purified with the Wizard SV Gel and PCR clean-up system (Promega, Madison, WI, United States), and pooled to equimolarity. Sequencing was carried out by Eurofins MWG Operon (Eurofins, Ebersberg, Germany) with an Illumina HiSeq 2500 system.

Bioinformatic Processing of 16S rRNA Gene Fragments

The generated 16S rRNA gene libraries were pre-processed using QIIME version 1.9.1 (Caporaso et al., 2010) and QIIME2 (version 2018.2, Bolyen et al., 2019). First, read quality was checked with FastQC,[2] reads were joined, and barcodes were extracted in QIIME1. Sequences were demultiplexed using the q2_demux plugin and denoised using q2_dada2 (Callahan et al., 2016) Taxonomy was assigned using a naïve Bayes taxonomy classifier (Bokulich et al., 2018) implemented in QIIME2. For taxonomic assignment, SILVA reference data base version 128 was used for bacteria and Silva 16S Archaeal database (349af–519ar 99, otusversion 128) for archaea with a 97 and 99% similarity cut-off, respectively (Quast et al., 2013). Amplicon sequence variants (ASVs) assigned to mitochondria or chloroplasts were removed using taxonomy-based filtering. ASVs were aligned with q2_mafft (Katoh and Standley, 2013), and a phylogenetic tree was constructed with q2_fasttree2 (Price et al., 2010). For estimating diversity metrics, sequence tables were rarefied to 1,210 ASVs (archaea) and 7,444 ASVs (bacteria). For evaluating alpha diversity, Kruskal-Wallis test (all groups and pairwise), alpha rarefaction, Shannon and Faith's phylogenetic diversity index (Faith, 1992) were calculated. Beta diversity was analyzed by principal coordinate analysis (PCoA) plots and ANOSIM based on phylogenetic distance metrics of weighted UniFrac distances (Lozupone et al., 2007) and visualized with the emperor plugin (Vázquez-Baeza et al., 2013). The ANOSIM test was performed with 999 permutations. To test for the influence of microhabitat and plant species, these variables were tested using the plugin Adonis (Anderson, 2001) for bacteria and archaea. To test for significant differences in abundances of identified antagonistic taxa, the bacterial dataset was analyzed using the LEfSe algorithm implemented in https://www.microbiomeanalyst.ca (Chong et al., 2020). The dataset was filtered using the default settings (minimum count for reads of 4, minimum prevalence in samples 20%, low variance filtered based on 10% interquantile range, LDA score = 2.0), rarefied to minimum library size and scaled using total sum scaling. Taxa were compared on family level between each plant species (all microhabitats combined) and soil, as well as within a single plant species between microhabitats. Cytoscape 3.3.0 software was used to visualize the bacterial distribution and network of the core genera (Shannon et al., 2003). ASVs, that were found in >75% of the plant samples, were assigned as interspecific core ASVs of the plant species. ASVs were assigned to genus level, and data of all four plants were combined. Taxa represented in ≥50% of samples across the

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

dataset were assigned as intraspecific core genera. Abundant sequences with a low taxonomical resolution were additionally assigned by using the nucleotide BLAST search.[3]

Nucleotide Sequence Accession Numbers

The 16S rRNA gene fragment amplicon library was submitted to the European Nucleotide Archive (ENA) and can be found under the accession number PRJEB39392.

RESULTS

General Community Structure of Prokaryotes Associated With Leafy Green Vegetables

Sequencing of the 16S rRNA gene fragments originating from the phyllosphere, root-endosphere, rhizosphere, and soil of the leafy greens blackjack, nightshade, okra, and spiderwisp resulted in a total of 10,688,730 high quality bacterial reads and 2,692,299 archaeal reads. After taxonomy-based filtering of mitochondria and chloroplast sequences, the datasets comprised 9,795,981 bacterial reads and 2,663,458 archaeal reads, clustered in a total of 27,697 and 2,995 distinct ASVs, respectively. Unassigned sequences remained in the dataset because we expected a considerable and potentially important part of microbes to be still unknown to science.

[3]https://blast.ncbi.nlm.nih.gov/Blast.cgi

The bacterial core microbiome revealed similarities and differences between the phytobiome composition in respect to the plant genotype and microhabitat (**Figure 1**). In the phyllosphere, *Enterobacteriaceae* (42.2%) and *Streptococcaceae* (14.4%) were dominant in the bacterial community, whereas in the root endosphere and rhizosphere *Enterobacteriaceae* (30.7 and 21.6%, respectively) and *Pseudomonadaceae* (28.0 and 19.0%, respectively) were predominant. In general, *Sphingomonadaceae* (4.2%), *Lactobacillaceae* (3.3%), *Bacillaceae* (2.9%), *Rhizobiaceae* (2.7%), *Comamonadaceae* (2.5%), *Flavobacteriaceae* (2.0%), and *Xanthomonadaceae* (1.5%) were ubiquitous but less abundant. In the phyllosphere of blackjack and Okra, *Streptococcaceae* were dominant, representing around a quarter of the core microbiome. Blackjack and spiderwisp both harbored *Lactobacillaceae* with 12.0–15.3% in the phyllosphere. *Bacillaceae* and *Pseudomonadaceae* were present in the core microbiome of each crop in each microhabitat (1.09–6.33%), with the exception in the spiderwisp phyllosphere, where no *Bacillaceae* were found. Throughout all microhabitats and crops, the fraction of families with an abundance lower than 1% ("others") was relatively high (13.9–21.6%). These bacteria mainly belonged to the families *Oxalobacteraceae* (0.9%), *Caulobacteraceae* (0.9%), unidentified *Acidobacteria* (0.9%), *Sphingobacteriaceae* (0.8%), *Paenibacillaceae* (0.8%), *Rhizobiales* (0.7%), *Chitinophagaceae* (0.7%), *Planctomycetaceae* (0.6%), *Enterococcaceae* (0.6%), and *Alcaligenaceae* (0.5%).

The archaeal communities (**Figure 2**) were clearly dominated by the phylum *Thaumarchaeota* (89.0%). In general, a high proportion of unassigned reads of up to 20.7% was detected,

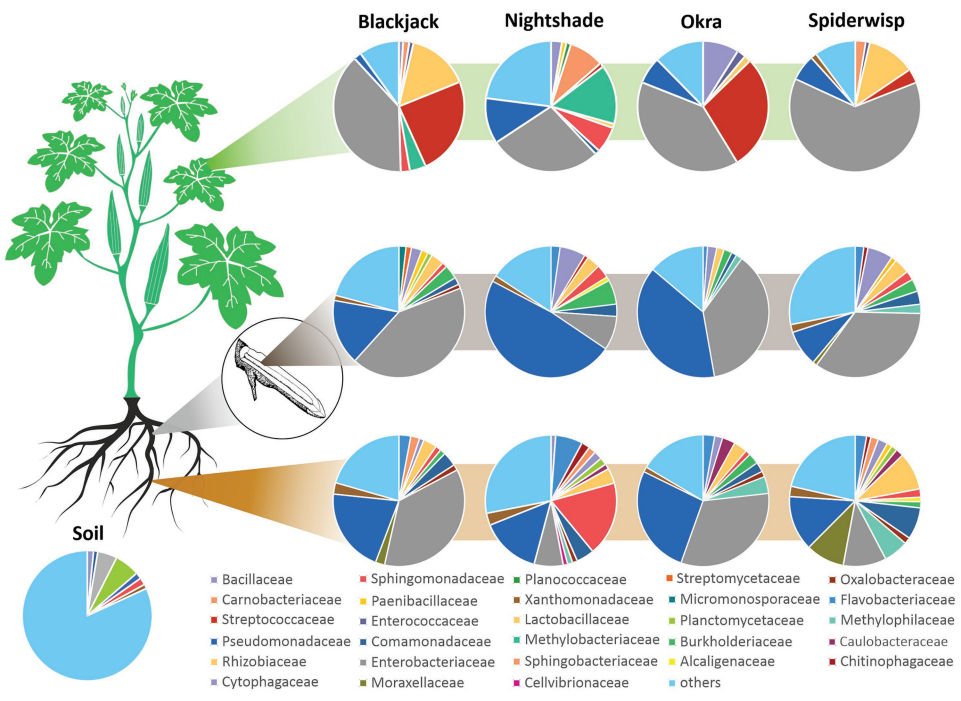

FIGURE 1 | Bacterial core microbiome of leafy greens. The composition of the microbiome of blackjack, nightshade, okra and spiderwisp, and their microhabitats, displayed at the family level: phyllosphere (green stripe), root-endosphere (gray stripe), and rhizosphere (brown stripe). Families with abundances below 1% of total microbiome are captured within "others."

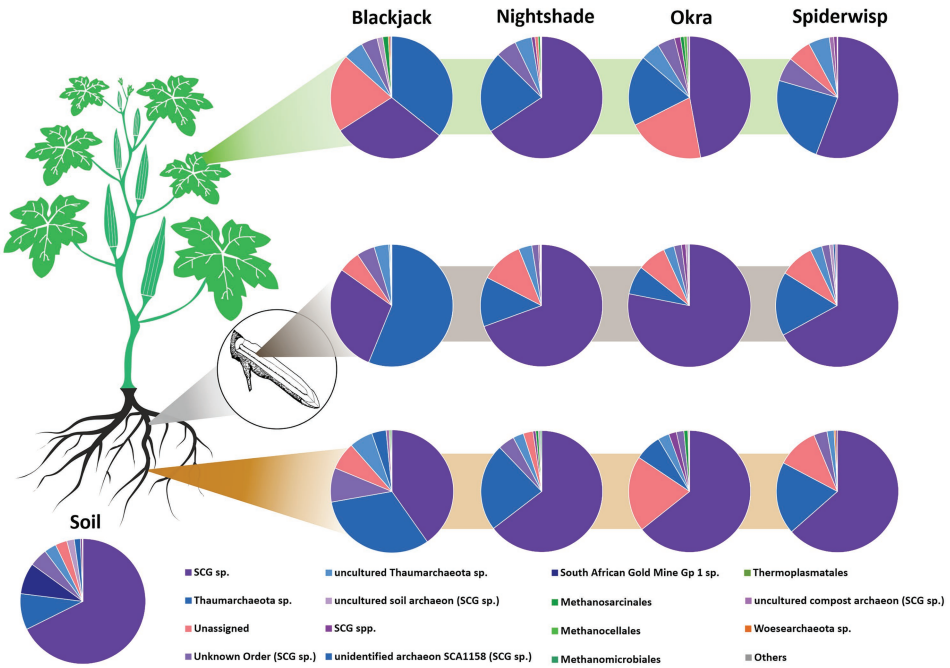

FIGURE 2 | Archaeal community in leafy greens. The composition of the archaeal community of blackjack, nightshade, okra and spiderwisp, and their microhabitats, displayed at the order level: phyllosphere (green stripe), root-endosphere (gray stripe), and rhizosphere (brown stripe).

which were especially associated with blackjack and okra phyllospheres. In all four leafy green crops, *Euryarchaeota* were present but in low relative abundances (0.7–1.0%), except in spiderwisp, in which no *Euryarchaeota* were detected. At the class level, archaea of the soil crenarchaeotic group (SCG) were relatively abundant (56.2%), followed by unassigned *Thaumarchaeota* (22.9%). Archaea of the SCG were especially abundant in nightshade and spiderwisp. Methanogenic archaea of the class *Methanomicrobia* were mainly found in phyllosphere and the root-endosphere samples, except in spiderwisp.

Bacterial Diversity Associated With Leafy Green Vegetables

Diversity metrics based on phylogeny were calculated with QIIME2 to determine similarities and dissimilarities of the bacterial community of the leafy green crops. Shannon's diversity in the plant-microhabitats decreased from rhizosphere to phyllosphere, with an exception for nightshade, which showed the least diversity in root endosphere. However, diversity in the rhizosphere of nightshade was the highest comparing all plants [$H_{(\text{nightshade})}$ = 7.81 ± 0.21]. Diversity within the bulk soil samples was higher [$H_{(\text{soil})}$ = 9.41 ± 0.42], however, compared to the plant species [$H_{(\text{mean})}$ = 6.91 ± 0.16; ranging from H = 5.31 to H = 6.24].

Between microhabitats, a cluster formation (**Figure 3I**) as well as a trend from rhizosphere to phyllosphere, was observed, whereas the rhizosphere bacterial community overlapped, to some extent, with the root endosphere. However, phyllosphere communities were more distinct, while the soil showed a clear cluster, which was significantly different to the other microhabitats with quantitative measures (ANOSIM: R = 0.504 and $p \leq 0.001$). When assigning the same communities to their respective plant species (**Figure 3II**), no distinct clustering could be detected (ANOSIM: R = 0.048 and p = 0.064). Only nightshade had a slightly different clustering pattern. When investigating relationships within and between plants (within-sample), the alpha diversity index was significantly different between microhabitats (**Figure 3III**; p = 0.001), but not between the four plant species (**Figure 3IV**; p = 0.080). The overall group of microhabitats differed in diversity, but with respect to pairwise investigations, this was due to differences in rhizospheres as well as root-endosphere to phyllosphere. Further, group statistics showed that bacterial alpha diversity of the tested leafy green crops was not plant species specific. However, PCoA and Kruskal-Wallis test revealed that microbial diversity was microhabitat-specific. The factor "habitat" explained more variance within the bacterial dataset than the factor "organism" in both Bray-Curtis and weighted UniFrac distances (**Supplementary Table 1**).

Archaeal Diversity Associated to Leafy Green Vegetables

Archaeal alpha diversity indices had similar values in all plant species [$H_{(\text{all})}$ = 4.51–4.95], with the highest archaeal diversity in nightshade [$H_{(\text{nightshade})}$ = 4.95 ± 0.21]. Within plant-associated communities, the diversity of the microhabitats differed only slightly, between the root-endosphere [$H_{(\text{endosphere})}$ = 4.42 ± 0.37] and the rhizosphere [$H_{(\text{rhizosphere})}$ = 4.92 ± 0.23; **Figure 4III**]. Alpha diversity of archaeal communities in bulk soil was higher than in plant-associated communities [$H_{(\text{soil})}$ = 5.26 ± 0.27; **Figure 4IV**].

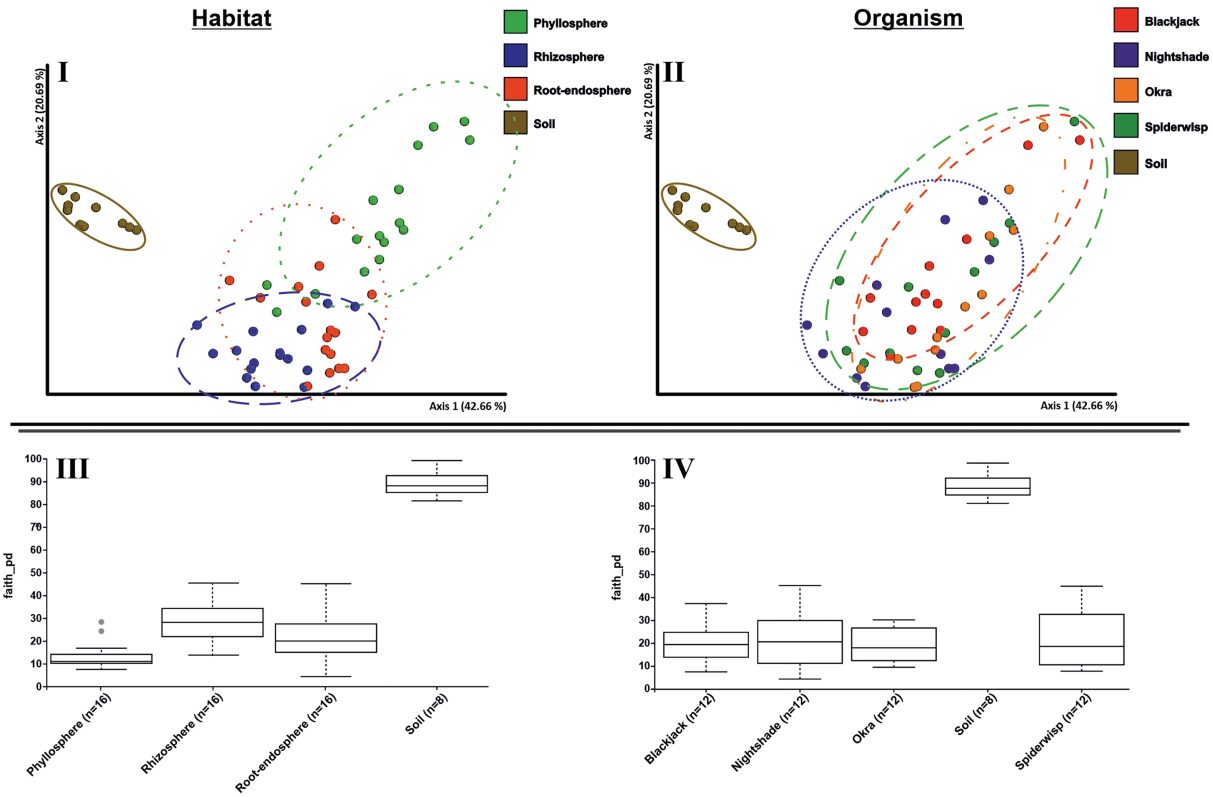

FIGURE 3 | Bacterial alpha and beta diversity of leafy green vegetables. Principal coordinate analysis (PCoA) plots of the 16S rRNA amplicon datasets of four crops (blackjack, nightshade, okra, and spiderwisp) constructed based on phylogenetic distance metrics (weighted UniFrac). The distance between the data points negatively correlates with the similarity of the communities. **(I)** Clusters of the communities based on microhabitat (phyllosphere, rhizosphere, root-endosphere, and soil), and **(II)** based on plant species (blackjack, nightshade, okra, spiderwisp, and soil). Comparison of bacterial alpha diversity based on Faith's phylogenetic diversity of the microhabitats **(III)** and plant species **(IV)**.

The alpha and beta diversity indices of the archaeal community are presented in **Figure 4**. In a PCoA-plot (**Figure 4I**), soil and the rhizosphere communities were clustered, whereas diversities in the root endosphere and phyllosphere were more dispersed. Again, a pattern, from rhizosphere to phyllosphere, was evident, as indicated by the overlapping clusters, with the soil diversity occurring within the rhizosphere diversity. In general, microhabitats showed significant differences in diversity (ANOSIM: $R = 0.226$; $p = 0.001$), with soil showing the highest diversity (**Figure 4III**). Analyzing the beta diversity for plant-type-specific differences, a cluster formation of nightshade and spiderwisp could be seen (**Figure 4II**). These plant species specific differences were confirmed by ANOSIM-test ($R = 0.131$; $p = 0.002$) and were found to be due to nightshade and blackjack ($q < 0.05$) based on pairwise comparison. However, spiderwisp and okra showed similarities ($q > 0.377$). Further, alpha diversity analysis with Kruskal-Wallis (all groups and pairwise) confirmed that archaeal diversity differed, depending on the microhabitat ($p = 0.001$) as well as the plant species ($p = 0.01$), which is due to the significantly different diversity of nightshade (**Figures 4III,IV**). However, pairwise comparison did not establish any differences between plant species ($q > 0.08$). Soil archaeal diversity was significantly different to all plant-associated microhabitats ($q < 0.004$), as well as the phyllosphere to root-endosphere ($q = 0.038$). However, the factors microhabitat and plant species explain <35% of the variance within the archaeal dataset (**Supplementary Table 2**), indicating other important factors determining archaeal community composition.

Analysis of the Core Microbiota of Leafy Green Vegetables

Microbial core communities across blackjack, nightshade, okra, and spiderwisp were cross-linked based on taxonomic analysis at the family level and were visualized as a network (**Figure 5**). In total, 91 features were identified on genus level, with just one belonging to *Archaea*. A large core microbiome of 18 families, such as *Bacillus*, *Sphingobium*, *Comamonadaceae* gen., *Pseudomonas*, and *Rhizobiaceae* gen. (including the archaeal SCG), mainly assigned to *Proteobacteria*, were shared between all four crops. An additional 11 families, also mostly *Proteobacteria*, were common in blackjack, okra, and spiderwisp, thus communities associated with nightshade were more specific. Nightshade and okra shared specific taxa of the genus *Carnobacterium*, while blackjack and spiderwisp both shared *Weissella* and *Acinetobacter*. Each crop was associated with specific bacterial families that were unique in the core microbiome

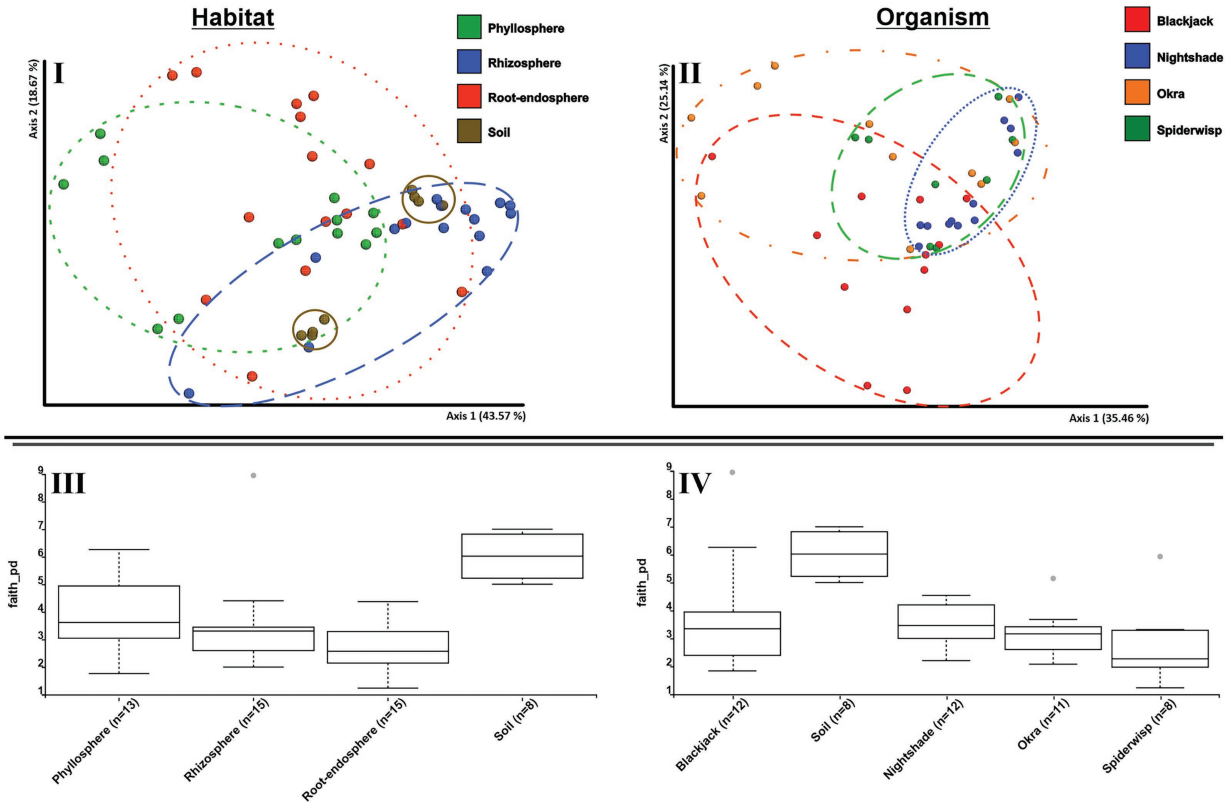

FIGURE 4 | Alpha and beta diversity analyses of archaeal communities associated to leafy green vegetables. PCoA plots based on weighted UniFrac distance metrics show the archaeal community of phyllosphere, rhizosphere, root-endosphere, and soil microhabitats **(I)** of the four leafy green crops blackjack, nightshade, okra, and spiderwisp **(II)**; comparison of alpha diversity based on Faith's phylogenetic diversity of the microhabitats **(III)** and plant species **(IV)**.

of the respective plant species. The number of such distinctive communities were ranged from five (spiderwisp) to nine (blackjack and nightshade) and 11 (okra).

Screening and Identification of Bacterial Antagonists Against Biotic and Abiotic Stresses

Of the 512 randomly selected bacterial isolates taken from the four leafy green crops and bulk soil, 108 isolates showed a high antagonistic activity (clear halo between fungi and bacteria $\geq$ 5 mm) against at least one pathogen (*B. cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii*, and *V. dahliae*), and 23 isolates against four pathogens (**Figure 6**). Screening test results against *V. dahliae* needed a separate evaluation category as the culturing of the fungi required a different procedure and was, therefore, not included into the Venn diagram. A total of 44 bacterial isolates were highly active against *V. dahliae*. Based on these results, a selection of 24 antagonists, mostly antagonistic against all tested pathogens, were chosen for further characterization; 12 of the isolates originated from soil, nine were isolated from root endosphere, and three from the rhizosphere. Genetic characterization of the 24 antagonistic isolates undertaken using BOX-PCR and 16S sequencing identified 16 isolates as *Bacillus* sp. with suggested species *B. siamensis*, *B. velenzensis*, *B. amyloliquefaciens*, *B. methylotrophicus*, *B. vallismortis*, and *B. subtilis*. A further eight isolates were assigned to *Sphingomonas* sp. with hits for *S. echinoides* and *S. glacialis*. Combining the alignment results with similarity pattern of BOX PCR bands, isolates were clustered into five similarity groups (**Supplementary Table 3**).

The resistance to abiotic stresses of antagonistic bacterial strains was further characterized in order to evaluate their potential for application as future biocontrol agents (BCAs). Therefore, abiotic stress tests comprising reactive oxygen species stress tests were conducted (**Table 1**).

The desiccation assay showed that all tested bacterial isolates were highly resistant to drought with a CFU/ml of above 10^5 after 88 days. The ability to resist reactive oxygen could not be shown in the tellurite assay, but when using hydrogen peroxide as stressor three isolates could still be cultured. Isolate "Soil-I-14" showed H_2O_2-tolerance as well as salt tolerance to high levels of NaCl after 24 h. Other isolates needed a longer period to adapt to higher NaCl concentrations and showed tolerance only after an adaption phase of 72 h (**Table 1**).

Further characterization of the mechanism of antagonism using two clamp VOC assays (TCVAs) showed no antagonistic effects of the bacterial isolates against the pathogens, based on VOCs.

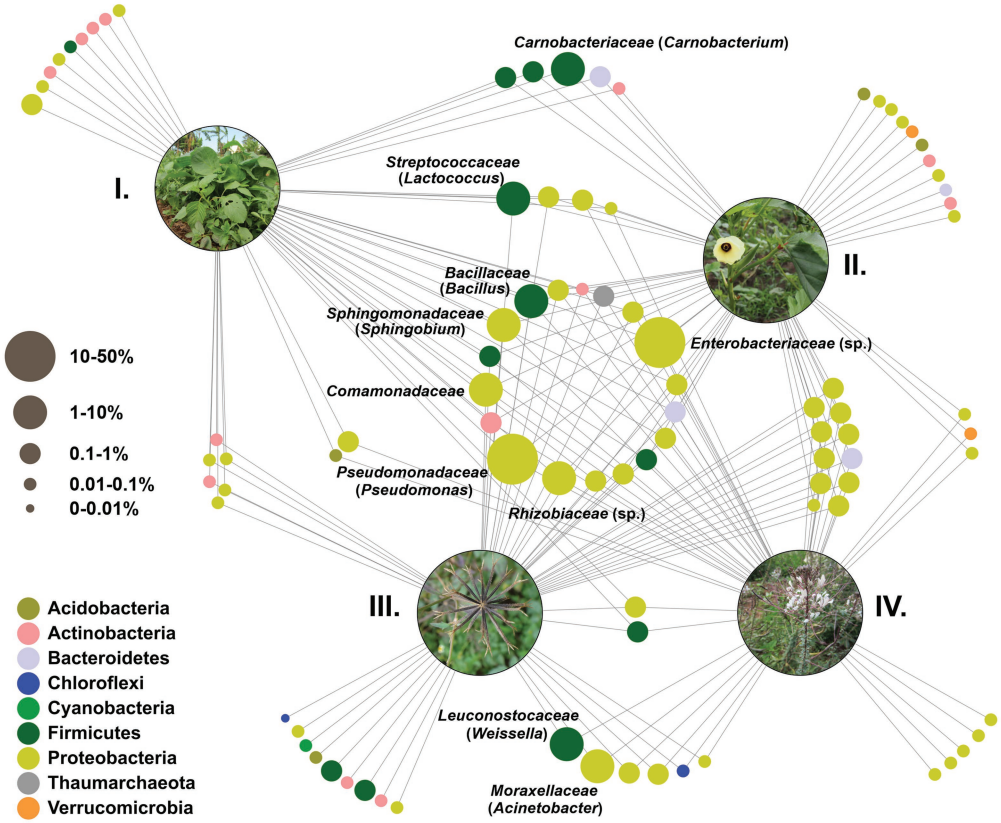

FIGURE 5 | Feature network based on taxonomic analysis at the genus level. Each node represents a family of the core microbiome and is colored according to its phylum. If families were only represented by one genus within the core microbiome, the corresponding genus is added in brackets. Cross-linked nodes express families shared between the plants blackjack, nightshade, okra, and spiderwisp. **(I)** Nightshade. **(II)** Okra. **(III)** Blackjack. **(IV)** Spiderwisp.

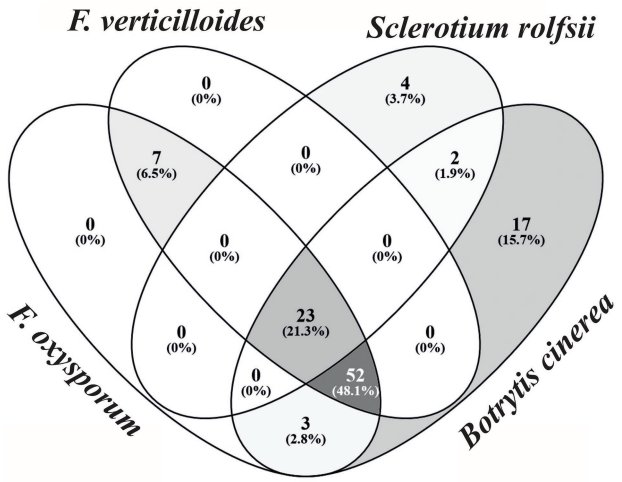

FIGURE 6 | Number of bacterial isolates with antagonistic effects against fungal pathogens. Fungal pathogens included *Fusarium oxysporum*, *Fusarium verticillioides*, *Sclerotium rolfsii*, and *Botrytis cinerea*. Only bacterial antagonists showing high antagonistic activity were assigned to their respective fungi. Graph was generated using VENNY 2.1.0 (https://bioinfogp.cnb.csic.es/tools/venny/).

Plant Growth Promotion of Bacterial Antagonists

Priming of tomato seeds with the six bacterial antagonistic strains resulted in a significantly increased fresh biomass of leaves (p = 0.039) and whole seedlings (p = 0.020) relative to the control when using *Bacillus* strains. Priming with *Sphingomonas* species showed no significant effect on both root and leaves growth (**Table 1**). The strongest plant growth-promoting effect was observed when using a *Bacillus* strain derived from roots of nightshade (strain NSRE-37). None of the bacterial isolates showed signs of phosphate solubilization.

Localization of Antagonists Within the Microbial Network of Leafy Green Vegetable Crops

The distribution and abundance of bacterial families comprising taxa with high antagonistic activity toward fungal phytopathogens (*Bacillaceae* and *Sphingomonadaceae*) within the microbiome of the leafy green crops were compared in order to highlight possible links to the robustness of the plant host. Bacterial families that were isolated from Ugandan tomato and were shown to comprise nematicidal effects to plant-pathogenic nematodes in earlier studies (Wolfgang et al., 2019) were included.

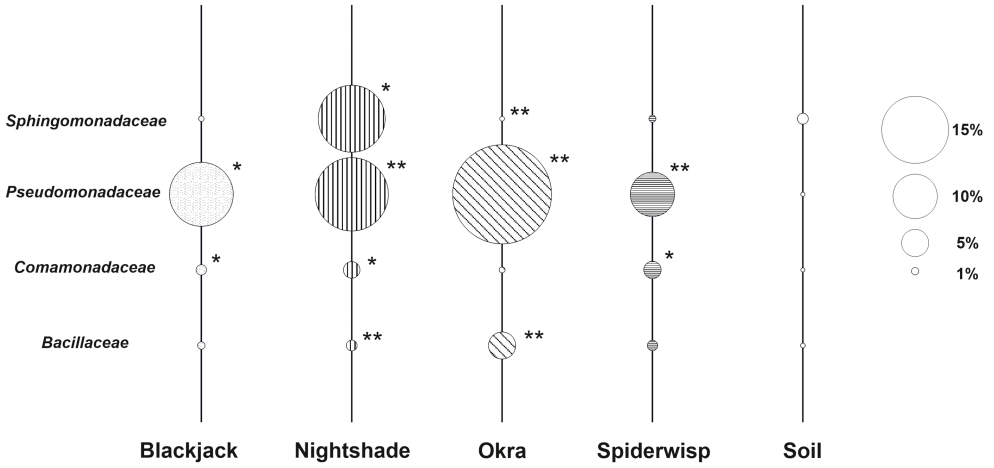

FIGURE 7 | Relative abundance of antagonistic families of bacteria associated with leafy green vegetables. The diameter of the bubble represents the abundance of each family within the microbiome of each leafy green crop and soil. Soil is used as a reference. Families, which were found to produce nematicidal VOCs in Ugandan tomatoes (Wolfgang et al., 2019) – namely *Comamonadaceae* and *Pseudomonadaceae* – are included. Asterisks indicate significant differences in relative abundance compared to soil based on LEfSe (*$p \leq 0.05$; **$p \leq 0.001$).

Most families were significantly enriched in plants compared to soil (**Supplementary Table 4**), although plant-specific differences were observed (**Figure 7**); while *Pseudomonadaceae* was the most abundant antagonistic family across all plant species with the highest relative abundance in okra, *Sphingomonadaceae* were higher abundant in soil except for nightshade. *Sphingomonadaceae* account for 9.6% (range 0.6–21.9) relative abundance in nightshade. Additionally, abundances of *Sphingomonadaceae* significantly differ within nightshade, with a higher relative abundance in rhizosphere and phyllosphere than in root-endosphere (**Supplementary Table 5**). Bacterial communities of nightshade consisted of the highest share of antagonistic families (31.2%), followed by okra (25.5%), blackjack (16.3%), and spiderwisp (14.8%). Within soil, antagonistic families comprised only 4.5% of all recorded microorganisms.

DISCUSSION

Research on plant-associated microbiomes in tropical regions is still in its beginning. Many factors known to influence crop-associated bacterial communities, e.g., soil quality, plants life cycle, or agronomic practices (Philippot et al., 2013), were not addressed in this study. Nevertheless, this study is a first step for understanding microbial communities in crops that are usually understudied in tropical agricultural research, but have a high relevance to local people. When investigating four locally popular leafy green vegetables (blackjack, nightshade, okra, and spiderwisp) in Uganda, we identified a microbiome that has both common and specific components between plant species. The general taxonomic composition was comparable with many other plant and crop species as well as the abundance of microbes (Bulgarelli et al., 2013; Berg et al., 2016). The rhizosphere was confirmed as the microbial hot spot for plants (Berg et al., 2006; Berg and Smalla, 2009) as well as the rhizosphere effect, which describes the selective enrichment visible in the composition of the microbiota (Foster et al., 1983; Buée et al., 2009). Interesting specific components were also observed. For instance, the microbiota of indigenous leafy greens were characterized by: (I) an unusually large core microbiome with only minor differences between plant species; (II) a high diversity of bacteria and archaea forming a network of potentially copiotrophic bacteria and oligotrophic archaea; and (III) a high proportion (15–31%) of potential plant beneficial microbes. The latter were identified in our culture collection, and can be potentially employed as biologically-based options for protection against stresses.

Leafy Green Vegetables Harbor Common Bacteria With Copiotrophic Lifestyle

The diversity and community structure of bacteria and archaea in four leafy greens was found to be microhabitat-specific, rather than plant genotype-specific. The extent of the impact of numerous variables (e.g., plant genotype, plant organ, habitat, developmental stage, and soil quality) is a persistent question in microbial ecology across studies. However, in studies focused on natural vegetation in particular, the plant genotype seem to be the most important factors to determine plant-associated bacterial communities, followed by soil traits (Berg and Smalla, 2009; Bulgarelli et al., 2013). Recent studies revealed a decrease in diversity of crop-associated microbial communities through breeding practices (Cardinale et al., 2015; Mendes et al., 2019). The less pronounced impact of the plant genotype can be explained by the life strategy of plants. Three (blackjack, nightshade, and spiderwisp) of the four leafy greens in the current study were naturally occurring, and in general are ubiquitous, mostly invasive, produce many seeds and are, therefore, categorized as *r*-strategists, which often have a copiotrophic lifestyle (Andrews and Harris, 1986). This life

strategy might also affect the composition of their associated microorganisms, which may have even co-evolved with them (Cordovez et al., 2019). For example, invasive plants, such as cheatgrass (*Bromus tectorum* L.), knapweed (*Centaurea stoebe* L.), and leafy spurge (*Euphorbia esula* L.), enrich copiotrophic bacteria in their associated rhizosphere (Gibbons et al., 2017). In our study, the microbiome associated with the leafy greens was neither specific nor depended on the plant genotype. We found that the most abundant bacterial phyla followed the same copiotrophic life strategy as their host, such as *Proteobacteria*, *Firmicutes*, *Bacteriodetes*, and *Actinobacteria* (Ho et al., 2017). In contrast, the archaeal phyla are considered to be oligotrophic, especially *Thaumarchaeota* (Uksa et al., 2015; Youssef et al., 2015). This indicates high substrate specificity and supports the assumption of a niche-colonization by archaea, including their role as followers of bacteria. Interestingly, a rhizosphere effect was also observed for archaea, especially for nightshade. The enrichment may depend on quality and composition of the root exudates, as demonstrated for archaea in tomato plants (Simon et al., 2005; Taffner et al., 2020). Altogether, copiotrophic bacteria and oligotrophic archaea appear to form a potent trophic network on leafy greens, which would warrant further investigation.

High Microbial Diversity of Leafy Green Vegetables Compared to Cultivated Crops

The four leafy greens studied all showed high and relatively similar values for Shannon's indices, with *H*-values ranging from 5.31 and 4.51 (okra) to 6.24 and 4.95 (nightshade), for bacteria and archaea, respectively. When comparing the Shannon's indices in bacterial phyllosphere communities [ranging from $H_{(okra)}$ = 4.4 to $H_{(nightshade)}$ = 5.74], the diversity of the leafy greens in this study is considerably higher than in some commercially cultivated leafy greens, such as spinach [*Spinacia oleracea* L.; $H_{(spinach)}$ = 3.15, Lopez-Velasco et al., 2013]. This also applies for rhizosphere: maize (*Zea mays*, L.) rhizosphere displayed a distinctly lower alpha diversity [$H_{(maize)}$ = 3.42; García-Salamanca et al., 2013] than the diversity of the rhizosphere of the leafy greens [$H_{(mean\_rhizosphere)}$ = 6.91]. Further, the archaeal diversity in rhizosphere of leafy greens tested was found to be higher [$H_{(mean\_rhizosphere)}$ = 4.51–4.95] than in other cultivated crops, such as rice [*Oryza sativa*, L., $H_{(rice)}$ = 4.08–4.43], Barbados nut [*Jatropha curcas*, L., $H_{(barbados\_nut)}$ = 3.16] and tomato [$H_{(tomato)}$ = 3.4; Lee et al., 2015; Dubey et al., 2016]. This large disparity in microbial diversity is attributed to the overbreeding of our main crops (Pérez-Jaramillo et al., 2016), whereas natural leafy greens have received much less attention and remain less intensively bred, having only recently attracted interest in agriculture. Further, agricultural practices affect microbial diversity. Comparing organic farming with conventional intensive farming, significant differences in the microbiome of maize, melon (*Citrullus lanatus* Thunb.), pepper (*Capsicum annuum* L.), and tomato, as well as the soil, were prominent (Hartmann et al., 2015; Xia et al., 2015). Given the large difference in diversity indices between the uncultivated leafy greens from Uganda with intensively cultivated field crops, we could assume that highly focused breeding programs, as well as intensive agricultural practices have led to a reduction and loss of diversity in the microbiome of these crops (Pérez-Jaramillo et al., 2016). Naturally occurring vegetables, such as leafy greens, have a high microbial diversity, which is directly correlated with healthier, more robust plants that are less vulnerable to pathogenic outbreaks (Berg et al., 2016).

High Proportion of Plant-Beneficial Bacteria of the Microbiome in Leafy Green Vegetables

A broad range of the taxa recovered from the core microbiome of leafy greens are well-known plant growth promoters, such as members of *Enterobacteriaceae* and *Pseudomonadaceae*, which occur frequently on leafy greens (Hayat et al., 2010). They are also known to be antagonistic against phytopathogenic fungi, either through competition or production of antimicrobial metabolites (Haas and Défago, 2005). However, *Enterobacteriaceae* also include human enteric pathogens, some of which, through occupation of crops *via* roots enter human digestive systems and have been associated with stimulating immune responses or acting as a "natural vaccination" as opposed a pathogen (Brandl, 2006; Berg et al., 2015). Further, *Actinobacteria* and *Proteobacteria* species were broadly distributed throughout the microbiome of the four leafy greens, both of which have previously been associated with host plant protection against fungal infections (Mendes et al., 2011). Besides the dominant families mentioned, some of the less common members of the microbiome showed growth promoting properties. *Bacillaceae* species such as *Bacillus subtilis*, *B. amyloliquefaciens* and *B. cereus*, and *Oxalobacteraceae* species such as *Herbaspirillum seropedicae* are known for supporting plant growth (Hayat et al., 2010), as well as members of the families *Xanthomonadaceae*, *Paenibacillaceae*, *Sphingobacteriaceae*, *Chitinophagaceae*, and *Alcaligenaceae* (Berg, 2009; Yang et al., 2014). One interesting fact is the relatively high abundance of *Sphingomonadaceae* in nightshade rhizosphere and phyllosphere (**Figures 1, 7**), compared to the other leafy greens. A high relative abundance of *Sphingomonadaceae* was frequently measured in other *Solanum*-associated communities, namely in rhizosphere (12%), root endosphere (5%), fruits (12–24%), and flowers (2–12%) of tomato (Allard et al., 2016; dataset of Wolfgang et al., 2019), rhizosphere of potatoes (*Solanum tuberosum* L., Pfeiffer et al., 2017), and rhizosphere of eggplant (*Solanum melongena* L., Li et al., 2019). *Sphingomonadaceae* comprise members with remarkable biotechnological potential, for instance degraders of aromatic or metalorganic compounds (Asaf et al., 2020). The high relative abundance of *Sphingomonadaceae* may be attributable to the diverse secondary metabolites (e.g., alkaloids) found in *Solanaceae*. However, further studies have to confirm the connection between *Sphingomonadaceae* and *Solanum*.

The archaeal community was clearly dominated by *Thaumarchaeota*, which are common colonizers of leafy greens, such as arugula (Taffner et al., 2019). This phylum consists mostly of ammonia oxidizing archaea (AOA), which are important for nitrogen cycling (Francis et al., 2007), and therefore, for supporting nutrient supplies to the plant. Further, recent studies

show that archaea have the potential to directly support plant growth *via* auxin biosynthesis, a plant growth hormone (Taffner et al., 2018). Besides *Thaumarchaeota*, methanogens of the phylum *Euryarchaeota* were observed. These are also common in plants, colonizing anoxic niches in the rhizosphere, such as on maize or arugula (Chelius and Triplett, 2001; Taffner et al., 2019). However, there was a high relative abundance of taxonomically unassigned archaeal features, although an up-to-date established pipeline was used for the bioinformatic analysis. This limitation is well-known for archaea, especially in novel, less studied habitats such as Uganda, and is mainly due to poorly defined reference databases. We can conclude therefore that the core microbiome of leafy greens contained several taxa with the potential to support plant growth and protection against pathogenic fungi, and thereby contribute to the robustness and health of plant hosts.

Promising Key Species for Future Biocontrol Agents

In the core microbiome of the leafy greens, we identified *Bacillus* spp. and *Sphingomonas* spp. playing a pivotal role in suppressing the key pathogenic fungi *B. cinerea*, *F. oxysporum*, *F. verticillioides*, *S. rolfsii*, and *V. dahliae*. *Bacillus* spp. have previously been shown to produce antimicrobial compounds, such as mycosubtilin and lipopeptides produced by *B. subtilis* (Leclère et al., 2005), or the antagonistic compound bacillomycin by *B. amyloliquefaciens* (Mülner et al., 2020). *Sphingomonas* are mainly known for their ability to degrade refractory contaminants, but have also been reported to be antagonistic against bacteria and fungi (White et al., 1996; Innerebner et al., 2011). These highly effective antagonists further showed resistance to abiotic stresses and plant-growth promotion capabilities in the current study. One isolate of *Bacillus* was able to tolerate high levels of hydrogen peroxide, which is a major abiotic stress factor for plants. Further, all isolates could grow under saline conditions up to 10%. Salinity reduces water-uptake efficiency and photosynthesis rate in plants, but microorganisms capable of dealing with such osmotic stress may confer resistance in plants to salt stress (Mayak et al., 2004). Furthermore, episodic drying and re-wetting of soil causes fluctuations in the soil's water potential and challenges microbes. We showed that all our selected isolates were highly resistant to desiccation. Effective consortia of biological control agents, therefore, should include bacteria that support plant growth in addition to antagonistic species. In our study, priming of tomato seeds with *Bacillus* isolates resulted in significant plant-growth promotion of up to 70%, whereas *Sphingomonas* isolates did not show any effect. However, *Sphingomonas* are known to promote plant growth by producing gibberellic acids (GAs) and indole acetic acid (IAA), improving crop productivity, which have also been reported for *Bacillus* spp. (Khan et al., 2014). Although *Bacillus* strains have previously been reported as solubilizers of inorganic phosphate (Hayat et al., 2010), we could not identify phosphate solubilizers among our isolates. This provides confirmation of the high specificity of plant beneficial traits at strain level (Berg, 2009). The antagonistic and plant-growth-promoting characteristics of the *Bacillus* and *Sphingomonas* isolates tested make them promising candidates for their application as biological control agents against fungal infections and for increasing robustness and plant health in Ugandan agriculture.

CONCLUSION

In our study, we found a unique, diverse, and robust microbiome occurring on natural leafy green vegetables in Uganda. Blackjack, okra, nightshade, and spiderwisp harbored microbes with strong antagonistic activities against pathogenic fungi, as well as promoting plant growth and demonstrating properties to enable host plants to withstand abiotic stresses. Six isolates in particular, assigned to the families *Sphingomonadaceae* and *Bacillaceae*, proved to be promising key-candidates for future sustainable biocontrol agents, toward supporting crop production in smallholder production systems in Sub-Saharan Africa. The biocontrol approach provides a more environmentally sustainable opportunity to produce crops and reduce or even replace excessive pesticide use. Identification of microbial isolates that are indigenous and adapted to African smallholder production systems will enable the development of technologies to support smallholders and improve human and environmental health.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ebi.ac.uk/ena, PRJEB39392.

AUTHOR CONTRIBUTIONS

JT, DC, AW, and GB performed the sampling. JT and OL conducted experiments in the laboratory and drafted the manuscript. JT, OL, and AW performed bioinformatics analyses. 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.2020.585690/full#supplementary-material

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Wine Terroir and the Soil Bacteria: An Amplicon Sequencing–Based Assessment of the Barossa Valley and Its Sub-Regions

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A wines' terroir, represented as wine traits with regional distinctiveness, is a reflection of both the biophysical and human-driven conditions in which the grapes were grown and wine made. Soil is an important factor contributing to the uniqueness of a wine produced by vines grown in specific conditions. Here, we evaluated the impact of environmental variables on the soil bacteria of 22 Barossa Valley vineyard sites based on the 16S rRNA gene hypervariable region 4. In this study, we report that both dispersal isolation by geographic distance and environmental heterogeneity (soil plant-available P content, elevation, rainfall, temperature, spacing between row and spacing between vine) contribute to microbial community dissimilarity between vineyards. Vineyards located in cooler and wetter regions showed lower beta diversity and a higher ratio of dominant taxa. Differences in soil bacterial community composition were significantly associated with differences in fruit and wine composition. Our results suggest that environmental factors affecting wine terroir, may be mediated by changes in microbial structure, thus providing a basic understanding of how growing conditions affect interactions between plants and their soil bacteria.

Keywords: terroir, vineyard soil bacteria, Barossa Valley, Illumina, 16SrRNA, soil microbiome

INTRODUCTION

Wine price differs considerably depending on its quality (e.g., flavor, color, and typicity), which is largely determined by the interactions between the grape and the growing conditions including climate, soil, topography, agricultural management, and the wine making process (Bokulich et al., 2016). These interactions influence the expression of wine's terroir (Bokulich et al., 2016; Fabres et al., 2017). Research on the drivers of terroir have predominantly focused on abiotic

environmental factors, such as climate, soil, viticultural management and wine making process, studied individually (Mira de Orduña, 2010; Vega-Avila et al., 2015; Romero et al., 2016) and simultaneously (Van Leeuwen et al., 2004). Research has been conducted on the association between soil microbiome (fungi and bacteria) and wine's terroir (Burns et al., 2015; Bokulich et al., 2016. For a recent review see Liu et al., 2019). In Australia, such studies have focused in the characterization of the microbial communities within a single vineyard (Gupta et al., 2019) to those present in vineyards planted in different growing regions (Liu et al., 2020). However, there is still a lack of large studies designed to understand whether vineyard microbiomes exhibit distinct patterns of distribution at small geographic scales (e.g., neighboring vineyards), and how such patterns are associated with a wine's terroir.

Soil microbiomes, especially bacterial species, have been found to be qualitatively and quantitatively different between vineyard systems (Vega-Avila et al., 2015). Environmental factors, such as topography, climate, soil properties, cultivars and agricultural management, combine to affect soil microbial communities (Castro et al., 2010; Reeve et al., 2010; Lamb et al., 2011). It has been shown that climate and topography, including rainfall pattern and temperature, affect these communities through their impacts on soil (Burns et al., 2015). Soil properties such as soil texture, nitrogen (N) content, phosphorus (P) content, carbon to nitrogen (C:N) ratio, water content, and pH show significant effects on the diversity and composition of microbial communities (Girvan et al., 2003; Frey et al., 2004; Rousk et al., 2010; Fierer and Jackson, 2006). Management practices, land use and varying degrees of stress and disturbance influence the soil microbiome markedly due to specific management objectives (Crowder et al., 2010; Reeve et al., 2010; Sugiyama et al., 2010; Lumini et al., 2011). Although environmental cues are the main drivers of the plant microbiota composition, it is now well established that host factors also contribute to the shaping of these communities. Plant genotypes exert an influence on the structural and functional diversity of soil microbiomes by varying root exudates and rhizodeposition (Broeckling et al., 2008; Dias et al., 2013; Philippot et al., 2013). Additionally, soil-plant compartments (bulk soil, rhizosphere and endorhizosphere) have been found more dominant in shaping fungal communities diversity and composition than spatial variability (Martínez-Diz et al., 2019).

Soil microbiomes interact with the vines, and thus affect wine quality (Burns et al., 2015; Bokulich et al., 2016). The interaction between soil microorganisms and plants includes the facilitation of nutrient uptake/utilization, stabilization of soil structure, reduction of disease prevalence by out-competing soil-borne pathogens or increase of disease prevalence by microbial pathogen invasion (Edwards et al., 2014; Zarraonaindia et al., 2015). Soil microbiomes also contribute to the wine fermentation flora, ultimately affecting wine quality (Compant et al., 2011; Barata et al., 2012; Martins et al., 2013). However, microbial assemblage function is intrinsically difficult to measure and define because of its highly changeable nature (Nannipieri et al., 2003). Additionally, due to the complex interactions between soil microbes, the influence of certain microbial communities can be substituted by other microorganisms with the same ecological function (Nannipieri et al., 2003; Wittebolle et al., 2009; Crowder et al., 2010; Lamb et al., 2011).

The primary aim of this project was to assess if there is a relationship between soil bacteria and terroir. To achieve this, we asked the following questions:

(i) Do wine sub-regions have distinct soil bacterial communities?;
(ii) What environmental conditions and agricultural practices shape soil bacterial community of vineyards?; and
(iii) Do differences in the soil bacterial community correlate with berry and wine characteristics?

In order to answer these questions, we undertook a soil bacteria survey in an iconic wine region, the Barossa in South Australia. The Barossa has a winemaking history of over 160 years and because of its importance as a growing region, has been chosen as a model to investigate terroir previously (Wolf et al., 2003; Edwards et al., 2014; Xie et al., 2017). Besides, the environmental characteristics of the Barossa, including climate, soil and topography have been previously characterized in detail (Robinson and Sandercock, 2014). However, to date, no study has analyzed the soil bacteria of the Barossa wine region or the possible influence on wine properties. Thus, determining how soil bacteria diversity and composition are influenced by environmental factors, and how bacteria differences correlate with differences in fruit/wine composition, will provide a starting point from which to better understand the (potential) functional role of soil microbial communities in terroir.

MATERIALS AND METHODS

Experimental Design and Plant Material

Twenty-two Barossa vineyards (**Supplementary Figure 1**), planted with own-rooted Shiraz (*Vitis vinifera* L.) and representative of the climate, soil and management practices of six Barossa sub-regions (i.e., Eden Valley, Northern Grounds, Central Grounds, Eastern Edge, Western Ridge, Southern Grounds) were selected for this study. Three to four vineyards per sub-region were included and nine vines from three rows from each vineyard were selected for measurement and sampling. Vines within the same row were adjacent to each other. Vines adjacent to missing vines, end of row vines and border rows were excluded from the selection.

Soil Sampling Protocol

Three soil cores (0–10 cm soil layer) were collected using a (20 mm diameter) soil auger from around each individual plant (approximately 10 cm from the trunk) and combined, giving a total nine soil samples per row. A total of 594 soil samples were collected (27 soil samples from each vineyard) on the 2nd of November (Austral Spring) 2015. At this time, vines have broken dormancy and are in a stage of rapid growth. Microbes present in the vineyard soil at this stage will have a more prolonged effect on the vines that those becoming more prominent later in the growing season. Additionally, focusing on the taxa present

early in the growing season should reduce the possibility of taxa found to be associated to differences in fruit/wine traits to be in reality driven by the environmental factors that induce those differences. Soil samples were immediately stored at 4°C and returned to the laboratory on the same day of collection. Soil samples from the same row were thoroughly mixed to obtain three samples per vineyard, and a total of 66 samples across the study. Coarse debris was removed from each soil sample using a 2 mm sieve, and each sample was then divided into two sub-samples (approximately 850 cm^3 each). The first subsample was air-dried until a constant mass was achieved and used for analysis of soil texture, pH, electrical conductivity, and plant-available (Colwell) P (phosphorus), as described previously (Cavagnaro, 2016). The second subsample was stored at -80°C for DNA extraction and downstream genomic analysis (see below).

Vineyard Physical Characterization

In this study, the climate was characterized on the basis of rainfall and temperature. The influence of topography was studied through elevation above sea level and vineyard orientation. Soil texture was determined following (Giddings, 2015). Soil pH and electrical conductivity were determined on a 1:5 soil/water mixture and then measured using pH/salinity meter (WP-81 Conductivity-Salinity-pH-mV Meter, v6.0, TPS Pty Ltd.). Plant-available phosphorus was extracted and measured using Colwell P method (Rayment and Higginson, 1992) (**Supplementary Table 1**). The remaining soil, topographic and climatic data was obtained from the Barossa Grounds project (Robinson and Sandercock, 2014) (**Supplementary Table 2**). Vineyard management information (including irrigation, midrow management, under vine management, planting year, vine density, pruning method, space between rows, row orientation, and canopy management) was collected from participating growers (**Supplementary Table 2**).

Fruit and Wine Chemical Analysis

Fruit juice pH and total acidity (TA) was measured using an autotitrator (Crison instruments Barcelona, Spain) (Iland et al., 2013). Total soluble solids (TSS) of juice samples were tested with a digital refractometer (BRX-242 Erma inc. Tokyo, Japan). A sample of 50 berries from random bunches on were collected from the same vines selected for soil microbiome analysis and frozen at −20°C for anthocyanin, phenolic and tannin analyses. Total grape tannins were measured by the methyl cellulose precipitable (MCP) tannin assay (Sarneckis et al., 2006) using the protocol of Mercurio et al. (2007). Total anthocyanin and phenolics were determined according the method of Iland et al. (2013) (**Supplementary Table 3**).

One bottle of commercial wine (2016 vintage) per vineyard was used for the chemical analysis. Wine pH and TA was determined as described by Iland et al. (2013). Final alcohol levels were determined using an Alcolyzer Wine ME (Anton Paar, Graz, Austria). Wine color was determined using the modified Somers assay using a high throughput method in 96 well plates [98]. Wine tannin concentration was determined using the methyl cellulose precipitable (MCP) tannin assay of Mercurio et al. (2007) and is expressed as epicatechin equivalents (mg/L) using an 8-point epicatechin standard curve Sarneckis et al. (2006). The modified Somers assay was used to determine; wine color density (WCD), SO2-corrected WCD, degree of anthocyanin ionization, phenolic substances and anthocyanins (in mg/L) (**Supplementary Table 4**).

Non-targeted metabolomic analysis of the wine samples was performed using LC-MS/MS. The metabolites were isolated from bottled wine samples using solid-phase extraction (SPE) with Phenomenex Strata-X 33 um 85 Å polymeric reverse-phase 60 mg/3 mL cartridges. A 2 mL aliquot of each sample was evaporated to dryness under nitrogen at 30°C. SPE conditions are presented in **Supplementary Table 5**. A pooled mix of all samples was prepared and used to monitor instrument performance. The analysis was performed on an Agilent 1200SL HPLC coupled to a Bruker microTOF-Q II in ESI negative mode. The operating conditions are described in **Supplementary Tables 5–7**.

Following data acquisition, mass calibration was performed on each file using Bruker Daltonic's DataAnalysisViewer4.1 "Enhanced Quadratic" calibration method (Bruker Singapore, The Helios, Singapore). Each file was exported from DataAnalysis in the mzXML generic file format for further processing. The files were processed using R (statistical programming environment) v3.1.0 and Bioconductor v2.14 under a Debian Linux 64-bot environment. Molecular features were extracted for each file using xcmx package and features that possessed a common mass and retention time across samples were grouped together.

16S rRNA Gene Next Generation Sequencing Library Preparation

DNA extractions from soil 66 samples were carried out at the Australian Genome Research Facility (AGRF) (Adelaide node) using Mo Bio Powersoil kit (Mo Bio Laboratories, Inc.) following the manufacture's protocol. DNA concentrations were estimated using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) and normalized to 5 ng/μl using nanopure water.

We prepared 16S rRNA amplicon libraries by following guidelines for the Illumina MiSeq System. Primers 515F and 806R (Bates et al., 2011; Caporaso et al., 2011) specific for the Bacterial 16S rRNA gene hypervariable "V4" region (expected amplicon and approximate size 390 bp, expected insert and approximate size 259 bp) were used for PCR amplification of extracted DNA and to prepare amplicon libraries. 515F worked as a universal forward primer for all the samples and 806R included 12-base sample specific barcodes to allow downstream de-multiplexing (**Supplementary Table 8**).

Three replicated PCR reactions were performed for each of the 66 samples. Each of these runs included one negative control as 'sample67' with no template DNA added. PCR reactions included 10ng of extracted DNA, 12.5 μl Q5 high-fidelity 2*master mix (New England Biolabs), 8.5 μl dH_2O, 1 μl forward and reverse primers (10 μM) in 25 μl reaction system. The PCR thermocycler (Bio-Rad T100) program was 95°C for 6 min, followed by 38 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 1 min and 30 s.

Success of PCR reactions was verified by agarose gel (1.5% w/v) electrophoresis. Samples exhibiting poor or no PCR

amplification (i.e., yielding faint or no visible bands on the agarose gel) were reamplified by adjusting the amount of DNA template. The triplicate reactions were then pooled into 67 pools. Individual pools were quantified by Qubit fluorometric double stranded DNA assay (Invitrogen, Carlsbad, CA, United States) and then mixed on an equimolar base to generate six pools each with 11 samples (each containing 5 μl of the water control pool). Pools were size-selected to remove unused primers using Agencourt AMPure XP (Beckman Coulter, Brea, CA, United States) following the manufacturer's protocol and mixed to equimolar concentrations to make one final pool. Library concentration and fragment size were estimated using TapeStation (Agilent, Santa Clara, CA, United States) and sequenced on the Illumina MiSeq platform (300 bp PE) (Illumina, San Diego, CA, United States) at the Australian Genome Research Facility-Adelaide node (AGRF).

Bioinformatics Analysis

Raw Illumina sequencing data was demultiplexed at AGRF-Adelaide node. Forward and reverse sequences were merged using *bbmerge* (Bushnell et al., 2017). Merged reads passing QC30 filter were analyzed using Quantitative Insight Into Microbial Ecology (QIIME) (QIIME version 1.8.0) (Navas-Molina et al., 2013). Operational taxonomic units (OTUs) were clustered using open-reference picking with the default *uclust* method (Edgar, 2010) based on 97% sequence similarity to the 16S rRNA Greengenes database (DeSantis et al., 2006; McDonald et al., 2012). OTUs were aligned to the Greengenes core reference database using *PyNAST* (Caporaso et al., 2010). Ribosomal Database Project (RDP) classifier was used to assign taxonomy (Wang et al., 2007). Both closed-reference OTU picking and open-reference OTU picking were performed for later analyses.

Alpha diversity (within-sample species richness and evenness) was measured using non-phylogenetic (including the observed number of OTUs, the Chao 1 and Shannon index) and phylogenetic (Faith's Phylogenetic Diversity) indices (Faith, 1992). Phylogenetic beta diversity (between-sample diversity) was calculated using both weighted and unweighted UniFrac (Lozupone and Knight, 2005) and three-dimensional principal coordinates analysis (PCoA) plots were built through Emperor (Vázquez-Baeza et al., 2013). We then constructed a neighbor joining ultrametric tree in QIIME from the beta diversity UniFrac distance matrix. The generated tree file, as well as the Barossa Valley geographical map, vineyard locations and taxa summary files, were input into GenGIS (Parks et al., 2009, 2013) to visualize the relationship between soil bacterial beta diversity and vineyard location. The statistical significance of this relationship was determined using the Mantel test based on 9,999 random permutations and implemented on GenAlex v6.5 (Peakall and Smouse, 2012).

To identify the association of environmental variables and grape and wine properties (**Supplementary Tables 1–7**) with soil bacterial microbiome, bacterial community dissimilarities were visualized with non-metric multidimensional scaling (nMDS) plots. All correlation analyses were done at species level, however, for simplicity during result visualization we used the highest taxonomical level available for the OTUs identified as significantly correlated to the trait of interest. Variables were fitted to the ordination plots using the function *envfit* in the package *Vegan version 2.5-2* (Oksanen et al., 2013) implemented in R version 3.5.0 (R Core Team,, 2013). Spearman's rank correlation coefficients were measured between individual taxon abundance and fruit and wine traits using the function *rcorr* in the package *Hmisc*. Grape traits included those from sensory, basic chemistry analyses, while wine traits included basic chemistry, wine fermentation products and amino acids concentration. Those traits and taxa with a significant (p-value < 0.05) correlation coefficient larger than 0.80 or lower than −0.80 were deemed as significantly associated.

To identify which variables are important in explaining the composition of the soil microbial community, we performed distance-based redundancy analysis (dbRDA), a form of multivariate multiple regression that we performed directly on a Bray-Curtis dissimilarity matrix of OTUs using the ADONIS function in *Vegan*. We used automatic model building using the function *step* in R. The step function uses Akaike's Information Criterion (AIC) in model choice, which is based on the goodness of fit. The model building proceeds by steps until the 'best' fit is identified. If two predictor variables were highly correlated (>0.85) one, typically that which was more difficult to measure, was removed as well as variables with missing replicates (Variables included in the automatic model building are marked with * in **Supplementary Tables 2–8**). Differential statistic functions within the *edgeR* package (Chen et al., 2008) was used, as in Weiss et al. (2017) to determine the significantly different taxa between vineyards separated by the main environmental drivers of beta diversity (i.e., soil type and soil phosphorous content). In order to avoid the influence of taxa showing low counts, a minimum threshold was set up at 100 counts per million.

RESULTS

Analysis of Soil Properties

Of the three soil physicochemical properties tested, plant-available phosphorous (P) and electrical conductivity (a measure of soil salinity), differed significantly (Kruskal–Wallis: p-value < 0.05) between sub-regions of the Barossa (**Supplementary Table 1**). Plant-available P was lowest in the Northern Grounds (11.5 ± 2.7 μg P/g soil) and highest in the Eastern Edge (39.0 ± 14.2 μg P/g soil). Electrical conductivity ranged from 111.0 μS/cm (Northern Grounds, SE = 34.2) to 302.5 μS/cm (Central Grounds, SE = 123.5). Soil pH did not differ between sub-regions, ranging from 6.2 (Eden Valley, SE = 0.4) to 6.8 (Southern Grounds, SE = 0.5).

Barossa Valley Soil Bacteria Community Composition

After quality filtering of the raw sequencing results, an average of 130,949 paired sequences remained per sample. Of these an average of 86,835 paired-end sequences per sample (66.3%) could be joined using *bbmerge* (**Supplementary Table 9**).

Both bacterial and archaeal DNA was detected in all soil samples. A total of 98.9% of sequences were classifiable at the phylum level (**Figure 1A**) and 95.2% at the genus level. Of those classifiable at the phylum level, 96.5% were assigned to one of nine dominant groups (relative abundance ≥1.0%) in the samples namely: Actinobacteria (26.9%), Proteobacteria (26.7%), Acidobacteria (12.0%), Planctomycetes (6.2%), Chloroflexi (5.6%), Firmicutes (5.3%), Gemmatimonadetes (3.9%), Bacteroidetes (3.5%), Verrucomicrobia (2.5%) (**Figure 1A**). The only dominant Archaea group was Crenarchaeota (4.0%). The overall dominant Bacteria and Archaea groups were consistently present in the six regions, but at different ratios (**Figure 1A**). The phylogenetic inference of bacteria composition differences between sub-regions showed three clusters with Central and Northern Grounds, and Eden Valley and Western Ridge sharing the more similar microbial profiles (**Figure 1A**).

The number of observed OTUs (**Figure 1B**) showed significant differences (*t*-test: p-value < 0.05) between the OTU rich sub-regions (Northern and Central Grounds) and the relatively OTU poor sub-regions (Eden Valley and Western Ridge) (**Supplementary Table 10**). Similarly, the Chao1 metric showed that Northern and Central Grounds presented higher levels of OTU richness while Eden Valley and Western Ridge had the lowest (**Figure 1C**). Pairwise comparison of alpha

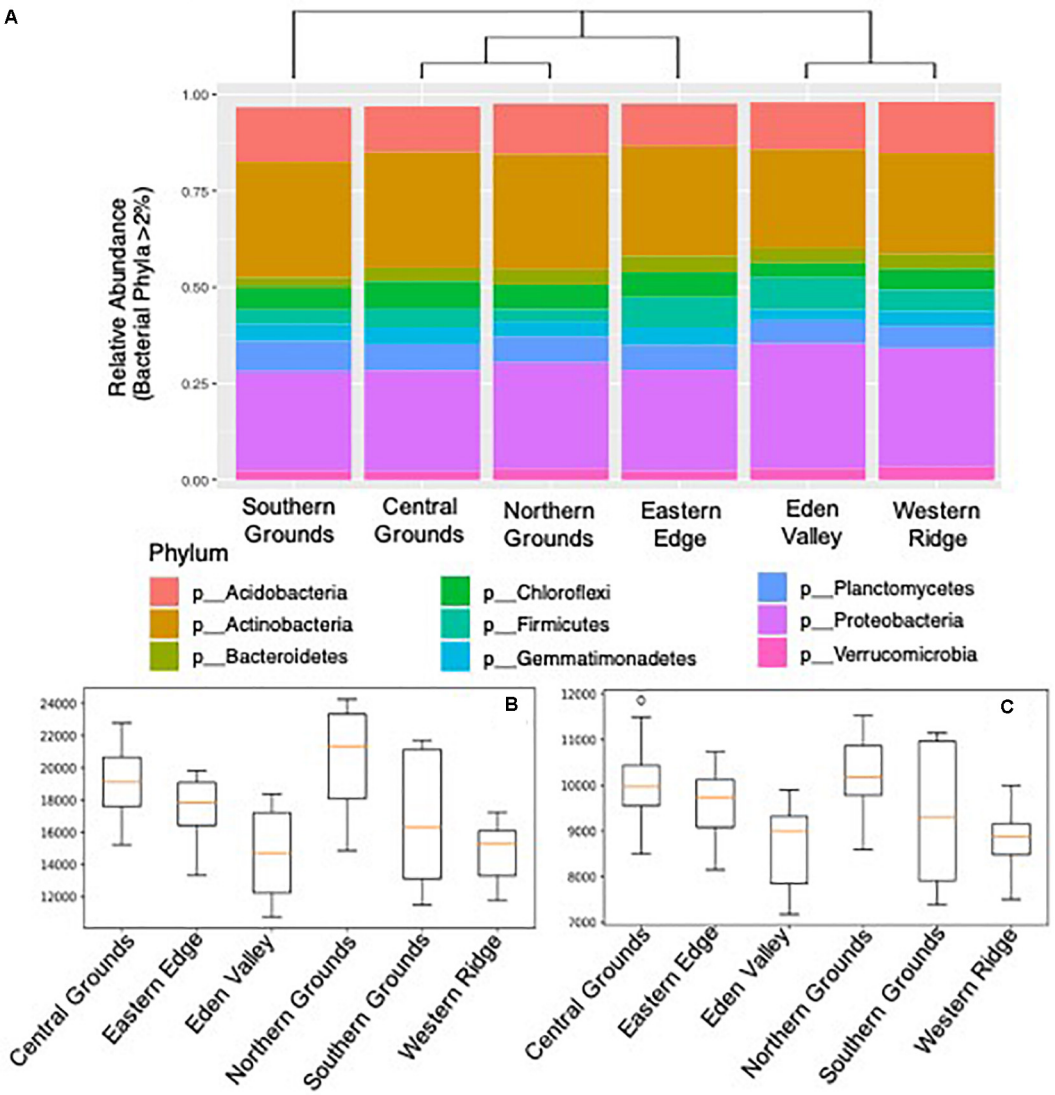

FIGURE 1 | Soil bacteria community composition and diversity in 6 Barossa sub-regions. **(A)** Phylogenetic inference of microbiome composition differences between Barossa sub-regions. Bar plots show bacterial taxa with greater than 2% relative abundance at the phylum level. Neighbor joining tree was generated with weighted UniFrac distances calculated with sequences classifiable at the phylum level (98.9% of total). **(B)** Alpha diversity: Chao1 diversity comparison, **(C)** and observed species diversity comparison (Observed number of OTUs). Alpha diversity values were calculated based on rarefied data was established using 16S sequencing reads from 3 soil samples per vineyard.

diversity between sub-regions showed significant differences (*t*-test, *p*-value < 0.05) between Northern Grounds and Eden Valley and Western Ridge and between Central Grounds and Eden Valley and Western Ridge (**Supplementary Table 11**). Pairwise Shannon diversity analysis did not show significant differences between any subregion (**Supplementary Table 12**).

A BIOM file was generated after OTU picking, then OTUs identified in the negative control samples were removed from soil sample OTUs, leaving between 37,176 and 114,777 OTUs per sample (mean = 60,147 OTUs). Data with and without rarefaction were used for alpha diversity and beta diversity analyses. 37,176 OTUs (the lowest amount of OTUs in one sample) were randomly selected from each sample for rarefaction. Dissimilarities in microbial communities between samples (i.e., beta diversity) were calculated as weighted and un-weighted UniFrac distances and both methods showed similar patterns, and so only analyses based on weighted results are shown here. For the most part, the three replicates from within a given vineyard were closely grouped on the ordination plot (**Figure 2A**), indicating that bacterial communities were consistent within sites. Pairwise analysis of the differences between groups (vineyards and sub-regions) showed that all vineyards and sub-regions are significantly different to each other (Adonis, *p*-value < 0.001). Mantel test analysis of the association between bacteria compositional differences and geographic distance, showed a small but significant correlation (rxy = 0.315; *p*-value = 0.0001) (**Figure 2B**).

To further explore dissimilarities among and within regions, neighbor joining analysis was used to cluster samples and to generate a similarity tree in QIIME. This information, along with a geographical map of the regions and their locations, were combined using the GenGIS software package (Parks et al., 2009). This approach showed a low level of clustering of vineyards according to their geographic location (**Figure 2C**).

TABLE 2 | Main drivers of soil microbiome differentiation between Barossa Region vineyards.

Step	Df	Deviance	Resid. Df	Resid. Dev	AIC
1	NA	NA	21	75.29566	29.06836
Soil P	−1	8.170918	20	67.12475	28.54123
Soil texture	−1	6.620899	19	60.50386	28.25662

*Variables that explained the greatest variation in the soil microbiome through model selection. The correlation test was carried out on environmental variables following the removal of the highly correlated variables (>0.85) using the function ordisten, in the package Vegan. *p < 0.05, **p < 0.01, and ***p < 0.001.*

Drivers of Soil Bacteria Differentiation

Model selection was used to identify the combination of variables that explained the greatest variation in the soil bacteria. This approach consistently selected soil plant-available

TABLE 1 | Fruit and wine characteristics significantly associated with microbial community composition in Barossa Region vineyards.

Variables		NMDS1	NMDS2	*r*2	Pr(>r)
Basic berry properties	50 berries weight	−0.87544	−0.48332	0.1612	0.008**
	TA berry	0.9369	0.3496	0.1119	0.029*
	Average color	0.76859	0.63974	0.1337	0.008**
	Average total phenolics berry	0.76558	0.64334	0.135	0.015*
	Malic acid	−0.90493	0.42557	0.104	0.03*
Basic wine chemistry	Total phenolics	0.83761	0.54627	0.2132	0.002**
	Total anthocyanins	0.99519	0.09801	0.2507	0.001***
	Color density (so2 corrected)	0.72894	0.68457	0.1449	0.006**
	Hue	−0.78985	0.61331	0.1314	0.011*
Wine amino acids	Alanine	0.11831	0.99298	0.124	0.017*
	Asparagine	0.55124	0.83435	0.1156	0.023*
	Glutamate	0.39571	0.91837	0.103	0.031*
	Glycine	0.62968	0.77685	0.1847	0.002**
	Serine	0.54731	0.83693	0.0936	0.04*
	Threonine	0.20213	0.97936	0.0934	0.049*
	Tryptophan	0.56228	0.82695	0.119	0.025*
Wine ferment. products	Acetic acid	−0.99914	−0.04153	0.1689	0.003**
	Propanoic acid	−0.96827	0.24991	0.118	0.012*
	3-methylbutanol	0.99079	0.13538	0.1184	0.02*
	2-methylbutanol	0.98968	0.14332	0.108	0.034*
	Butanoic acid	−0.89212	0.4518	0.1298	0.013*
	2-phenyl ethyl ethanol	0.70731	0.7069	0.2064	0.001***
	2-phenyl ethyl acetate	0.82725	0.56183	0.1249	0.013*

Table shows the envfit output that was carried out the correlation test between grape and wine characteristics variables that fitted onto an ordination of non-metric multidimensional scaling (nMDS) plots of microbial community data from soils in 22 vineyard sites. Analysis was conducted using 999 permutations with variables deemed significant where p-value < 0.05.

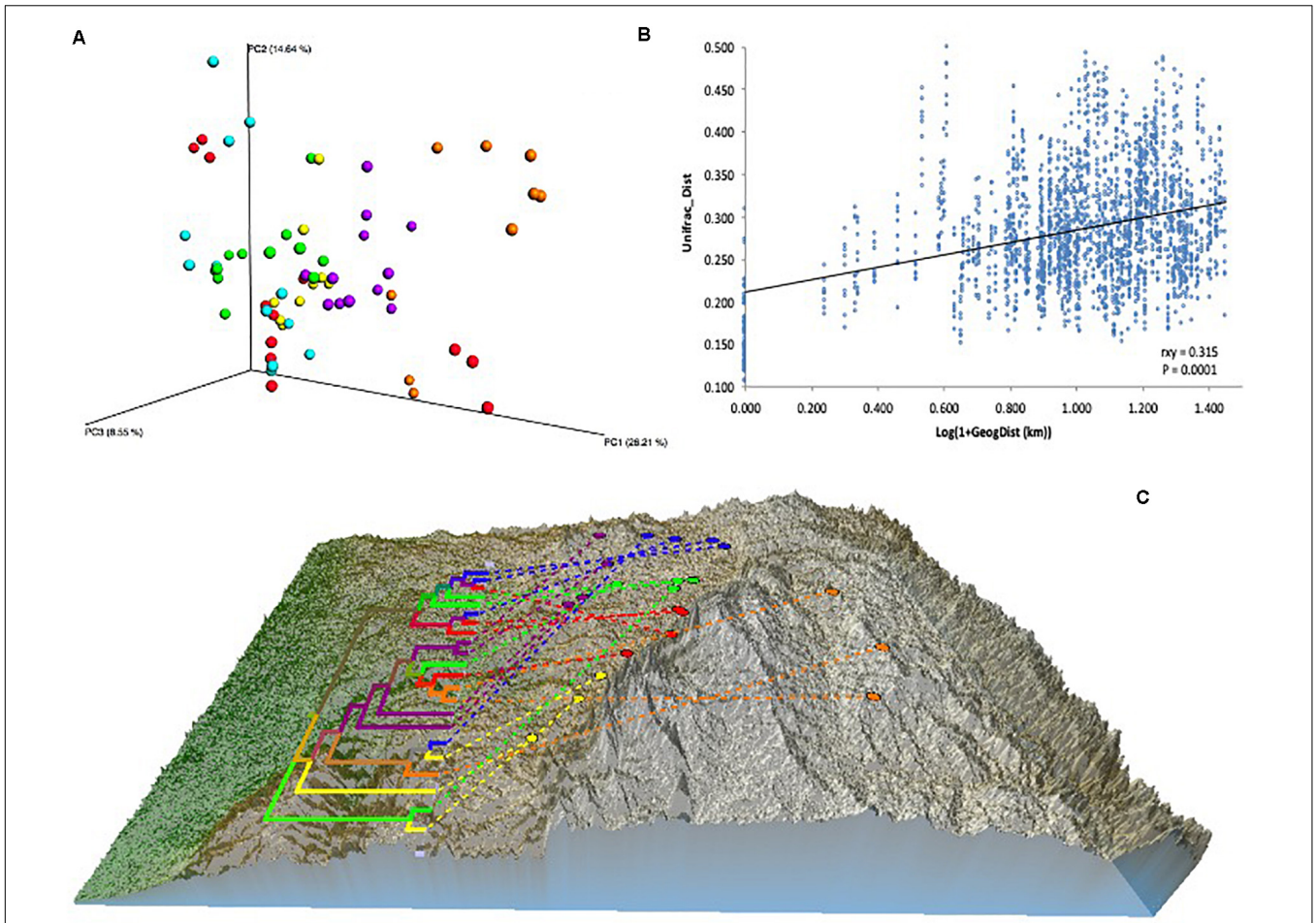

FIGURE 2 | Effect of vineyard location on soil microbiome differentiation. **(A)** PCoA based on Beta diversity of soil bacterial communities calculated using weighted UniFrac distances. Values were calculated based on rarefied data to 37,176 sequences per sample. **(B)** Relationship between phylogenetic Beta diversity and geographic distance. Unifrac_dist indicates weighted UniFrac distances. Geographic distances were calculated from latitude/longitude coordinates using GenAlex v6.5 *geographic distance* function implemented as Log(1 + distances in Km). The relationship was tested using Mantel's correlation coefficient (rxy) with its probability estimate for significance (P) based on 9,999 random permutations and implemented using GenAlex v6.5. **(C)** Neighbor joining ultrametric tree calculated from Beta diversity weighted UniFrac distance matrix between 22 vineyards located in six sub-regions: Northern Grounds (blue); Southern Grounds (yellow); Central Grounds (green); Eastern Edge (red); Western Ridge (purple); Eden Valley (orange). Tree was overlayed with the Barossa Region elevation map using GenGIS. Beta diversity was established using 16S sequencing reads from 3 soil samples per vineyard.

phosphorus (P) and soil texture as the main drivers (Model: p-value = 0.001) of soil bacteria in the Barossa vineyards tested (**Figure 3**). Together, both variables explained 19.7% of the observed variability. Independent pairwise analysis of UniFrac distances of vineyards grouped by these soil characteristics, showed that microbial communities in clay soil types were significantly dissimilar from those in sandy soils (PERMANOVA: p-value < 0.001, **Figure 4A**). Microbial communities in soils with high plant-available phosphorus ($P > 30$ mg/kg) were also dissimilar from those with low plant available phosphorous (PERMANOVA: p-value < 0.001, **Figure 4B**). Three and eight taxa were significantly more abundant in clay and sandy soils, respectively (**Figure 4C**), while eight taxa were found significantly associated with low plant available phosphorous content, and three associated high levels of plant available phosphorous in soil (**Figure 4D**).

Envfit analysis identified a number of other environmental factors as individually associated with microbial community composition (**Figure 5**). Aside from plant available phosphorous ($r^2 = 0.3706$, p-value < 0.001), these variables were: elevation ($r^2 = 0.3609$, p-value < 0.001), growing season rainfall ($r^2 = 0.2499$, p-value < 0.001), mean annual rainfall ($r^2 = 0.1621$, p-value $= 0.004$), spacing between rows ($r^2 = 0.1512$, p-value $= 0.006$) and between vines ($r^2 = 0.1561$, p-value $= 0.011$), and growing season mean temperature ($r^2 = 0.1113$, p-value $= 0.022$).

Analysis of the correlation between individual environmental and vineyard management variables and taxa abundance, identified 4 positive (Spearman's > 0.80, p-value < 0.001) and 3 negative (Spearman's < -0.80, p-value < 0.001) significant correlations (**Supplementary Figure 2**). Positive

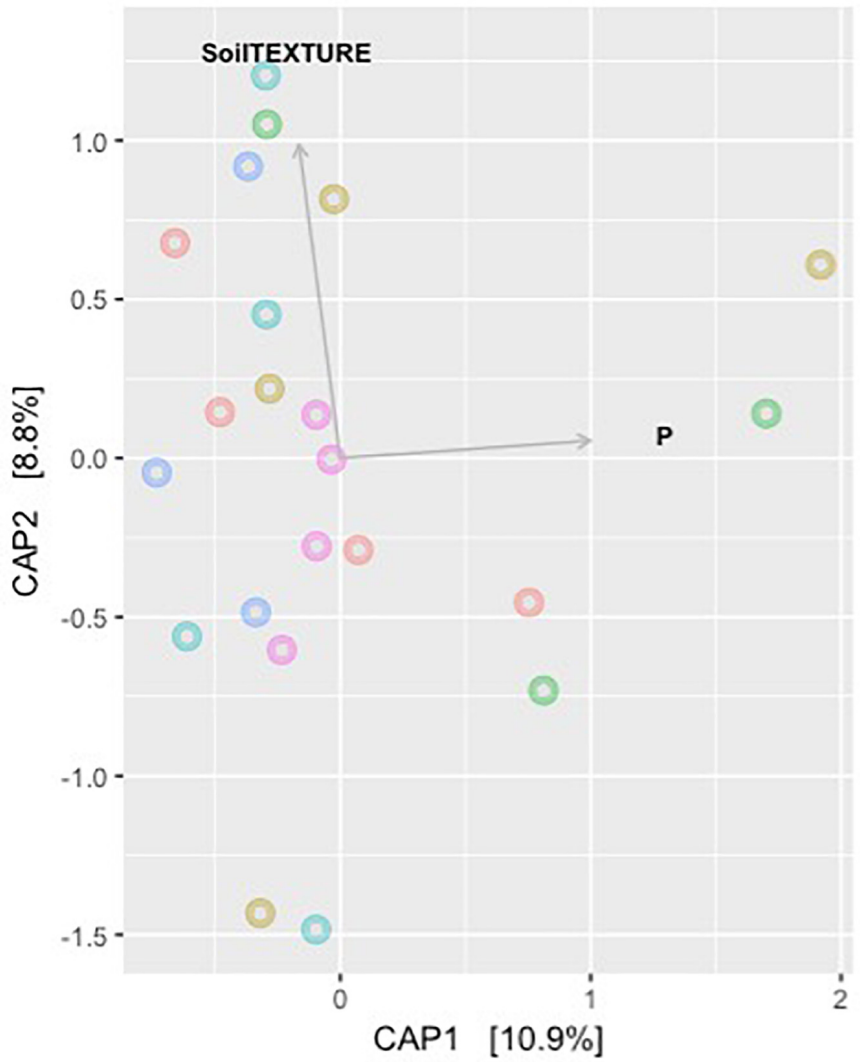

FIGURE 3 | Main drivers of soil microbiome differentiation between Barossa Region vineyards. The observed important soil factors that affect soil microbial community groups in combinations. CAP plot displays the combination of variables that explained the greatest variation in the soil microbiome through model selection (full results **Table 2**). The correlation test was carried out on environmental variables following the removal of the highly correlated variables (>0.85) using the function *ordisten*, in the package *Vegan*. The variables implemented in the final model were soil phosphorous and soil texture, which explained 19.7% of variation in the soil microbiome. Distance based redundancy analysis (dbRDA) with Bray-Curtis dissimilarity matrix of OTUs was used to examine the influence of these predictor variables using the function capscale in the package Vegan in R.

correlations with individual taxa included, pH (order iii1-15 and family Pirellulaceae), elevation (family Isosphaeraceae), and plant age (family Hyphomicrobiaceae); while negative correlations included P (family OPB35), elevation (family Conexibacteraceae), and the spacing between vines on the same row (family Haliangiaceae).

Terroir and Vineyard Soil Bacteria

Twenty four of the 75 grape and wine characteristics included in the study displayed a significant correlation with the soil microbial community composition (**Table 1**). The strongest associations identified for each of the four groups of traits tested were: 50 berry weight and average color per berry (basic berry properties); total anthocyanins and total phenolics (basic wine chemistry); Glycine and Alanine (wine amino acids); and 2-phenyl ethyl ethanol and acetic acid (wine fermentation products).

Significant positive correlation (Spearman's $>$ 0.80, p-value $<$ 0.05) were identified between the abundance of one taxon (order IS_44) and the average level of total phenolics mg/g berry weight (**Supplementary Figure 3A**). Similarly, six wine traits showed positive correlations with the abundance of six microbial taxa (**Supplementary Figures 3B–F**). Briefly, the genus *Rhodoplanes* (Order Rhizobiales; family Hyphomicrobiaceae) was positively associated with the level of wine total phenolics and the family Chitinophagaceae (Order Chitinophagales)

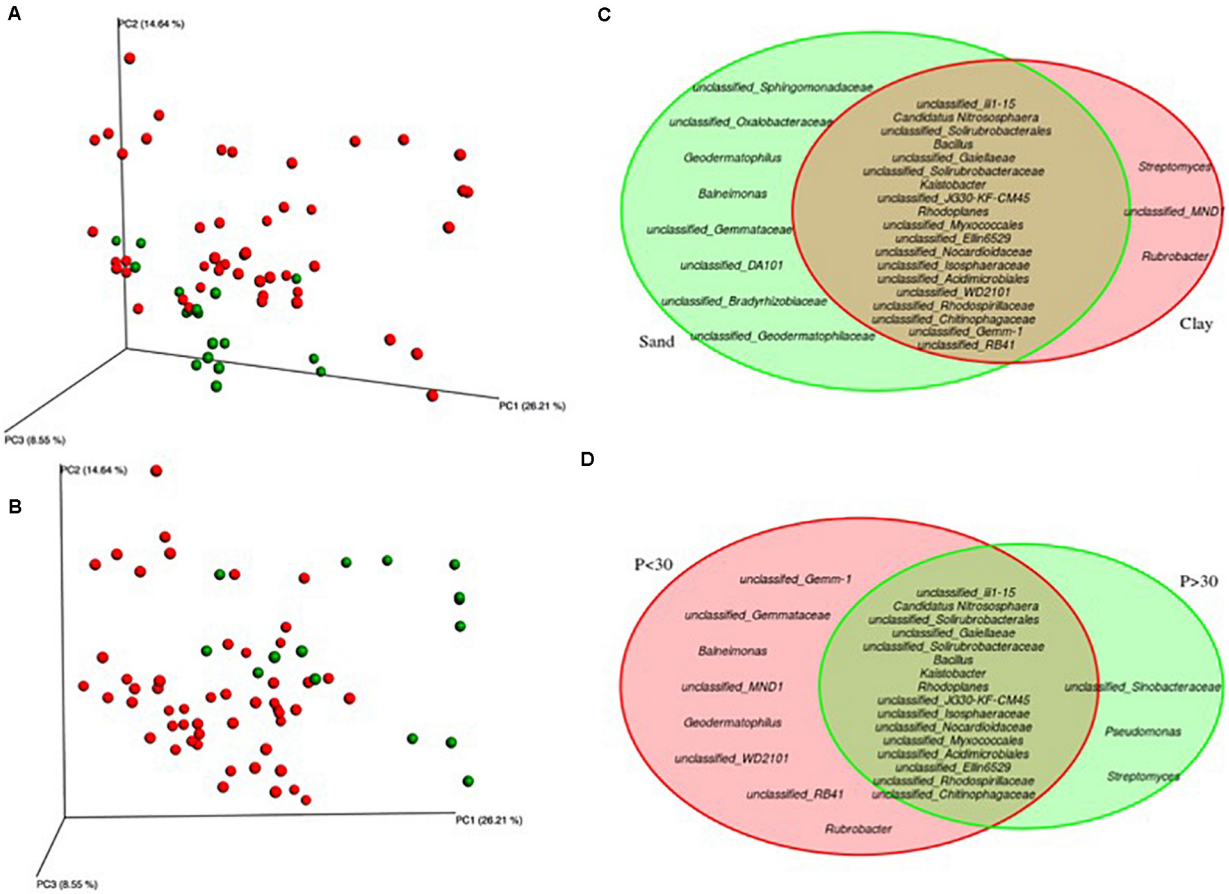

FIGURE 4 | Identification of microbial genera associated to soil texture and plant-available phosphorous in Barossa Region vineyards. Principal coordinate analysis plots display weighted UniFrac distances of soil samples from 22 vineyards in six sub-regions of Barossa Valley. Venn Diagrams show significantly different ($P > 0.01$) genera. Plots and diagrams are grouped by **(A,C)** soil type [clay (red) versus sandy soils (green)], and **(B,D)** plant-available Phosphorous (P) [$P < 30$ μg P/g soil (red), $P > 30$ μg P/g soil (green)]. Beta diversity was established using 16S sequencing reads from 3 soil samples per vineyard.

was associated with color density of SO_2 corrected wine and with the level SO_2 resistant pigments in wine, while the family Kouleothrixaceae (Order Roseiflexales) was positively correlated with wine color density.

DISCUSSION

Previous studies have shown that environmental factors (e.g., climate and soil properties) and crop management may affect microbial populations in vineyards (Burns et al., 2015; Gupta et al., 2019; Liu et al., 2020). To date, the largest number of vineyards included in a single vineyard microbiome study is 15 (Liu et al., 2020). Here the authors made a thorough examination of the contribution of microbial communities to wine regionality at a supra-regional level (up to 400 km) and identified the fungal microbiome as a potential driver of terroir. To better understand how these variables contribute to vineyard microbial communities and how microbial diversity and composition correlate with fruit and wine quality traits at a regional and subregional level, we studied the soil bacteria composition of 22 commercial Shiraz vineyards representative of the Barossa Valley wine region of South Australia, Australia.

Vineyard Soil Bacteria Composition and Diversity

With over 37,176 sequences per sample we reached a sequencing depth deemed sufficient to describe patterns in bacterial alpha and beta diversities (e.g., Caporaso et al., 2010; Lundin et al., 2012). From a species composition point of view, our results indicate that vineyard soil bacteria present similarities across the six sub-regions studied. All soils analyzed presented both bacteria and archaea. A total of 96.5% of the all identified sequences were allocated in one of ten main dominant phyla (relative abundance ≥1.0%). Of these, nine (Actinobacteria, Proteobacteria, Acidobacteria, Planctomycetes, Chloroflexi, Firmicutes, Gemmatimonadetes, Bacteroidetes, and Verrucomicrobia) were Eubacteria, while only one dominant taxon was from Archeabacteria (Crenarchaeota). Although dominant phyla were consistently found in the six regions tested, they were present in different ratios. This finding

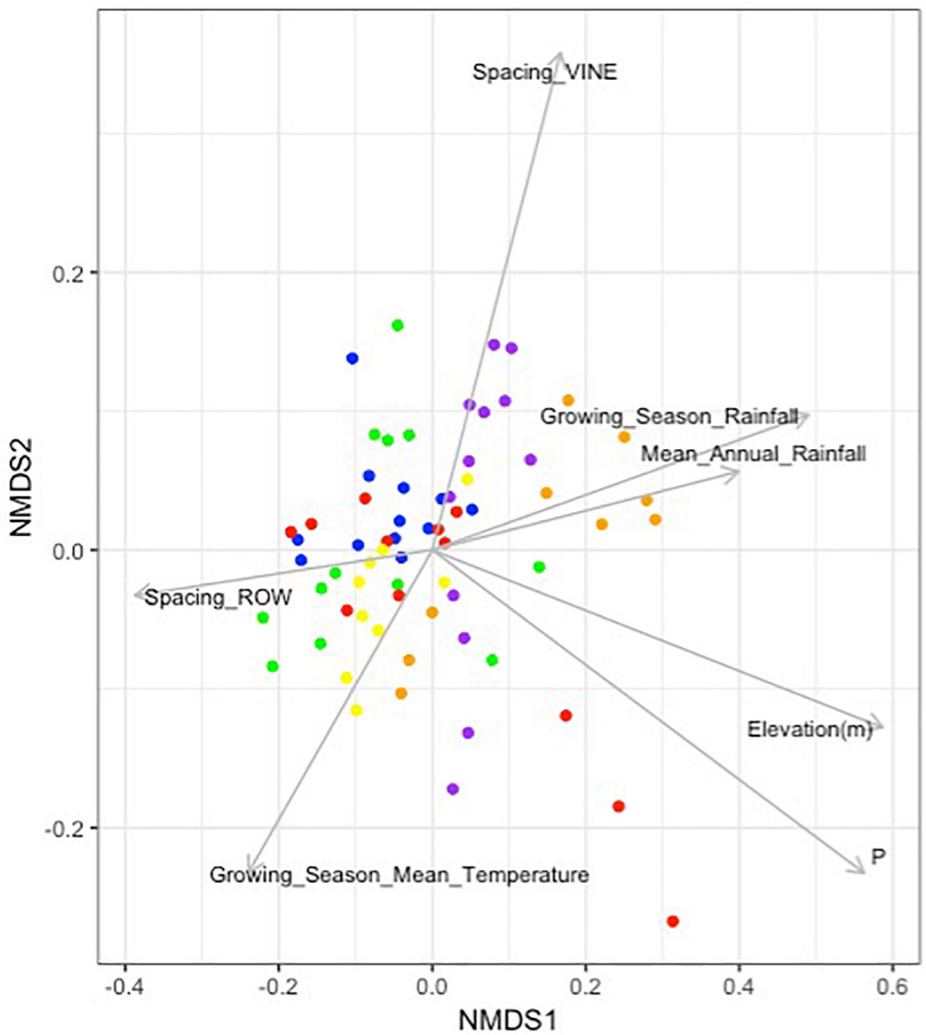

FIGURE 5 | Environmental and vineyard management factors significantly associated with soil microbial community composition in Barossa Region vineyards. Non-metric multidimensional scaling plot displays the microbial community composition of 22 vineyards located in six sub-regions: Northern Grounds (blue); Southern Grounds (yellow); Central Grounds (green); Eastern Edge (red); Western Ridge (purple); Eden Valley (orange). Vector arrows indicate the association with environmental variables with p-value < 0.05. Arrow heads indicate the direction and length indicates the strength of the variable and nMDS correlation. Analysis was conducted using 999 permutations with variables deemed significant where p-value < 0.05.

is similar to earlier work; for example, investigating Pinot Noir vineyards in regions of Victoria and South Australia, Australia, Liu et al. (2020) found the same nine top dominant bacteria groups. However, in Australian agricultural soils, Bissett et al. (2010) found top six dominant soil bacteria groups (>3% occurrence) are slightly different and Cyanobacteria replacing Planctomycetes, Chloroflexi, Gemmatimonadetes, and Verrucomicrobia (Bissett et al., 2010). Interestingly, studies investigating vineyard and agricultural soils outside of Australia found similar results as soil samples from Australian vineyards. Burns et al. (2015) found the same nine top dominant bacteria groups in Napa Valley American Viticultural Area (AVA). Similarly, Liu et al.'s (2014) analysis of agricultural black soils in northeast China found almost the same dominant bacterial groups. However, analysis of non-agricultural soils outside Australia by Lauber et al. (2009) and Faoro et al. (2010) identified the same dominant groups, with the exception of Verrucomicrobia which was replaced by Nitrospira (Faoro et al., 2010) and TM7 and Cyanobacteria replacing Planctomycetes and Chloroflexi (Lauber et al., 2009).

Location, Soil Properties, Climate and Vineyard Management Are Associated With Soil Microbial Community Dissimilarity in the Barossa

Although dominant taxa were constant at a regional level, soil bacteria diversity and composition seemed to be a better factor separating soil bacteria from different sub-regions. The phylogenetic inference of bacteria composition differences

between sub-regions showed that OTU richer sub-regions (Northern and Central Grounds) clustered independently from the OTU poorer ones (Eden Valley and Western Ridge).

Previous studies have shown that the major factors determining compositional dissimilarities of soil bacteria between sites are dispersal constraints (which predicts that more distant soils should have greater phylogenetic dissimilarity) and environmental heterogeneity (Fierer, 2008; Liu et al., 2014; Burns et al., 2015). Analysis of the influence of geographical distance on soil bacteria composition differences between Barossa Valley Region vineyards showed a small significant correlation between both parameters. It could be argued that such small contributions to vineyard soil bacteria composition differences could be associated with the relatively small distances between the vineyards in this study (Average distance 11.7 km, minimum distance 0.7 km and maximum distance 26.5 km). However, this correlation was similar to that observed by Burns et al. (2015) when studying 19 vineyards of the Napa Valley AVA that were separated by up to 53 km. This suggests that dispersal constraints contribute to soil bacteria differences at a much smaller scale than previously perceived.

Environmental heterogeneity has been largely claimed to be more important than geographic distance in shaping bacterial community at different geographical scales (Fierer and Jackson, 2006; da et al., 2009; Ranjard et al., 2013; Hermans et al., 2017; Miura et al., 2017). The main contributors to environmental associated variability in soil communities are differences in climatic conditions, topography, soil properties, and cultivation practices (Burns et al., 2015; Mezzasalma et al., 2018). Bacteria composition similarity analysis results did not show a clear clustering of vineyards according to their geographic location, indicating that even at a close geographic distance, environmental heterogeneity is the dominant factor shaping soil bacteria composition. Bissett et al. (2010) pointed that in Australian agricultural soils, the correlations between bacterial communities and both environmental factors and geographic distance depend critically on the taxonomic resolution used to evaluate microbial diversity, as well as life history of the taxa groups being investigated. For example, geographic distance had more influence over community structure of bacteria known to be poor dispersers/colonizers than good dispersers/colonizers (Bissett et al., 2010). Furthermore, previous work on a more detailed analysis on the effect of soil-plant compartment, as previously done for grapevine fungal microbiomes (Martínez-Diz et al., 2019), is adamant to fully understand the diversity and composition of grapevine microbiomes.

To determine which environmental factors contribute to the observed differences in soil microbial communities we used an automatic model building approach. This analysis revealed that when taken in combination, plant-available phosphorous and soil texture were the major contributors to soil bacteria differences between vineyards (approximately 20% of the total observed variability). Gupta et al. (2019) found in an Australian vineyard planted on silty loams over clays with some areas of sandier soils, P and sand percentage showed significant correlations with bacterial community variation. Soil particle size has been previously negatively correlated with bacteria community alpha diversity (Sessitsch et al., 2001) indicating that both variables could be affecting bacteria composition in an, at least partially, independent manner. Moreover, while genera *Streptomyces*, *Rubrobacter* (both Actinobacteria) and *unclassified MND1*, were especially prevalent in clay soils, genera *Streptomyces*, *Pseudomonas* and *unclassified Sinobacteraceae* were found in soils with plant-available phosphorous content higher than 30 μg/g soil. *Pseudomonas*, are inorganic P solubilizing bacteria (Awasthi et al., 2011; Goswami et al., 2013; Schmalenberger and Fox, 2016). Conversely, P levels negatively correlated with the abundance of the organic P mineralizing taxon OPB35. Pairwise analysis of individual taxa and environmental variables also identified previously reported strong and positive correlations between soil pH and order iii1-15 (acidobacteria-6) and family Pirellulaceae (Rousk et al., 2010; Hermans et al., 2017; Wu et al., 2017).

Previous studies have shown that climatic variables such as rainfall (Wildman, 2015) and temperature (Cong et al., 2015) are major shapers of soil microbial population composition and activity. Our results indicate that cooler and wetter regions (Western Ridge and Eden Valley; mean annual rainfall: mean = 663.18 mm, *SD* = 0; growing season rainfall: mean = 245.26 mm, *SD* = 0; mean January temperature: mean = 21.3°C, *SD* = 0.9; growing season mean temperature: mean = 18.19°C, *SD* = 0.8) had relatively lower soil microbial diversity, and a higher ratio of dominant species, than the warmer and drier sites (mean annual rainfall: mean = 585.85 mm, *SD* = 71.60; growing season rainfall: mean = 224.00 mm, *SD* = 15.56; mean January temperature: mean = 21.6°C, *SD* = 0.65; growing season mean temperature: mean = 18.68°C, *SD* = 0.66). Additionally, elevation, which negatively affects air temperature, showed a positive correlation with the families Isosphaeraceae and an unsurprising negative correlation with the thermophilic taxon Conexibacteraceae (Wagner and Wiegel, 2008).

Agricultural lands tend to show similar patterns of dominant bacteria (Lauber et al., 2009; Faoro et al., 2010; Liu et al., 2014; Burns et al., 2015), indicating that microbial community composition can be profoundly affected by cropping practices (Hartman et al., 2018). Our results show that, both spacing between row and vine (**Supplementary Table 2**), which determine the vineyard's planting density (between 772 and 1,792 vines/ha in our study), are significantly associated with global differences in soil microbial community. Work in oil palm plantations has shown that planting density affects soil bacteria by altering the level of solar light incidence on soils, which can have dramatic effects on soil temperature and moisture (Tripathi et al., 2016). Pairwise comparisons between agronomical practices and individual taxa showed a negative correlation between spacing among vines on the same row and the abundance of representatives of the Haliangiaceae family. These are mesophilic organisms previously identified to be sensitive to agricultural practices (e.g., Ding et al., 2014; Kim and Liesack, 2015; Wang et al., 2016), which abundance could be favored by lower soil temperatures in densely planted vineyards. This highlights the importance of temperature, shown above, in the formation of soil bacterial communities. However, vine density and the use of under-vine cover crops could also cause

different levels of interactions between plant roots and soil microbes. This is particularly prominent when comparing sites with similar topography and soil texture, in which spatial patterns of soil biota are assumed to be structured primarily by plant growth, age, growth form and density (Ettema, 2002). Our results indicate that the abundance of taxa from the bacterial family Hyphomicrobiaceae is positively correlated with the vineyard age. Plant age has previously been linked to differences in soil bacterial communities in annual crops (Marques et al., 2014; Walters et al., 2018) and in wild plant species (Wagner et al., 2016; Na et al., 2017). However, how composition and diversity of rhizosphere communities shift with plant age in perennial, long-living crops has received less attention and needs to be investigated in the future.

Correlations Between Soil Bacterial Communities and Berry and Wine Parameters

Berry parameters were found to be significantly associated with both the composition and diversity of soil bacteria and with the abundance of single taxa. A total of six fruit traits correlated with differences in bacterial community composition and diversity, while one fruit trait (total phenolics in berry) was found significantly associated with the abundance of specific taxa. Plant–microbe interactions are known to modify the metabolome of *Arabidopsis thaliana* plants grown under controlled conditions (Badri et al., 2013), however, the modulating effect of soil bacteria on the metabolome of commercial crops is unexplored. Unfortunately, the non-intervention nature of this research impedes us determining if the relationships observed between vineyard soil bacteria and fruit traits are causal or simply mere correlations.

Soil microbes have previously been described as a contributor to the final sensory properties of wines by affecting wine fermentation. Soil (Gupta et al., 2019) and grape must (Bokulich et al., 2016; Liu et al., 2020) microbiota were found to be correlated to regional metabolite profiles and was suggested to be potential predictor for the abundance of wine metabolites. In what, to this date, is possibly the most thorough analysis of the contribution of vineyard soil microbiomes to wine regionality, Liu et al. (2020) found that soil and must fungal communities are affected by the vineyard's edaphic and climatic characteristics and, in turn, associated to wine regionality. Similarly, through the analysis of soil bacterial communities in 22 vineyards, our study identified 18 wine traits correlated with differences in bacterial community composition and diversity, and four correlated with the abundance of specific taxa. Vineyard soils may serve as a bacterial reservoir since bacterial communities associated with leaves, flowers, and grapes share a greater proportion of taxa with soil communities than with each other (Zarraonaindia et al., 2015). Liu et al. (2020) proposed the xylem sap as one mechanisms joining the soil and the fruit microbial communities. Unfortunately, the non-intervention nature of this research, the lack of replicability and the use of commercially produced wines (each of these wines was made commercially by different producers so there is potential for a certain level of winemaking effect), preclude us from determining if the relationships observed between vineyard soil bacteria and fruit/wine traits are causal or simply mere correlations. Future work should be aimed at experimentally testing the true nature of the observed correlations.

CONCLUSION

Taken collectively our results show that geographic separation between vineyards contributes to bacterial community dissimilarities at a much smaller scale than previously reported. Environmental variables (e.g., climatic, topography, soil properties, and management practices) were the greatest contributor to such differences. Particularly, we found that soil variables are the major shapers of bacterial communities. Also, we show that variables highly affected by soil anthropogenisation (pH, plant available Phosphorous) and agricultural management variables (plant age, planting density) have strong correlations both with the community composition and diversity and the relative abundance of individual taxa. Our results provide an important starting point for future studies investigating the potential influence of bacterial communities on the metabolome of grapevines in general, and on the definition of local Terroirs. Future studies should include the analysis of fungal communities, which has been shown to be strongly associated with wine regionality. It will also be important to study a wider range of soil physicochemical properties, and vineyard floor vegetation, on the soil microbiome.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA601984.

AUTHOR CONTRIBUTIONS

TC, JS, AM, MG, JB, CC, and CR conceived and planned the experiments. CC and RDB contributed to the design of the research project, vineyard selection, and fruit and wine chemical characterization. JZ conducted soil physicochemical analysis and the 16S rRNA gene laboratory work. JZ and TN conducted the bioinformatics analysis. JZ and CR took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and 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.2020.597944/full#supplementary-material

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The Structure of Rhizosphere Fungal Communities of Wild and Domesticated Rice: Changes in Diversity and Co-occurrence Patterns

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The rhizosphere fungal community affects the ability of crops to acquire nutrients and their susceptibility to pathogen invasion. However, the effects of rice domestication on the diversity and interactions of rhizosphere fungal community still remain largely unknown. Here, internal transcribed spacer amplicon sequencing was used to systematically analyze the structure of rhizosphere fungal communities of wild and domesticated rice. The results showed that domestication increased the alpha diversity indices of the rice rhizosphere fungal community. The changes of alpha diversity index may be associated with the enrichment of *Acremonium*, *Lecythophora*, and other specific rare taxa in the rhizosphere of domesticated rice. The co-occurrence network showed that the complexity of wild rice rhizosphere fungal community was higher than that of the domesticated rice rhizosphere fungal community. Arbuscular mycorrhizal fungi (AMF) and soilborne fungi were positively and negatively correlated with more fungi in the wild rice rhizosphere, respectively. For restructuring the rhizomicrobial community of domesticated crops, we hypothesize that microbes that hold positive connections with AMF and negative connections with soilborne fungi can be used as potential sources for bio-inoculation. Our findings provide a scientific basis for reshaping the structure of rhizomicrobial community and furthermore create potential for novel intelligent and sustainable agricultural solutions.

Keywords: domestication, rhizosphere, soil-borne fungi, co-occurrence patterns, arbuscular mycorrhizal fungi

INTRODUCTION

The rhizosphere is inhabited by the taxonomically structured fungal community that plays an essential role in absorbing nutrients and provides resistance against pathogen invasion and other abiotic stresses associated with their respective host crops (Stringlis et al., 2018; Perez-Jaramillo et al., 2019). For instance, arbuscular mycorrhizal fungi (AMF) can establish mutualistic

endosymbiosis with their respective host crop to improve the absorption of mineral nutrients (predominantly phosphate) and enhance the resistance to pathogens, as well as abiotic stresses within the host plant (Akiyama et al., 2005; Luginbuehl et al., 2017; Wang et al., 2017; Jia et al., 2019; Gao et al., 2020). Contrary to AMF, some soilborne fungi are causative factors for creating disease at the crop level (Delgado-Baquerizo et al., 2020). For example, *Passalora rosicola* and *Fusarium oxysporum* in the rhizosphere were associated with common leaf spot and wilt or root rots (Fravel et al., 2003; Feres et al., 2017). As an important feature of the rhizosphere fungal community, the dynamics of fungi–fungi interactions are recognized to carry out important symbiotic microbial functions for maintaining crop health. Characterizations of these interactions have shed new light on the mechanism of pathogen infection and AMF symbiosis (van der Heijden et al., 2008; Agler et al., 2016). However, at the present time, little is known regarding the fungi–fungi interactions within rhizosphere fungal community. As a result, it is of great interest to reveal the mechanism of pathogen infection and AMF symbiosis.

As an important economic crop, rice (*Oryza* species) is the principal food for half of the world's population (Wang et al., 2018), and rice domestication has been regarded as a critical development in the history of crop domestication. The existing domesticated rice *Oryza sativa* ssp. *japonica*, *O. sativa* ssp. *Indica*, and *Oryza glaberrima* varieties were domesticated from the wild rice species *Oryza rufipogon*, *Oryza nivara*, and *Oryza barthii*, which originated from China, India, and West Africa, respectively (Huang et al., 2012; Wang et al., 2014). The different origins of these materials make the selection highly oriented, and the influence mechanism of habitat factors is also another major factor affecting the genetic context of the germplasms at the gene level. The rhizomicrobial community shares a long history of coevolution with host rice species (Gosling et al., 2006; Kovach et al., 2007; Bin Rahman and Zhang, 2016; Leff et al., 2017; Chang et al., 2021). Studies showed that wild accessions of crops are more tolerant to stress (i.e., disease tolerance and cold tolerance) than domesticated accessions, which may be associated with the interactions among the rhizomicrobial community (Perez-Jaramillo et al., 2017; Choudhary et al., 2018; Tian et al., 2019). Specifically, our team found that the structures of the bacterial and fungal community of wild rice rhizosphere were more stable than those of domesticated rice after the stimulation of exogenous factors to the rhizosphere, such as application of *Magnaporthe grisea* (Shi et al., 2018, 2019; Xu et al., 2019). Recent investigations have addressed the selective effect on the structure of rhizosphere bacterial community during rice domestication. Furthermore, changes of bacterial communities affecting plant growth and development, and further altering plant traits, have been investigated (Mendes et al., 2013; Shenton et al., 2016; Tian et al., 2017). However, little attention has been given to the complicated changes and the interactions among the rhizosphere fungal community during rice domestication.

The major purpose of the current study was to reveal the selective effect of wild and domesticated rice on the structure of the rhizosphere fungal community. Furthermore, we investigated the interactions of fungi–fungi in the wild and domesticated rice rhizosphere, with emphasis on elucidating the interactions between soilborne fungi, AMF, and other fungi community members. We hypothesized that the domestication of rice decreased the cooperative interactions between AMF and other fungi and further limited the beneficial effect to plant growth. To test the hypothesis, we selected rhizosphere soil samples of 12 accessions of wild and domesticated rice to compare the structure of wild and domesticated rice rhizosphere fungal communities using internal transcribed spacer (ITS) amplicon sequencing.

MATERIALS AND METHODS

Plant Materials

Twelve accessions of wild and domesticated rice were used in this study. Wild rice accessions were obtained from the International Rice Research Institute (IRRI), and domesticated rice accessions were obtained from the Philippines and Jiangxi Academy of Agricultural Sciences, China. Five accessions of wild rice included African wild rice (*O. barthii*) IRGC 106238, common wild rice (*O. rufipogon*) IRGC 106286, common wild rice (*O. rufipogon*) IRGC 106452, Indian wild rice (*O. nivara*) IRGC 86655, and Indian wild rice (*O. nivara*) IRGC 88949. Seven accessions of domesticated rice include African domesticated rice No. 2 (*O. glaberrima*), African domesticated rice No. 3 (*O. glaberrima*), African domesticated rice No. 4 (*O. glaberrima*), Asian domesticated rice japonica Jiangxi (*O. sativa* ssp. *japonica*), Asian domesticated rice japonica Daohuaxiang (*O. sativa* ssp. *japonica*), and Asian domesticated rice cultivars indica 106 and Meitezhen (*O. sativa* ssp. *indica*).

Sample Collection

The experimental plots were located in the rice experimental station of the Chinese Academy of Sciences (18°19′57 N, 109°27′ E) in Sanya, Hainan Province, and were designed in the standard sized plots for rice cultivation. This area is representative of a typical tropical ocean monsoon climate with a mean annual precipitation of 1,347.5 mm and an annual average temperature of 25.7°C. The experiment was arranged in a randomized block design, and there is a 30-cm distance between each of the plots (1 m^2) to avoid interference. Before experiments were conducted, five soil samples were sampled from 10-cm soil layer of each plot, and the data confirmed soil property homogeneity. The soil texture of the experimental plots was sandy loam. The soil chemical properties of the experimental plots, including pH, soil organic matter, total nitrogen, total phosphorus, available nitrogen, and available phosphorus were 5.22, 25.61 g/kg, 1.54 g/kg, 0.51 g/kg, 162.72 mg/kg, and 20.64 mg/kg, respectively. The samples were collected on November 5–7, 2017, and the experimental plots were kept flooded with a water depth of 10 cm. Wild rice was shown to have longer root length, higher plant height, and better resistance to numerous biotic and abiotic stresses than domesticated rice, such as lodging and drought tolerance (Tian et al., 2018;

Nasir et al., 2019; Yu et al., 2020). As comparison, domesticated rice was reported to have high root biomass, more lateral roots, and higher yield than wild rice (Raju et al., 2014; Shi et al., 2019). The trypan blue method was used as described by Phillips and Hayman (1970) to observe the root colonization of the arbuscular mycorrhiza fungi. The rhizosphere samples (soil adhering to root in 1 mm) were collected at the flowering stage of rice under flooding conditions. Five samples from one experimental plot were well mixed as a biological replicate, and there were five replicates for each rice accession, with the exception of common wild rice (*O. rufipogon*) IRGC 106452 which had four replicates. A total of 59 samples were collected, including 35 from domesticated rice and 24 wild rice samples. The loose soil adhering to the roots was shaken off, and the 1-mm root was subsequently immersed in the tube containing 5 ml of sterile water and vortexed to collect the rhizosphere soil that was tightly attached to the root (Edwards et al., 2015). After a short centrifugation at a relative centrifugal force (RCF) of 10,000 *g* for 30 s and removal of the supernatant, each rhizosphere soil sample was stored at −80°C (Edwards et al., 2015).

DNA Extraction and Amplicon Sequencing

A 0.5-g rhizosphere soil sample was ground into powder in liquid nitrogen, and DNA was extracted from the soil according to the instructions of the Fast DNA SPIN Kit (Catalog No. 6560-220, MP Biomedicals, Germany). DNA concentrations were measured using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States). The quality of DNA was evaluated by 1.2% agarose gel electrophoresis, and only DNA exhibiting a clear resolved band was chosen for further sequencing. Fungal ITS1 was amplified by using the primers ITF5F (GGAAGTAAAAGTCGTAACAAGG) and ITS1R (GCTGCGTTCTTCATCGATGC). Sample-specific 7-bp barcodes were incorporated into the primers for multiplex sequencing. The PCR amplicons were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN, United States) and subsequently quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, United States). The 250-bp paired-end sequencing was performed by using the Illumina MiSeq platform. The raw sequencing data were processed by using QIIME v2.0.0 (Quantitative Insights Into Microbial Ecology)[1] (Caporaso et al., 2012), and paired-end reads were merged by using FLASH v1.2.7 (Magoč and Salzberg, 2011). Sequences shorter than 300 bp, average quality scores of <20, with ambiguous bases, and with mononucleotide repeats of >8 bp were filtered using Trimmomatic v0.33 (Bolger et al., 2014). Chimeric sequences were identified and removed using UCHIME v4.2.0 (Edgar et al., 2011). The remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at a 97% sequence identity by UPARSE v7.0.1001 (Edgar, 2013). A total of 1,689,479 raw fungal reads were obtained, and after quality filtering, a total of 1,687,277 clean reads were obtained. The rarefaction curves already reached a plateau, indicating that the sequencing coverage was valid to quantify the majority of species (**Supplementary Figure S1**). A representative sequence was selected from each OTU using default parameters. OTU taxonomic classification was conducted by BLAST searching the representative sequence set against and UNITE database (version 5.0)[2] (Koljalg et al., 2013). Potential plant pathogens for fungal communities and AMF were determined using the FUNGuild database.[3]

Statistical Analysis

Permutational multivariate analysis of variance (PERMANOVA) was used to detect significant changes in the microbiota structure using pseudo-F value as a proxy for the strength of an individual factor (rice domestication, rice species, or origins) on the fungal community composition (Tkacz et al., 2020). For the PERMANOVA of domestication factor, all samples were separated into two groups: wild and domesticated rice. For the PERMANOVA of accessions factor, all samples were separated into 12 groups of rice species as described in section "Materials and Methods." For the PERMANOVA of origins factor, all samples were separated into two groups: African rice and Asian rice.

Principal coordinates analysis (PCoA) was used to visualize the difference of fungal classification of wild and domesticated rice rhizosphere based on the Bray–Curtis dissimilarity matrix with the "vegan" package in R (v3.6.1). The statistical significances of the clustering patterns in ordination plots were subsequently evaluated by using PERMANOVA.

Richness (Chao1 index) and evenness (Shannon index) between samples were used for alpha diversity. Chao1 and Shannon indices were calculated by "vegan" and "picante" packages in R (v3.6.1) (Hughes and Hellmann, 2005). To evaluate the difference of Chao1 and Shannon index across wild and domesticated rice rhizospheres, a one-way analysis of variance was conducted with a Tukey's test ($P < 0.05$) using SPSS v20.0 (IBM, Chicago, IL, United States). To test the number of shared and unique OTUs across wild and domesticated rice rhizosphere, the "VennDiagram" package in R (v3.6.1) was used (Fouts et al., 2012).

To determine the co-occurrence patterns of fungal communities between wild and domesticated rice rhizospheres, we calculated SparCC's rank correlation coefficients (Python 2.6.1) of taxonomic genera. Here, all samples were grouped into wild and domesticated rice. This method randomly creates 100 simulation datasets from the original data and calculates the pseudo-*P* value by determining how many of the 100 datasets produce the same order of magnitude correlation with the original data (Friedman et al., 2012). For wild rice, 25 replicates were used to calculate the correlation coefficients, while 35 replicates were used for domesticated rice. The network was visualized by Gephi 8.0 [SparCC's r (absolute value) > 0.5, $P < 0.05$].

[1]http://qiime.org/

[2]https://unite.ut.ee/analysis.php/

[3]http://www.stbates.org/guilds/app.php

RESULTS

Influence of the Rice Domestication, Species, and Origins on Fungal Community Structure

We initially investigated the structure of fungal communities of 12 rice species, which are divided into two groups, wild rice and domesticated rice, with origins from China, India, and West Africa. PERMANOVA was used to determine the strongest influences on the fungal community. Rice domestication, species, and origins were found to significantly influence the composition of rhizosphere fungal community. Specifically, the strongest factor was rice domestication (pseudo-F: 21.60) (**Figure 1A**); the second strongest factor was the rice species (pseudo-F: 15.29), and the least important factor was the origins (pseudo-F: 6.22).

Effect of Domestication on the Composition and Diversity of Fungal Community

To elucidate the effects of rice domestication on the structure of rhizosphere microbiota, PCoA based on Bray–Curtis dissimilarity matrix was performed using 12 accessions that were grouped into wild and domesticated rice. The rhizosphere fungal communities of wild and domesticated rice were significantly

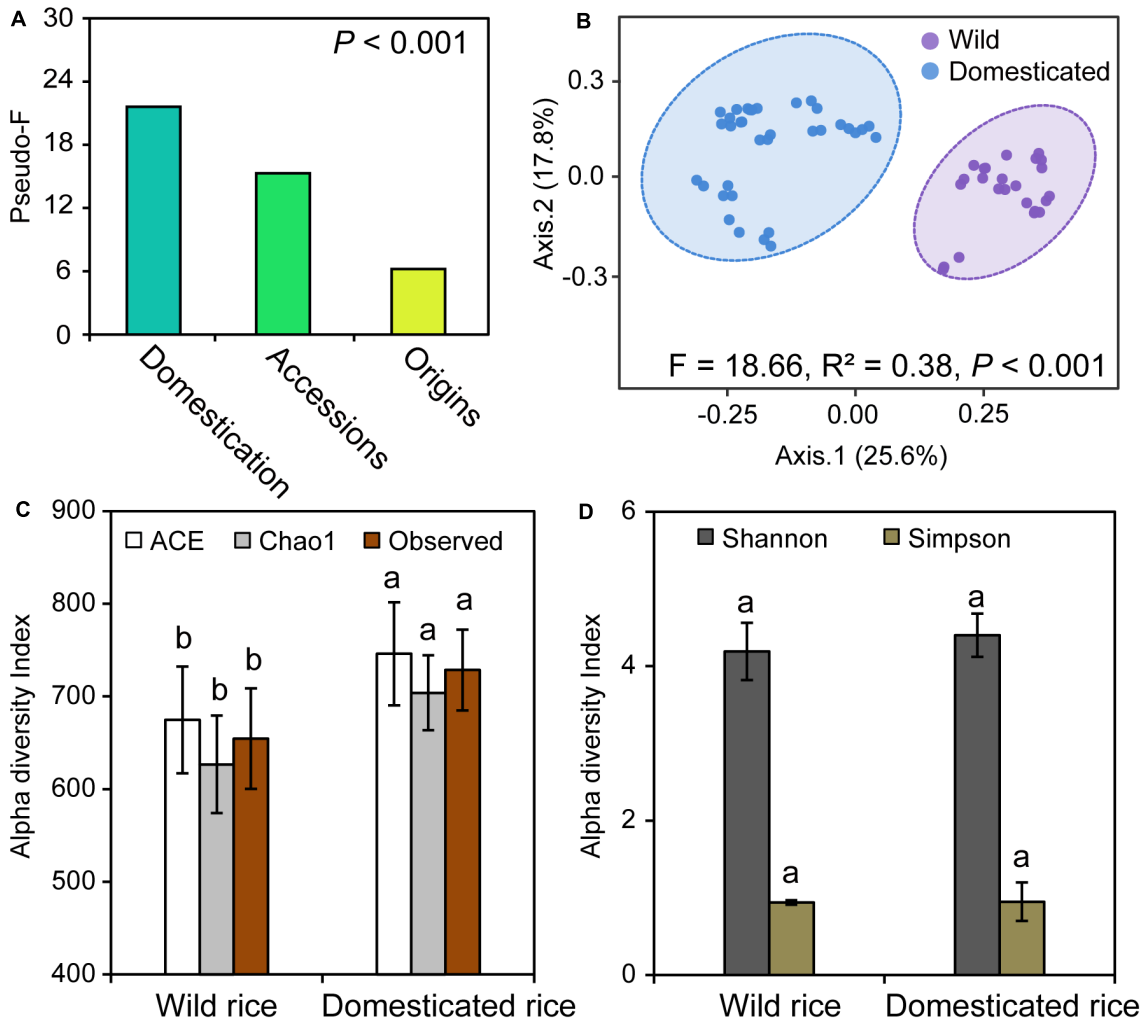

FIGURE 1 | (A) The permutational multivariate analysis of variance (PERMANOVA) output showing the importance of rice domestication, rice species, and origins factors shaping the rhizosphere fungal community. **(B)** Compositional changes of fungal communities in the rhizospheres of wild and domesticated rice. **(C,D)** Alpha diversity indices of fungi in the rhizospheres of wild and domesticated rice. The pseudo-F value was used as a proxy for the importance of the factor **(A)**. For the PERMANOVA of domestication factor, all samples were separated into two groups: wild and domesticated rice. For the PERMANOVA of accessions factor, all samples were separated into 12 groups: 12 rice species as described in section "Materials and Methods". For the PERMANOVA of origins factor, all samples were separated into two groups: African rice and Asian rice. Principal coordinates analysis (PCoA) was performed based on the Bray–Curtis dissimilarity matrix at operational taxonomic units (OTUs) level across all the samples **(B)**. Ellipses demonstrate the mean ± 1 SD, wild rice in purple and domesticated rice in blue. In panels **(C,D)**, wild rice rhizosphere samples, $n = 24$; domesticated rice rhizosphere samples, $n = 35$. Different letters within each column indicated significant differences between wild and domesticated rice based on a one-way ANOVA with a Tukey test at the $P < 0.05$ level.

different (PERMANOVA, R^2 = 0.28, P < 0.001), and the percentages of variation explained by axis 1 and axis 2 were 29.6 and 17.4%, respectively (**Figure 1B**). To identify the effects of the diversity of the fungal community, we investigated the alpha diversity. Chao1 index, ACE index, and Observed index of the rhizosphere fungal community of domesticated rice were significantly higher than those of wild rice (**Figure 1C**; $P < 0.05$). There was no significant difference in the Shannon and Simpson indices between wild and domesticated rice (**Figure 1D**; $P > 0.05$). Additionally, 1,685 shared OTUs accounted for 73% of the total OTUs of wild and domesticated rice rhizosphere (**Supplementary Figure S2**). The numbers of unique OTUs of fungal communities of wild and domesticated rice rhizospheres were 249 and 373, respectively; accounting for 10.8 and 16.2% of totally wild and domesticated rice OTUs.

Differences in the main proportion of microbiota were subsequently tested, and the dominant fungal phyla across wild and domesticated rice rhizospheres were Ascomycota, Basidiomycota, and Zygomycota. These data presented relative abundances ranging from 29.32 to 47.79%, 27.24 to 28.70%, 10.81 to 17.32%, respectively (**Figure 2A**). The relative abundance of Zygomycota in the domesticated rice rhizosphere was higher than that of wild rice. On the other hand, the relative abundances of Ascomycota and Basidiomycota in the rhizosphere of wild rice were higher than that of domesticated rice. AMF correspond to a monophyletic group included in the phylum Glomeromycota, and there was no significant difference in the relative abundance of Glomeromycota between wild and cultivated rice. Results indicated that the AMF were able to colonize both wild and domesticated rice root under flooding conditions (**Figures 3A,B**). And there was no significant difference between the root colonization of the AMF of wild and domesticated rice (**Figure 3C**). Further taxonomical classification at the genus level revealed that the relative abundance of *Acremonium* in the rhizosphere of domesticated rice (56.2%) was higher than that of wild rice (54.87%) (**Figure 2B**).

Effect of Domestication on the Correlations Between Fungal Communities

Co-occurrence analysis was used to investigate potential interactions between fungal taxa of wild and domesticated rice rhizospheres (**Figure 4**). The co-occurrence network of the wild rice rhizosphere consisted of 131 nodes and 1,046 edges, whereas those of domesticated rice consisted of 84 nodes and 346 edges (**Supplementary Table S3**). Besides, the network of wild rice rhizosphere had more balanced interactions, with 53.92% positive connections and 45.7% negative connections. The discrepancy in the total numbers of correlations among genera in the network input data and that of the co-occurrence network nodes suggests that a tighter association exists among genera in wild rice rhizosphere than what occurs within the domesticated rice rhizosphere.

Five genera of AMF (*Acaulospora*, *Claroideoglomus*, *Pacispora*, *Redeckera*, and *Scutellospora*) were significantly correlated with other fungi in the rhizosphere of wild rice, whereas three groups of AMF (*Claroideoglomus*, *Gigaspora*, and *Redeckera*) were significantly correlated with other fungi in the rhizosphere of domesticated rice. The number of connections of these AMF with other fungi of the wild rice rhizosphere was higher than that of the domesticated rice rhizosphere (**Supplementary Table S1**). Besides, there are 77% positive connections and 23% negative connections between AMF and other fungi of the wild rice rhizosphere, while the network in the rhizosphere of domesticated rice had 59% positive connections and 41% negative connections between AMF and other fungi. Furthermore, the number of connections between soilborne fungi and other fungi of the wild rice rhizosphere was 327 and higher than that of the domesticated rice rhizosphere (63 connections) (**Supplementary Table S1**). The network of the wild rice rhizosphere had 53% positive connections and 47% negative connections between soilborne fungi and other fungi.

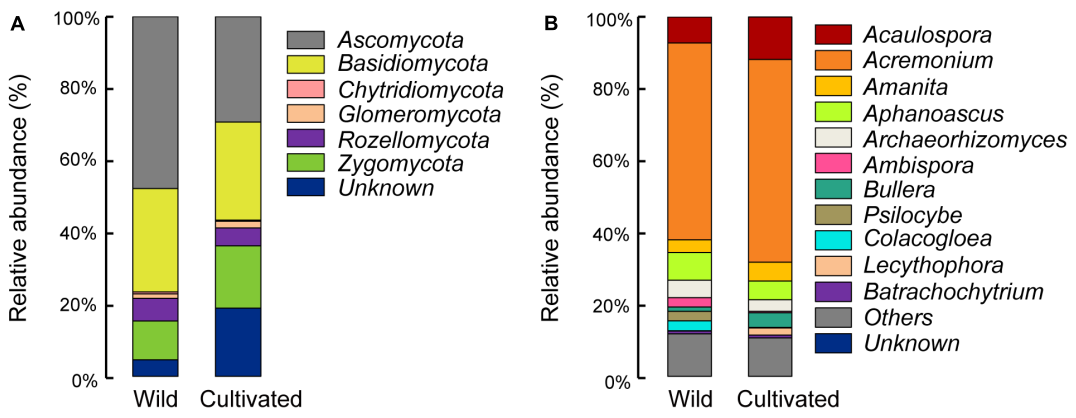

FIGURE 2 | Relative abundances of the dominant fungal phyla **(A)** and fungal genera **(B)** in the rhizospheres of wild and domesticated rice. Relative abundance of the dominant fungal phyla and genera were identified by BLAST searching the representative sequence set against the UNITE database, and the sequences not assigned to kingdom fungi were classified as "unknown." Treatments included the rhizospheres of wild and domesticated rice. The relative abundance of Zygomycota was highest in bulk soil, while the relative abundance of Ascomycota was highest in the rhizosphere of wild and domesticated rice. The relative abundance of *Acaulospora* was highest in bulk soil, while the relative abundance of *Acremonium* was highest in the rhizosphere of wild and domesticated rice.

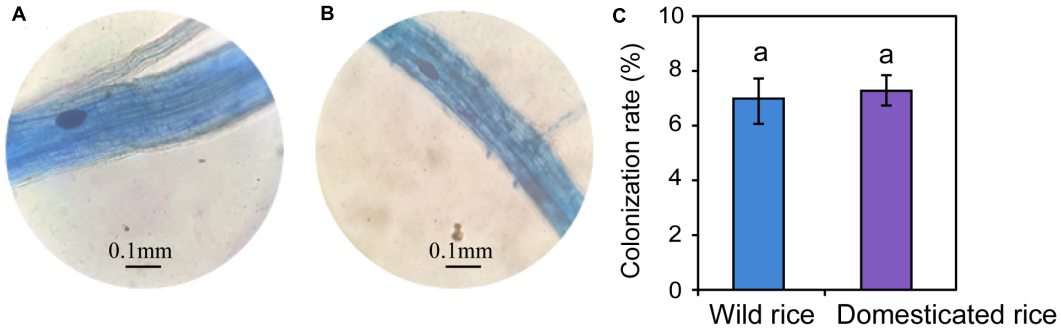

FIGURE 3 | Morphology of the arbuscular mycorrhizal fungi-colonized roots of wild **(A)** and domesticated **(B)** rice. Colonization rate of the arbuscular mycorrhizal fungi in roots of the rice plants **(C)**.

On the other hand, there were 68% positive connections and 32% negative connections between pathogens and other fungi of the domesticated rice rhizosphere.

The higher degree and betweenness centrality of the nodes in the network of the wild rice rhizosphere indicated that the connectivity among nodes is higher in the rhizosphere of wild rice in comparison to that of the domesticated rice rhizosphere. Here, the network hub genera showing a high degree and betweenness centrality were identified. For the wild rice rhizosphere, *Westerdykella* and *Amanita* were defined as the hub taxa and may play an important role in structuring the rhizosphere fungal community (**Figure 4C**). With the development of domestication, *Retroconis* and *Schizangiella* were defined as the hub taxa in the rhizosphere of domesticated rice (**Figure 4D**).

DISCUSSION

Plant genotype, soil, and environmental stresses have been studied intensively to reveal their influences on rhizomicrobiomes (Tkacz et al., 2015; Walitang et al., 2018). However, rhizomicrobiomes are also affected by plant domestication, species, and different origins (Bulgarelli et al., 2015; Leff et al., 2017). The current study was designed and deployed in an effort to determine the role of rice domestication, species, and origins factors in shaping the rhizosphere fungal structure. These efforts ultimately identified rice domestication as the most important factor. Studies on the rhizomicrobiomes of *O. sativa* and their relative wild accessions *O. rufipogon* have shown that the domesticated rice is different from wild rice due to its long domestication history and some distinct rhizomicrobial groups of wild rice that were gradually eliminated (Shenton et al., 2016; Tian et al., 2017; Chang et al., 2021). These observations are in accordance with the hypothesis that the plant host coevolved with their rhizomicrobiomes, and that rhizomicrobial communities are primarily determined by a series of changes due to domestication (Moran and Sloan, 2015; Sasse et al., 2017; Kim et al., 2020).

Rice domestication increased the richness index of the rhizosphere fungal community. There are some lines of experimental evidence demonstrating that the domestication of soybean (*Glycine* ssp.) and corn (*Zea mays* ssp.) also increased the richness index of the rhizosphere bacterial community (Szoboszlay et al., 2015; Chang et al., 2018). One possible explanation for this result might be that, after long-term directional selection, rice domestication resulted in a change of physiological characteristics and affected the levels of metabolites, which may recruit specific fungal species for colonization of the rhizosphere (Grigulis and Clément, 2013). This further proved by our result that *Lecythophora* was the specific fungal genus recruited by domesticated rice rhizosphere in comparison with the wild rice rhizosphere. The relative abundances of *Ambispora* and *Lecythophora* were significantly changed, showing that some microbial groups can be recruited and some microbial groups can be eliminated during rice domestication. Recently, Zhang et al. (2019) found that the duration of rice cultivation affected the alpha diversity of the soil fungal community. These data indicated that the fungal alpha diversity significantly increased when the duration of rice cultivation was less than 15 years and tended to be stable after 15 years. Our study extends this result and demonstrates that domestication leads to the increase of alpha diversity of the rhizosphere fungal community.

Although AMF has been reported to grow under aerobic conditions (Li et al., 2011; Maiti, 2015), we found that the AMF were able to colonize both wild and domesticated rice root under flooding conditions. Recent evidence showed that AMF could colonize rice root grown in flooded conditions, and the rice root colonization by AMF under flooded conditions was lower than that under non-flooded conditions (Hajiboland et al., 2009; Liu et al., 2013, 2014). One possible explanation for this result might be that rice root aerenchyma can transport oxygen to satisfy the aerobic respiration process of the root. Additionally, some of the oxygen will be released into the rhizosphere through the root axis during the transport process to support some members of the rhizomicrobiomes (Colmer, 2003). However, there was no significant difference between the root colonization of AMF between wild and domesticated rice.

It is plausible that the most important contribution of rice domestication might be an alteration in the interactions between

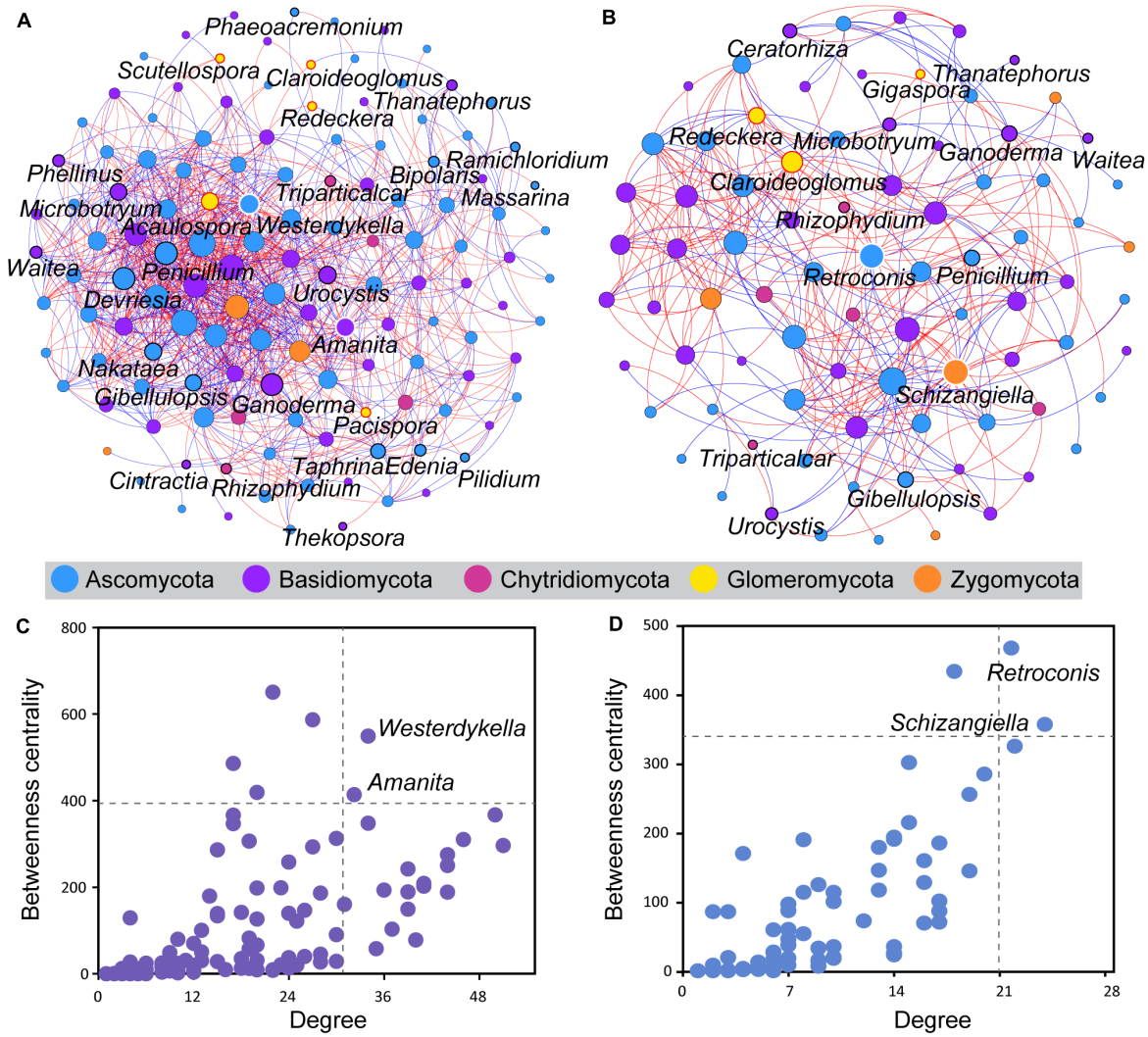

FIGURE 4 | Co-occurrence analysis of the rhizosphere fungal community of wild and domesticated rice. The co-occurrence networks of the rhizosphere fungal community of wild **(A)** and domesticated **(B)** rice were constructed based on the SparCC correlation coefficients. An edge stands for a significant positive correlation (SparCC $r > 0.5$, $P < 0.05$, red line) or significant negative correlation (SparCC $r < -0.5$, $P < 0.05$, blue line). The nodes represent taxa affiliated at the genus level with at least one significant correlation. The size of the node is proportional to the degree (the number of connections). Each node was colored by phyla. The nodes circled by red, white, and black outlines are arbuscular mycorrhizal fungi, hub fungi, and potential plant pathogens, respectively. The nodes representing hub fungi taxa were labeled. The hub taxa of the rhizosphere fungal community of wild **(C)** and domesticated **(D)** rice were nodes showing a high degree and betweenness centrality. The dashed lines indicated the threshold values (top 2%). The nodes representing hub fungi taxa were labeled.

microbes. As a consequence, this would further affect the function of the rhizomicrobial community or even the function of the rhizosphere micro-ecosystem. The results of the co-occurrence network-based analysis in this study showed that both node-level and network-level topological features differ between the wild and domesticated rice rhizospheres. The hub taxa of the co-occurrence network of the wild rice rhizosphere were *Westerdykella* and *Amanita*. Additionally, the hub taxa of the co-occurrence network of the domesticated rice rhizosphere were *Retroconis* and *Schizangiella*, which further indicates that the interactions were significantly changed during the domestication of rice. Genera characteristic of the domesticated rice rhizosphere had lower betweenness centrality values and degree values as compared to that of the wild rice rhizosphere. The betweenness centrality represents the importance of the control potential that an individual genus exerts over the interactions of other genera in that network (Greenblum et al., 2012). Additionally, the higher values of connectance, nodes, and correlations made the network of the wild rice rhizosphere more complex than that of domesticated rice rhizosphere. As a result, this enables the wild rice rhizosphere to be more stable against external environmental changes (Wang et al., 2006).

Our team revealed an interesting finding that wild types (including soybean and rice) may have evolved to recruit beneficial microbes in the rhizosphere that are capable of promoting nutrient requisition, biostasis, and disease

resistance (Chang et al., 2018; Shi et al., 2019). However, little is known about the interaction of beneficial microbes in the rhizosphere. The interactions between microbes can be the reflection of biological interactions in an ecosystem where species are associated with complicated positive (for example, commensalism and mutualism) and negative (for example, predation and competition) interactions (Faust and Raes, 2012; Jiang et al., 2017). In the current study, the co-occurrence network suggested that AMF of the wild rice rhizosphere are positively correlated with more fungi, while soilborne fungi of the wild rice rhizosphere are negatively correlated with more fungi. Additional commensalism and mutualism connections of AMF with other fungi may result in the characteristics of stress resistance of wild rice. Meanwhile, more and more fungi that compete or predate with soilborne fungi may inhibit the infection of pathogens to the host (Whipps, 2001). This result is in support of the hypothesis that rice domestication decreased the cooperative relationships between AMF and other fungi and further limited the beneficial effect to the growth of plants. Specific microbial groups that keep negative correlations with *Fusarium* spp. have been suggested to be used as candidate biocontrol agents to prevent or control the diseases caused by *Fusarium* (Cobo-Diaz et al., 2019). These findings served as the inspiration for our hypothesis that the microbial groups that hold positive connections with AMF and negative connections with soilborne fungi are likely to be used as potential biocontrol agents to restructure the domesticated rice rhizosphere.

In conclusion, domestication is a very important factor that increased the alpha diversity index (richness index) of the rice rhizosphere fungal community. The co-occurrence patterns demonstrated that the complexity of wild rice rhizosphere was higher than that of domesticated rice, and that AMF and soilborne fungi were positively or negatively correlated with more other fungi, respectively. These results suggested that rice domestication decreased the possibility of benefiting from AMF symbiosis and increased the possibility of infection by pathogens. From the perspective of restructuring the rhizomicrobial community of domesticated crops, we suggest that the microbial groups holding positive connections with AMF and negative connections with plant pathogens can be used as potential biocontrol agents. Our findings are expected to provide rational suggestions for reshaping the structure of rhizomicrobial community of domesticated rice and furthermore highlight the potential to create novel intelligent and sustainable agricultural solutions.

DATA AVAILABILITY STATEMENT

The datasets presented in this study are deposited at https://www.ncbi.nlm.nih.gov/, SRP298523.

AUTHOR CONTRIBUTIONS

CT conceived and designed the study. JC, LJ, and SL helped with the experiment design. FN and LT collected the samples. JC analyzed the data and prepared the figures and table. EK and YS helped with the improvement of 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.2021.610823/full#supplementary-material

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Effect of Low-Input Organic and Conventional Farming Systems on Maize Rhizosphere in Two Portuguese Open-Pollinated Varieties (OPV), "Pigarro" (Improved Landrace) and "SinPre" (a Composite Cross Population)

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Maize is one of the most important crops worldwide and is the number one arable crop in Portugal. A transition from the conventional farming system to organic agriculture requires optimization of cultivars and management, the interaction of plant–soil rhizosphere microbiota being pivotal. The objectives of this study were to unravel the effect of population genotype and farming system on microbial communities in the rhizosphere of maize. Rhizosphere soil samples of two open-pollinated maize populations ("SinPre" and "Pigarro") cultivated under conventional and organic farming systems were taken during flowering and analyzed by next-generation sequencing (NGS). Phenological data were collected from the replicated field trial. A total of 266 fungi and 317 bacteria genera were identified in "SinPre" and "Pigarro" populations, of which 186 (69.9%) and 277 (87.4%) were shared among them. The microbiota of "Pigarro" showed a significant higher ($P < 0.05$) average abundance than the microbiota of "SinPre." The farming system had a statistically significant impact ($P < 0.05$) on the soil rhizosphere microbiota, and several fungal and bacterial taxa were found to be farming system-specific. The rhizosphere microbiota diversity in the organic farming system was higher than that in the conventional system for both varieties. The presence of arbuscular mycorrhizae (Glomeromycota) was mainly detected in the microbiota of the "SinPre" population under the organic farming systems and very rare under conventional systems. A detailed metagenome function prediction was performed. At the fungal level, pathotroph–saprotroph and pathotroph–symbiotroph lifestyles were modified by the farming system. For bacterial microbiota, the main functions altered by the farming system were membrane transport, transcription, translation, cell motility, and signal

transduction. This study allowed identifying groups of microorganisms known for their role as plant growth-promoting rhizobacteria (PGPR) and with the capacity to improve crop tolerance for stress conditions, allowing to minimize the use of synthetic fertilizers and pesticides. Arbuscular mycorrhizae (phyla Glomeromycota) were among the most important functional groups in the fungal microbiota and *Achromobacter*, *Burkholderia*, *Erwinia*, *Lysinibacillus*, *Paenibacillus*, *Pseudomonas*, and *Stenotrophomonas* in the bacterial microbiota. In this perspective, the potential role of these microorganisms will be explored in future research.

Keywords: maize, microbiota, rhizosphere, organic and conventional farming system, open-pollinated populations, next-generation sequencing

INTRODUCTION

Maize (*Zea mays*) is one of the most important cereal crops in human and animal diets worldwide. Together with rice and wheat, it provides at least 30% of the food calories to more than 4.5 billion people in 94 developing countries (Shiferaw et al., 2011). Aside from providing nutrients for humans and animals, maize serves as a basic raw material to produce starch, oil, protein, alcoholic beverages, food sweeteners, and fuel (Wu and Guclu, 2013).

According to FAOSTAT, from 2012 to 2017, the average world production per year of corn was around 1,036,263,896 t. In Portugal, maize is the most important arable crop, occupying an area of approximately 150,000 ha (ANPROMIS, 2019), producing an annual average of 826,417 Mg from 2012 to 2017 (FAOSTAT, 2019).

Due to new challenges related to the expansion of the human and animal populations allied with the global climatic changes, all opportunities for sustainably increasing the yield are relevant and should be developed. For example, improvements on both agronomic practices and breeding related with microorganism interactions (e.g., control of root diseases) and organic management are appellative proposals since the rhizosphere microbial communities are critically important for soil nitrogen cycling and plant productivity (Schmidt et al., 2016; Emmett et al., 2017; Wille et al., 2018).

In this context, alternatives that promote more resilient farming systems, increasing the economic activity of rural areas as well as preventing the significant loss of biodiversity in these areas, would contribute to more sustainable agriculture. In line with this thought, the traditional varieties are extremely important and should not be neglected (Altieri, 2004) since they present a genetic reservoir that can be used to select for advantages associated with the adaptability and resilience of cultivars to low-input and a permanently changing environment. One of the characteristics of interest in these varieties is the possibility of maintaining genetic diversity through open pollination. New genetic combinations may present new features or capabilities that will allow the plant population to respond with more resilience toward pests, diseases, or even the most adverse weather conditions (Garcia-Tejero et al., 2015). Therefore, traditional varieties can adapt themselves to the environment, having greater adaptability to external factors, increasing the crop population fitness (Altieri and Merrick, 1987; Lane and Jarvis, 2007).

Although traditional Portuguese varieties do not have high yields, they are still cultivated due to their high yield stability even under unfavorable conditions like drought (Garcia-Tejero et al., 2015; Leitão et al., 2019). It is important to note that these varieties also play an important role in the country's rural economy, especially in the Central and Northern regions of Portugal, as their market value for bread making has increased thanks to their health benefits. This practice is also seen as a viable way to preserve the biodiversity of threatened farming systems (Vaz Patto et al., 2013).

Since 2002, knowledge about microbiota and the rhizosphere has increased exponentially; however, we are just beginning to understand the mechanisms of plant–microorganism interactions (Vandenkoornhuyse et al., 2015; Compant et al., 2019). According to Philippot et al. (2013), the rhizosphere is a hotspot of plant–microbe interactions with a profound influence on plant productivity, and all its functions are extremely important in terms of nutrition, health, and plant quality. Indeed, the rhizosphere is a critical interface that supports the exchange of resources between plants and their related soil environment. It is already known that several plants produce components that interact with the rhizosphere microbiota, thus forming a dynamic structure in which microbial diversity can be modified with soil composition, plant species, different genotypes within the same cultivar, and the development stage of the organism (Turner et al., 2013; Chaparro et al., 2014; Kandel et al., 2017). Moreover, the microbiota can help plants survive climate changes, modify tolerance to abiotic and biotic stresses, affect the plant–pathogen interactions, and change the nutrient contents inside the plant (Chadha et al., 2015). In addition to all these factors, management agricultural practices, the addition of fertilizers, the presence of pathogens, or extreme climatic conditions cause important effects on the microbial diversity composition (Andreote and Silva, 2017). All these factors are highly relevant to improve vigor, growth, and plant's health (Muller et al., 2016).

In this context, this study aimed to unravel the effect of genotype and farming system on structural diversity and putative functions of the microbial communities in the rhizosphere of two open-pollinated maize populations ("SinPre" and "Pigarro") cultivated under conventional and organic farming systems in

Portugal. With this approach, groups of microorganisms with the potential to modulate the soil quality and fertility were identified and linked to specific conditions, thus potentially contributing to the increasing crop tolerance for stress conditions and to minimize the use of synthetic fertilizers and pesticides.

MATERIALS AND METHODS

Germplasm Characteristics

A synthetic population of maize, "SinPre" (Sintético Precoce), was obtained through the crossing of 12 maize populations (10 Portuguese landraces and two American populations) using a polycross method based on "Nutica" experience (Mendes-Moreira et al., 2009). The 12 maize populations were open-pollinated in geographic isolation (from other maize) in 2009. The border was constituted by equal amounts of seeds of each population. Two equal sets of 12 rows were organized from the earliest to the latest flowering population and *vice versa* for the second set. With this spatial organization, the earliest populations were close to the latest populations. Per row, progenies were submitted to intensive selection among parents during continued cycles. Afterward, a bulk of the best ears was obtained and distributed to farms in different agroclimatic regions of Portugal. The purpose of this new synthetic population was to provide farmers with a new population, with high diversity that could be adapted to their needs, so that farmers can select the characteristics they appreciate the most (P. Mendes-Moreira, October 2019, personal communication). "Pigarro" is a Portuguese population used to produce maize bread that presents a strong emergency vigor, great tolerance at low temperatures, early flowering and fast drying of the grain, and with ripening group FAO 300. It has white and flint kernels and its ear is known for the high number of kernels per row (between 18 and 28) as well as a strong expression of fasciation (Mendes-Moreira et al., 2017).

Field Characterization and Agronomical Practices

Field trials were established at two locations in Coimbra, Portugal, that belong to Coimbra College of Agriculture: "Caldeirão" in organic (40°130′0.22″N, 8°260′47.69″W) and "Vagem grande" in conventional (40°13′16.2″N, 8°28′29.3″W), with a distance of approximately 2.4 km. Both locations have alluvial soils with a medium field texture; however, they can be differentiated between organic and conventional in the organic matter (1.8 vs. 0.8%), pH (6.4 vs. 6.7), available phosphorus (high vs. very high), and available potassium (very high vs. high) (**Supplementary Table 1**).

The maize seed used was produced in the organic location in 2018 and seeds were not treated. For both fields, the preceding crop was maize.

In conventional agriculture soil, tillage started on 15/05/2019 followed by the first fertilization on 21/05/2019. The fertilizer was distributed (12:20:12 NPK, 318 kg/ha) with a centrifugal fertilizer spreader and incorporated with a rotor tiller. On 24/05/2019, the fertilizer (12:20:12 NPK) was simultaneously incorporated at a rate of 592 kg/ha during sowing. Sowing was conducted with a single-seed sowing machine with nine plants per square meter, followed by a herbicide application (609.38 g/ha terbuthylazine + 121.88 g/ha mesotrione + 1,015.63 g/ha *S*-metolachlor). On 25/06/2019, a fertilizer application of 560 kg/ha with a composition of 40% nitrogen (N) and 14% sulfur (SO_3) was done, followed by the application of herbicides (25.13 g/ha nicossulfuron + 375 g/ha terbuthylazine + 165 g/ha sulcotrione). Finally, a pilling up through mechanical weed control with a harrow helped to control weeds.

In organic and low-input farming, soil tillage was done on 13/05/2019. Sowing occurred on 14/05/2019. Sowing was conducted with a single-seed sowing machine with six plants per square meter. Weed control was carried out manually since sowing, until late June, when pilling up was finally done. No fertilizer was applied.

Germplasm Characterization

For 20 randomly chosen plants from each population, an adaptation of the HUNTERS descriptor was used based on the field data collected during the monitoring of the maize crop: height (*H*), height of first ear insertion (H1E), uniformity (*U*), root (*R*%), and stalk lodging percentage (*S*%). IBMSPSS® statistics program was used for phenotyping data analyses (IBM Corp, 2020).

Sampling of Maize Rhizosphere

The soil was collected at the flowering stage in the two maize populations: "SinPre" and "Pigarro". For each population, samples were collected from the organic and conventional farming systems. Within each plot, three individual plants, separated by at least 5 m from each other, were selected. Entire plants were dug up with a soil monolith in the middle of them. Bulk soil was taken off the plant roots by vigorous shaking. Plant fine roots were collected from each plant, stored in cool temperature, and moved rapidly to the laboratory where rhizospheric soil samples (1–2 mm soil adhering to roots) were collected.

DNA Extraction and Sequencing

Total DNA was extracted using Nucleospin Soil Kit (Macherey Nagel, Düren, Germany) with Buffer SL1 in combination with Enhancer SX, according to manufacturer's instructions. Internal transcribed spacer 2 (ITS2) region amplicon libraries and Illumina 16S ribosomal RNA (rRNA) genes were generated and sequenced at Genoinseq (Portugal). The DNA was amplified for the hypervariable regions with specific primers and further reamplified in a limited-cycle PCR reaction to add sequencing adapters and dual indexes. The first PCR reactions were performed using a pool of forward primers—ITS3NGS1_F-5′-CATCGATGAAGAACGCAG-3′, ITS3NGS2_F-5′-CAACGATGAAGAACGCAG-3′, ITS3NGS3_F-5′-CACCGATGAAGAACGCAG-3′, ITS3NGS4_F-5′-CATCGATGAAGAACGTAG-3′, ITS3NGS5_F-5′-CATCGATGAAGAACGTGG-3′, ITS3NGS10_F-5′-CATCGATGAAGAACGCTG-3′—and reverse primer ITS4NGS001_R-5′-TCCTSCGCTTATTGATATGC-3′ for fungi (Tedersoo et al., 2014) and forward

primer Bakt_341F-5′-CCTACGGGNGGCWGCAG-3′ and reverse primer Bakt_805R-5′-GACTACHVGGGTATCTAATCC-3′ for bacteria (Herlemann et al., 2011; Klindworth et al., 2013). The second PCR reaction added indexes and sequencing adapters to both ends of the amplified target region according to the manufacturer's recommendations (Illumina, 2013). Negative PCR controls were included for all amplification procedures. PCR products were then one-step purified and normalized using a SequalPrep 96-well plate kit (Thermo Fisher Scientific, Waltham, United States) (Comeau et al., 2017), pooled, and pair-end sequenced in the Illumina MiSeq® sequencer with the V3 chemistry, according to the manufacturer's instructions (Illumina, San Diego, CA, United States) at Genoinseq (Cantanhede, Portugal).

In silico Functional Analysis

Prediction of functional bacterial and fungal diversity within the 16S rRNA and ITS2 sequence libraries was performed using PICRUSt (Langille et al., 2013) and FUNGuild (Nguyen et al., 2015), respectively. PICRUSt predicts the potential metagenomic gene content of a 16S amplicon library based on genomic information of the bacteria represented within the Greengenes 16S database. To perform the process within the PICRUSt program, samples derived from the QIIME2 process, before taxonomic assignment, were selected and grouped into 97% operational taxonomic units (OTUs) against the Greengenes database v.13.8. The nearest sequence taxon index (NSTI) within the PICRUSt pipeline was also calculated as a quality control to validate the accuracy of the predicted functional annotations. FUNGuild assigns trophic modes to fungal taxa based on a comparison to a curated database of fungal lifestyles (*sensu* Tedersoo et al., 2014): pathotroph, symbiotroph, and saprotroph. Trophic mode refers to the mechanisms through which organisms obtain resources, providing putative information on the ecology of such organisms (Nguyen et al., 2015). Functional assignments through FUNGuild are based on taxonomy and are possible only if the taxa have been classified at the genus level or if the taxa belong to a fungal group with an exclusive lifestyle. Input data for FUNGuild was the OTU table.

Statistical and Bioinformatics Analysis

Raw reads were extracted from the IlluminaMiSeq® System in fastq format and quality-filtered with PRINSEQ version 0.20.4 to remove sequencing adapters, reads with less than 100 bases for the ITS2 region and 150 bases for the 16S rRNA gene, and trim bases with an average quality lower than Q25 in a window of 5 bases (Schmieder and Edwards, 2011). The forward and reverse reads were merged by overlapping paired-end reads with Adapter Removal version 2.1.5 using default parameters (Schubert et al., 2016). After sequencing, the bacterial and fungal communities were analyzed using the QIIME software package. Chimeric sequences were removed using the consensus method and clustered in OTUs at 99% using a reference. Taxonomy was assigned to bacterial and fungal OTU sequences using Greengenes v13.8 and UNITEv.7.2, respectively. The phylogenetic classification was performed to the genus level. The rarefaction curves obtained were saturated for each sample, demonstrating that the OTUs recovered were representative of the bacterial and fungi diversity, supporting a robust analysis.

The alpha diversity indexes Shannon index (H'), Simpson (D), and Chao1 were calculated with the Phyloseq package to include in MicrobiomeAnalyst (Dhariwal et al., 2017). The statistical significance of grouping based on experimental factor was estimated using t-test/analysis of variance (ANOVA, $P < 0.05$) to determine differences in the alpha diversity indexes among variables: "SinPre" and "Pigarro" populations and conventional and organic farming systems. A non-supervised principal component analysis (PCA) was performed to compare the bacterial and fungal community structures. Statistical analyses were performed with ANOVA at $P < 0.05$ using R software v.4.0. Venn diagrams were generated with Venny 2.1 (Oliveros, 2007–2015) to identify shared and unique taxa of each population according to the farming system. To identify fungal and bacterial taxa that differed in the relative abundance among population genotypes and farming systems in the rhizosphere of maize, a linear discriminant analysis (LDA) was performed combined with effect size (LEfSe) using a graphical interface in Galaxy version 1.0 (The Huttenhower Lab, 2018). A P-value of < 0.05 and a score ≥ 2.0 were considered significant in Kruskal–Wallis and pairwise Wilcoxon tests, respectively.

RESULTS

Germplasm Agronomic Characterization

The averages of the data collected in the field according to the HUNTERS (Mendes-Moreira et al., 2017) descriptor for both populations ("SinPre" and "Pigarro") in conventional and organic farming are detailed in **Supplementary Table 2**. The phenotypic characterization indicates that "SinPre" in conventional farming was significantly higher than in organic farming for plant and first ear height, but no differences were observed for "Pigarro." Within farming systems (conventional and organic), there were no significant differences between the tested populations ("SinPre" × "Pigarro") for the measured parameters. Both populations showed values of uniformity between 2 and 4, and for the parameters angle (N), tassel (T), and ear insertion position (E), the values variated from 5 to 6. Root lodging (R) ranged from 0% ("SinPre" conventional) to 4.1% ("Pigarro" conventional). Stalk lodging (S) ranged from 10.5% ("Pigarro" organic) to 17.1% ("SinPre" conventional).

Fungal and Bacterial Rhizosphere Microbiota Associated With Two Maize Populations

The structural compositions of the fungi and bacterial communities associated with the rhizosphere soils of two traditional populations of maize grown were grouped into 973 fungal and 4,051 bacterial OTUs.

A total of five fungal phyla, 25 classes, 79, orders, 150 families, and 266 genera were identified (**Supplementary Table 3**). The most abundant and diverse phylum was Ascomycota (64%, comprising 144 genera), followed by

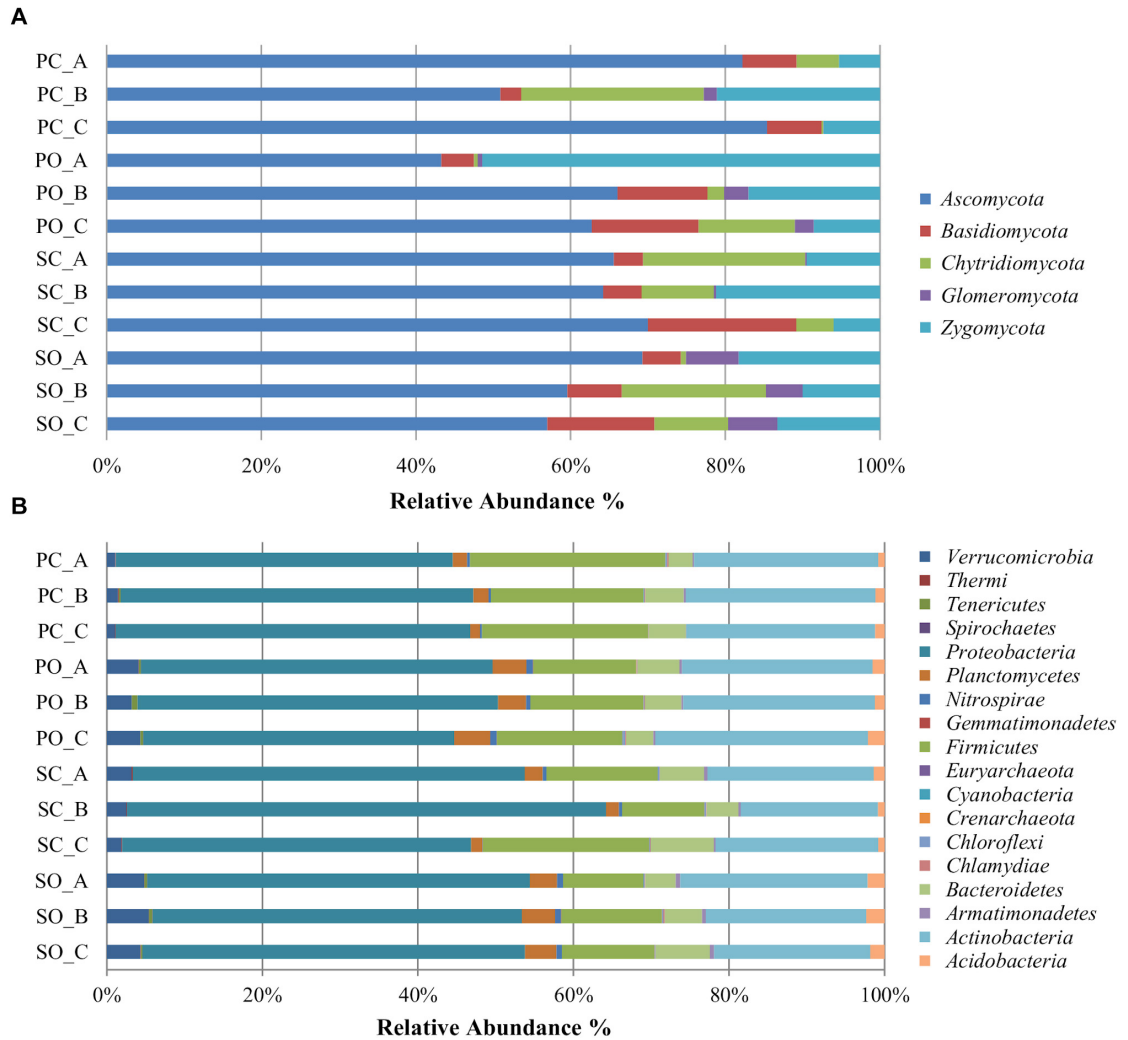

FIGURE 1 | Relative abundance of different fungal **(A)** and bacterial **(B)** phyla in the soil rhizosphere in both populations. *PC_A*, "Pigarro" conventional plant A; *PC_B*, "Pigarro" conventional plant B; *PC_C*, "Pigarro" conventional plant C; *PO_A*, "Pigarro" organic plant A; *PO_B*, "Pigarro" organic plant B; *PO_C*, "Pigarro" organic plant C; *SC_A*, "SinPre" conventional plant A; *SC_B*, "SinPre" conventional plant B; *SC_C*, "SinPre" conventional plant C; *SO_A*, "SinPre" organic plant A; *SO_B*, "SinPre" organic plant B; *SO_C*, "SinPre" organic plant C.

Zygomycota (18%, comprising 12 genera), Basidiomycota (8%, comprising 87 genera), Chytridiomycota (8%, comprising 11 genera), and Glomeromycota (2% comprising 12 genera) (**Figure 1A**). The most abundant genera within Ascomycota were *Odiodendron* (13%), *Fusarium* (12%), and *Aspergillus* (11%); within Basidiomycota were *Rhodotorula* (18%), *Puccinia* (13%) *Papiloterma* (12%), and *Conocybe* (9%); within Chytridiomycota the genera *Rhizoplictis* (65%), *Olpidium* (17%), and *Powellomyces* (8%); within Glomeromycota were *Dentiscutata* (45%) *Gigaspora* (22%), and *Paraglomus* (15%); and within Zygomycota were *Mortierella* (39%), *Rhizopus* (39%), and *Cunninghamella* (13%) (**Figure 2A**). Overall, the most abundant genera were *Oidiodendron* and *Fusarium* (with 8% each), *Rhizopus*, *Morteriella*, and *Aspergillus* (with 7% each), *Rhizophlyctis* (5%), *Alternaria* and *Penicillium* (with 4% each), *Podospora* and *Microdochium* (with 3% each), and *Cunninghamella*, *Coniochaeta*, *Exophiala*, *Talaromyces*, *Myrothecium*, and *Zopfiella* (with 2% each), accounting for approximately 68% of the total diversity. Thirty-seven (14%) genera were shared by all samples; nevertheless, 22% of these bacterial taxa were rare since each represented less than 1% of the total diversity (**Supplementary Table 3**).

For the bacterial microbiota, a total of 18 phyla, 41 class, 72 orders, 158 families, and 317 genera were identified (**Supplementary Table 4**). The most abundant and diverse bacterial phylum was Proteobacteria (47%, comprising 119 genera), followed by Actinobacteria (23%, comprising 83 genera), Firmicutes (16%, comprising 59 genera), and Bacteroidetes (5%, comprising 36 genera) (**Figure 1B**). At the genus level, the most abundant by phylum were *Kaistobacter* (26%), *Burkholderia* (10%), *Methylibium* (9%), *Sphingomonas* (8%), and *Rhodoplanes* (7%) within Proteobacteria; *Streptomyces*

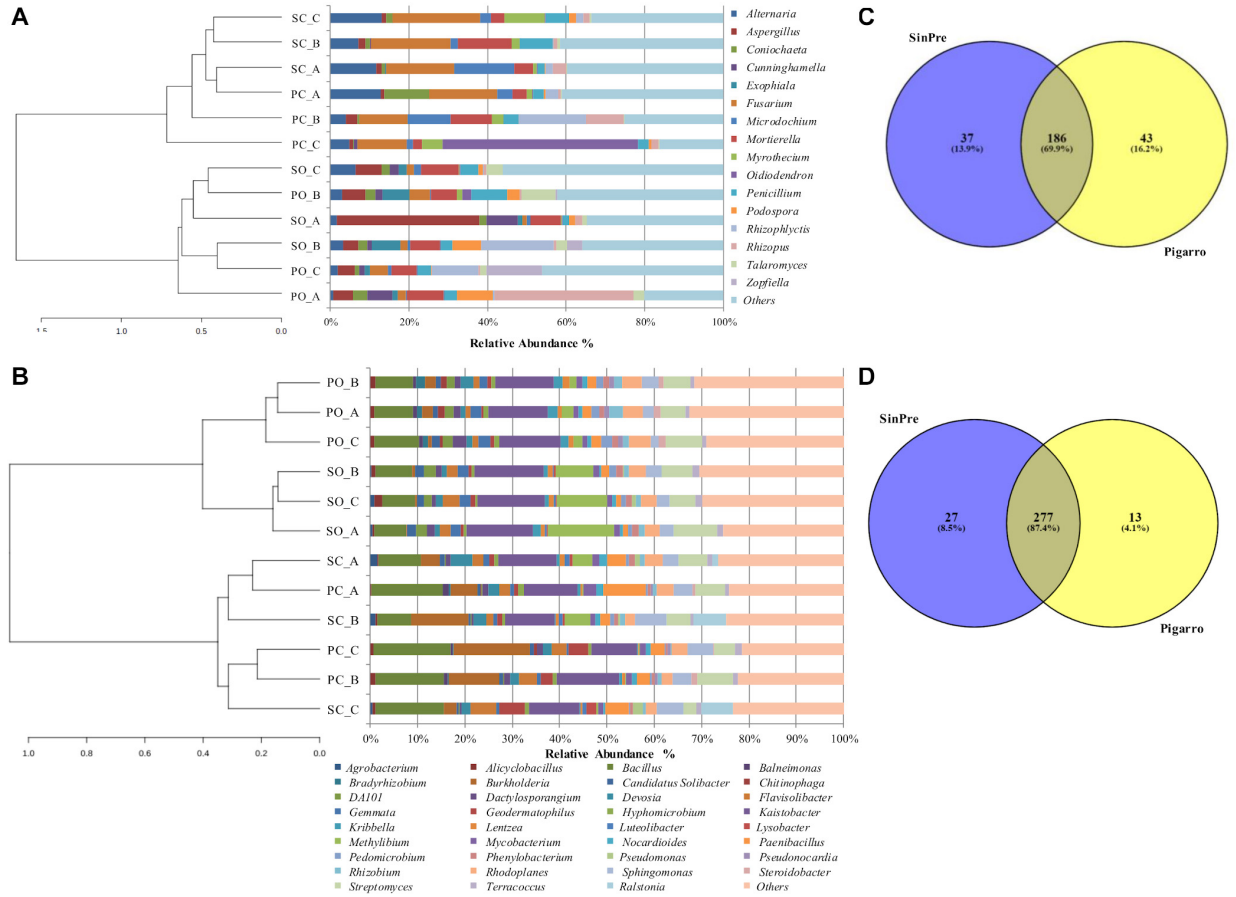

FIGURE 2 | Relative abundance of different fungal **(A)** and bacterial **(B)** genera in the soil rhizosphere in both populations representing the genera showing more than 1% relative abundance of all reads. The genera representing less than 1% of the total reads are grouped into "Others." Venn diagrams showing the common and exclusive fungal **(C)** and bacterial **(D)** genera of the rhizosphere of the maize populations "SinPre" and "Pigarro." *PC_A*, "Pigarro" conventional plant A; *PC_B*, "Pigarro" conventional plant B; *PC_C*, "Pigarro" conventional plant C; *PO_A*, "Pigarro" organic plant A; *PO_B*, "Pigarro" organic plant B; *PO_C*, "Pigarro" organic plant C; *SC_A*, "SinPre" conventional plant A; *SC_B*, "SinPre" conventional plant B; *SC_C*, "SinPre" conventional plant C; *SO_A*, "SinPre" organic plant A; *SO_B*, "SinPre" organic plant B; *SO_C*, "SinPre" organic plant C.

(26%), *Geodermatophilus* (7%), and *Mycobacterium* (6%) within Actinobacteria; *Bacillus* (65%), *Paenibacillus* (18%), and *Alicylobacillus* (5%) within Firmicutes; and *Flavisolibacter* (51%) and *Chitinophaga* (14%) within Bacteroidetes (**Figure 2B**). Overall, the most abundant genera were *Kaistobacter* (12%), *Bacillus* (10%), *Streptomyces* (6%), *Burkholderia* (5%), *Methylibium* and *Sphingomonas* (with 4% each), *Rhodoplanes*, *Paenibacillus*, and *Flavisolibacter* (with 3% each), and *Devosia*, *Geodermatophilus*, and *Gemmata* (with 2% each), accounting for approximately 55% of the total diversity. One hundred and thirteen (36%) genera were shared by all samples, while only 20 of these bacterial taxa were restricted to one sample (**Supplementary Table 4**).

Core Rhizosphere Microbiota Associated With Different Maize Populations

The rhizosphere of the maize populations showed specific fungal and bacterial OTUs for each population and a cluster of shared OTUs. Comparing the fungal and bacterial microbiota of the "Pigarro" and "SinPre" populations, 69.9 and 87.4% of the fungal and bacterial genera, respectively, were shared between populations, demonstrating the existence of a "core" maize phylogeny (**Figures 2C,D**).

Eighty-seven fungal genera composed the core fungal rhizosphere microbiota, affiliated with Ascomycota (54 genera), Basidiomycota (20 genera), Chytridiomycota (three genera), Glomeromycota (three genera), and Zygomycota (seven genera) (**Supplementary Table 5**).

The core rhizosphere bacterial microbiota present in both maize populations was composed of 183 genera, affiliated with Proteobacteria (64 genera), followed by Actinobacteria (54 genera), Firmicutes (22 genera), Bacteroidetes (17 genera), Verrumicrobia (10 genera), Planctomycetes (four genera), Chlamydiae (three genera), Acidobacteria (three genera), Chloroflexi (two genera), and Armatimonadetes, Crenarchaeota, Nitrospirae, and Thermi, with one genus each (**Supplementary Table 5**).

Specific fungal genera were only associated with the "SinPre" population (37 genera, corresponding to 13.9%) and with the "Pigarro" population (43 genera, corresponding to 16.2%). Regarding the bacterial microbiota, 13 genera (4.1%) were exclusive to the "SinPre" population and 27 to the "Pigarro" population (8.5%) (**Figures 2C,D**). The population-specific genera are shown in **Supplementary Table 6**.

Fungal communities of the rhizospheric soil samples did not differ between populations. Bacterial communities were only significantly different in the rhizosphere associated with the "Pigarro" genotype in the organic farming system ($F = 3.5$, $P < 0.05$). The maize genotype was a significant factor structuring the bacterial community in both farming systems, supported by Shannon (t-test = 3.0627, $P < 0.05$) and Simpson (t-test = −2.7668, $P < 0.05$) α diversity indexes (**Figures 3A,B** and **Table 1**). Principal coordinate analysis (PCoA) further evidenced that most of the variations on the dataset could be attributed to the population genotype (**Figures 3C,D**).

The LEfSe detected 64 fungal (12 PC_SC and 52 PO_SO) and 98 bacterial (24 PC_SC and 74 PO_SO) bacterial clades in the rhizosphere, which discriminated the microbial communities between populations (**Figure 4**). Annulatascaceae, Coniochaetaceae, Nectriaceae (class Sordariomycetes) were the major fungi families that contributed to differentiate the fungal communities associated with "Pigarro" and Rhizophydiaceae (class Chytridiomycetes) with "SinPre" under the conventional farming system. Under the organic farming system, the differences between genotypes were more pronounced due to the contributions of several other classes, namely, Agaricomycetes, Eurotiomycetes, Leotiomycetes, and Microbotryomycetes in "Pigarro" and Dothideomycetes, Glomeromycetes, Pucciniomycetes, and Ustilaginomycetes in "SinPre" (**Figure 4A**).

The main bacterial families that contributed to differentiate the communities associated with population genotypes in the conventional agricultural system were Alteromonadaceae, Bradyrhizobiaceae, Comamonadaceae, Cytophagaceae, Oxalobacteraceae, Pseudomonadaceae, Sphingobacteriaceae, and Xanthomonadaceae in "SinPre" and Chromatiaceae and Symbiobacteriaceae in "Pigarro." The differences between populations were more pronounced in the organic farming system because of the presence of members of the classes Alcaligenaceae Caulobacteraceae, Cellvibrionaceae, Chitinophagaceae, Intrasporangiaceae, Hyphomicrobiaceae, Opitutaceae, Planctomycetaceae, Rhodospirillaceae, and Sphingomonadaceae in "SinPre" and Acidobacteriaceae, Bradyrhizobiaceae, Caldicoprobacteraceae, Comamonadaceae, Cystobacterineae, Hyphomicrobiaceae, Micromonosporaceae, Nocardioidaceae, Nocardiopsaceae, Planococcaceae, Pseudonocardiaceae, Rhizobiaceae, Rhodanobacteraceae, Rhodospirillaceae, Solirubrobacteraceae, and Steroidobacteraceae in "Pigarro" (**Figure 4B**). Detailed information can be found in **Supplementary Figure 1**. Several genera were significantly different between population genotypes, as detailed in **Supplementary Table 7**.

Specific fungal and bacterial OTUs associated with the "SinPre" population ranged from 9.4 and 13.8% in the conventional and between 45.3 and 17.2% in the organic farming system, respectively. The core microbiota of the "SinPre" population corresponded to 45.3% of the fungal and 69% of bacterial total diversity. A similar trend was observed in the dataset from the "Pigarro" population, with specific fungal and bacterial OTUs comprising 12.7 and 11.8% in conventional and 39.3 and 18.4% in the organic farming system, respectively. The core microbiota of the "Pigarro" population corresponded to 48% of the fungi and 69.7% of the bacteria total diversity. The farming system-specific genera are shown in **Supplementary Table 8**.

Core Rhizosphere Microbiota Associated With Farming Systems

The results obtained showed that rhizospheric soil harbored a distinct microbiota according to the farming system ($P < 0.05$; **Table 1**). This was visible in the cluster-based analysis of the fungal and bacterial community structure and composition, where maize populations were clustered by the farming system (**Figure 2**). Indeed, the rhizosphere showed specific fungal and bacterial OTUs for each farming type and a cluster of shared OTUs. Comparing the total dataset of the fungal and bacterial microbiota of the conventional and organic farming systems, 49.2 and 74.8% of fungal and bacterial OTUs, respectively, were shared between the two systems. Specific OTUs were associated with the conventional farming system, 25 fungal and 28 bacterial genera, representing 9.4 and 8.8% of the community, respectively. Regarding the organic farming system, the relative abundance of specific fungal (110, 41.4%) and bacterial (52, 16.4%) OTUs was considerably higher.

Core Rhizosphere Microbiota Across Both Maize Populations

The farming system was a significant factor structuring the fungal community predicted by Chao1 diversity in the "Pigarro" (t-test = −17 014, $P < 0.05$) and "SinPre" (t-test = −3,939, $P < 0.05$) genotypes (**Figure 3A** and **Table 1**). Besides, the mycobiota associated with "SinPre" was significantly different between the farming systems ($F = 9.08$, $P < 0.05$). However, the farming systems had a much weaker influence on the rhizosphere-associated bacterial communities. The PCoA further evidenced that most of the variations on the dataset could be related to the farming system (**Figures 3C,D**).

At the phylum level, the impact of the farming system on fungal diversity associated with the "Pigarro" rhizosphere translated into a higher dominance of Ascomycota in the conventional system, partially replacing the Zygomycota in the organic system. In the "SinPre" population, the relative abundance of Ascomycota was similar in both farming systems. Importantly, a notably higher presence of mycorrhizae (Glomeromycota) was observed in both populations cultivated in the organic farming system, in clear contrast to the conventional farming system (**Figure 1A**).

The LEfSe detected 255 fungal (124 PC_PO and 131 SC_SO) and 266 bacterial (138 PC_PO and 128 SO_SC) clades in the rhizosphere, which discriminated the microbial communities between the farming systems (**Figure 5**). Dothideomycetes,

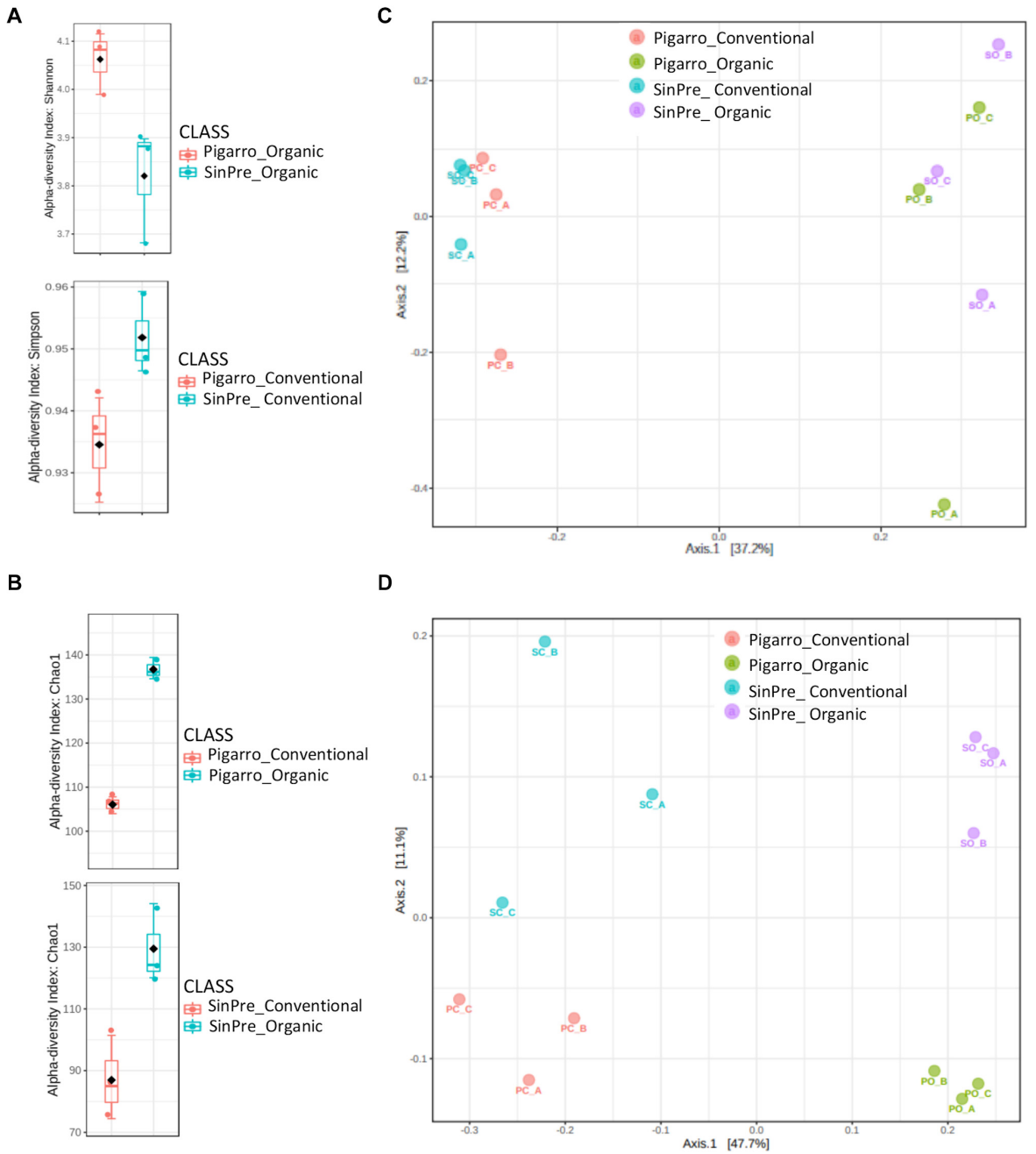

FIGURE 3 | Box plot illustrating statistically significant α diversity measures of the effect of farming system in fungal **(A)** and bacterial **(B)** communities in the maize rhizosphere. Principal coordinate analysis (PCoA) was performed using the prcomp package to illustrate the effect of farming system on the fungal **(C)** and bacterial **(D)** communities in the maize rhizosphere. The calculation is based on singular value decomposition of the fungal and bacterial communities for the farming system. A greater distance between the two samples indicates low similarity. The percentage of variation is explained by component 1 (PC1) and by component 2 (PC2).

Orbiliomycetes, Sordariomycetes, and Tremellomycetes were the major fungi classes that contributed to differentiate the fungal communities associated with the conventional farming system despite the considered population, while under the organic farming system the differences were due to the contribution of several other classes, namely, Eurotiomycetes, Mortierellomycetes, Saccharomycetes, and Ustilaginomycetes. Worth noticing is that Glomeromycetes and Pucciniomycetes were highly abundant in the "SinPre" population cropped in organic farming (**Figures 5A,B**). The main bacterial classes that contributed to differentiate the communities associated with farming systems were Bacilli and Verrucomicrobiae in the conventional farming system and Alphaproteobacteria, Anaerolineae, Fimbriimonadia, Mollicutes, Nitrospira, Opitutae,

TABLE 1 | Experimental factors predicting α diversity of the rhizosphere-associated fungal and bacterial communities in maize.

		Chao1		Shannon		Simpson	
		t-test	*P*	*t*-test	*P*	*t*-test	*P*
Fungi							
Genotype	PC_PO	−17.014	**0.0001**	−1.5369	0.1992	−0.8259	0.4663
	SC_SO	−3.939	**0.0171**	−2.1054	0.1503	−0.4810	0.6759
Farming system	PC_SC	2.4068	0.1330	−0.5873	0.6123	−0.6863	0.5625
	PO_SO	0.4327	0.9591	−0.1506	0.8880	0.0035	0.9974
Bacteria							
Genotype	PC_SC	0.3649	0.7338	−2.1922	0.1001	−2.7668	**0.0540***
	PO_SO	0.9858	0.3956	3.0627	**0.0530***	2.8629	0.0623
Farming system	PC_PO	−0.48906	0.6513	−7.3396	**0.0036**	−5.1265	**0.0171**
	SC_SO	−0.20527	0.8150	−0.50067	0.6437	0.5229	0.6288

*P, "Pigarro"; S, "SinPre"; C, conventional; O, organic. Statistics describe the linear random intercept models of Shannon and Simpson diversity and Chao1 richness in the rhizosphere. Alpha diversity analysis was performed using the phyloseq package. The statistical significance of grouping based on the experimental factor was estimated using t-test/ANOVA. Bold values indicate statistically significant results (P < 0.05). *Statistically significant.*

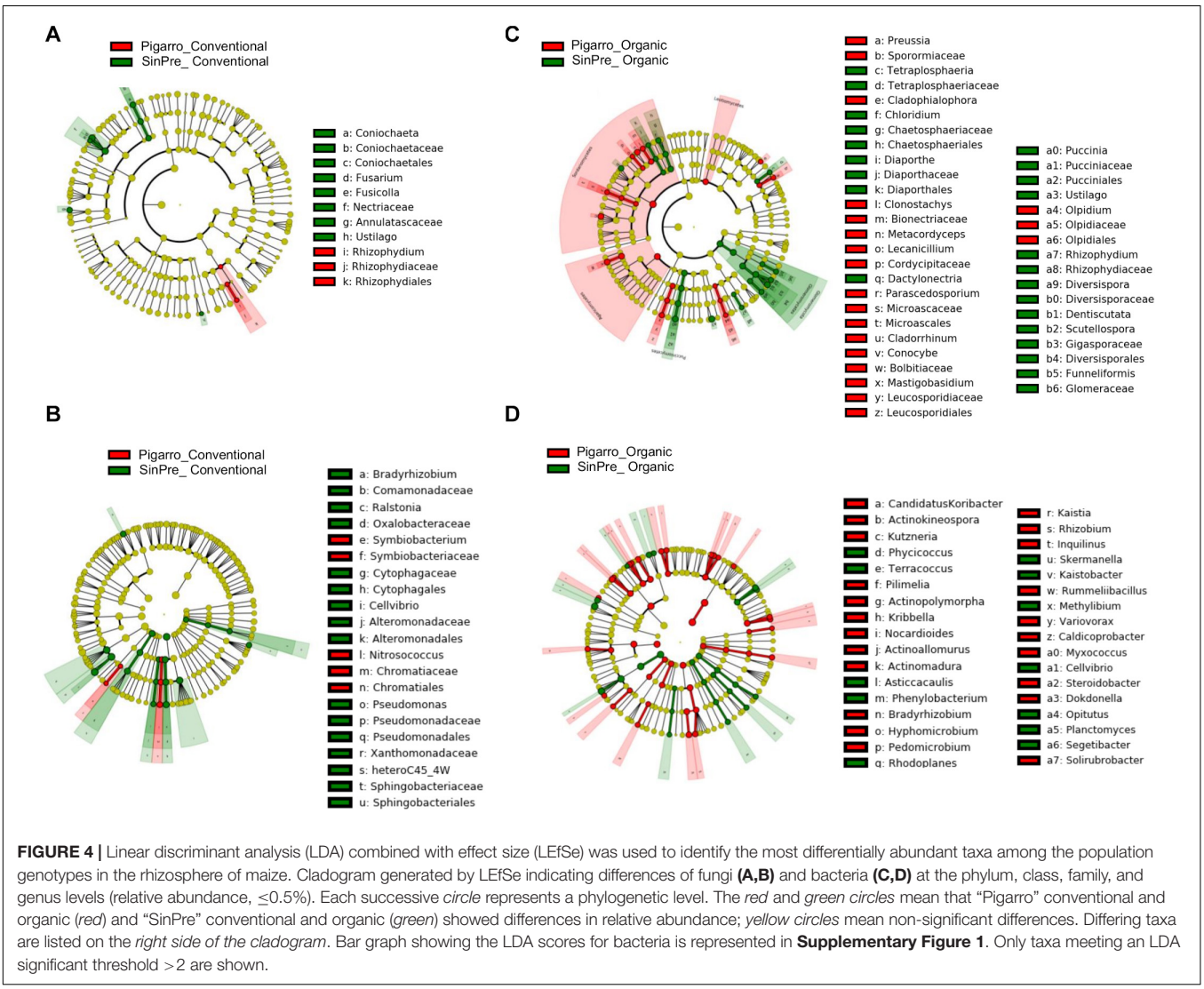

FIGURE 4 | Linear discriminant analysis (LDA) combined with effect size (LEfSe) was used to identify the most differentially abundant taxa among the population genotypes in the rhizosphere of maize. Cladogram generated by LEfSe indicating differences of fungi **(A,B)** and bacteria **(C,D)** at the phylum, class, family, and genus levels (relative abundance, ≤0.5%). Each successive *circle* represents a phylogenetic level. The *red* and *green circles* mean that "Pigarro" conventional and organic (*red*) and "SinPre" conventional and organic (*green*) showed differences in relative abundance; *yellow circles* mean non-significant differences. Differing taxa are listed on the *right side of the cladogram*. Bar graph showing the LDA scores for bacteria is represented in **Supplementary Figure 1**. Only taxa meeting an LDA significant threshold >2 are shown.

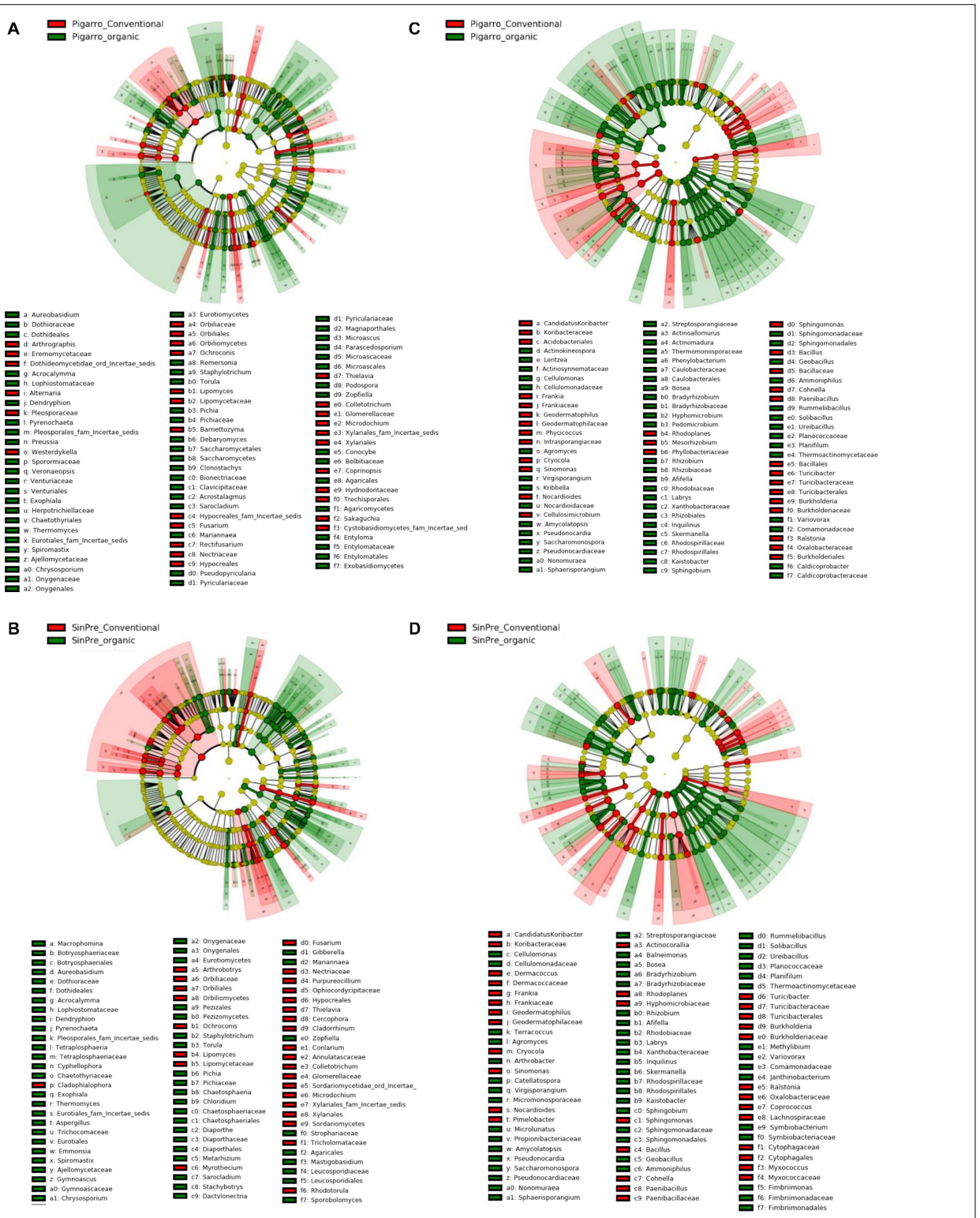

FIGURE 5 | Linear discriminant analysis (LDA) combined with effect size (LEfSe) was used to identify the most differentially abundant taxa among farming systems in the rhizosphere of maize. Cladogram generated by LEfSe indicating differences of fungi **(A,B)** and bacteria **(C,D)** at the phylum, class, family, and genus levels (relative abundance, ≤0.5%). Each *successive circle* represents a phylogenetic level. The *red* and *green circles* mean that conventional (*red*) and organic (*green*) showed differences in relative abundance; *yellow circles* mean non-significant differences. Differing taxa are listed on the *right side of the cladogram*. Bar graph showing the LDA scores for bacteria is represented in **Supplementary Figure 1**. Only taxa meeting an LDA significant threshold >2 are shown.

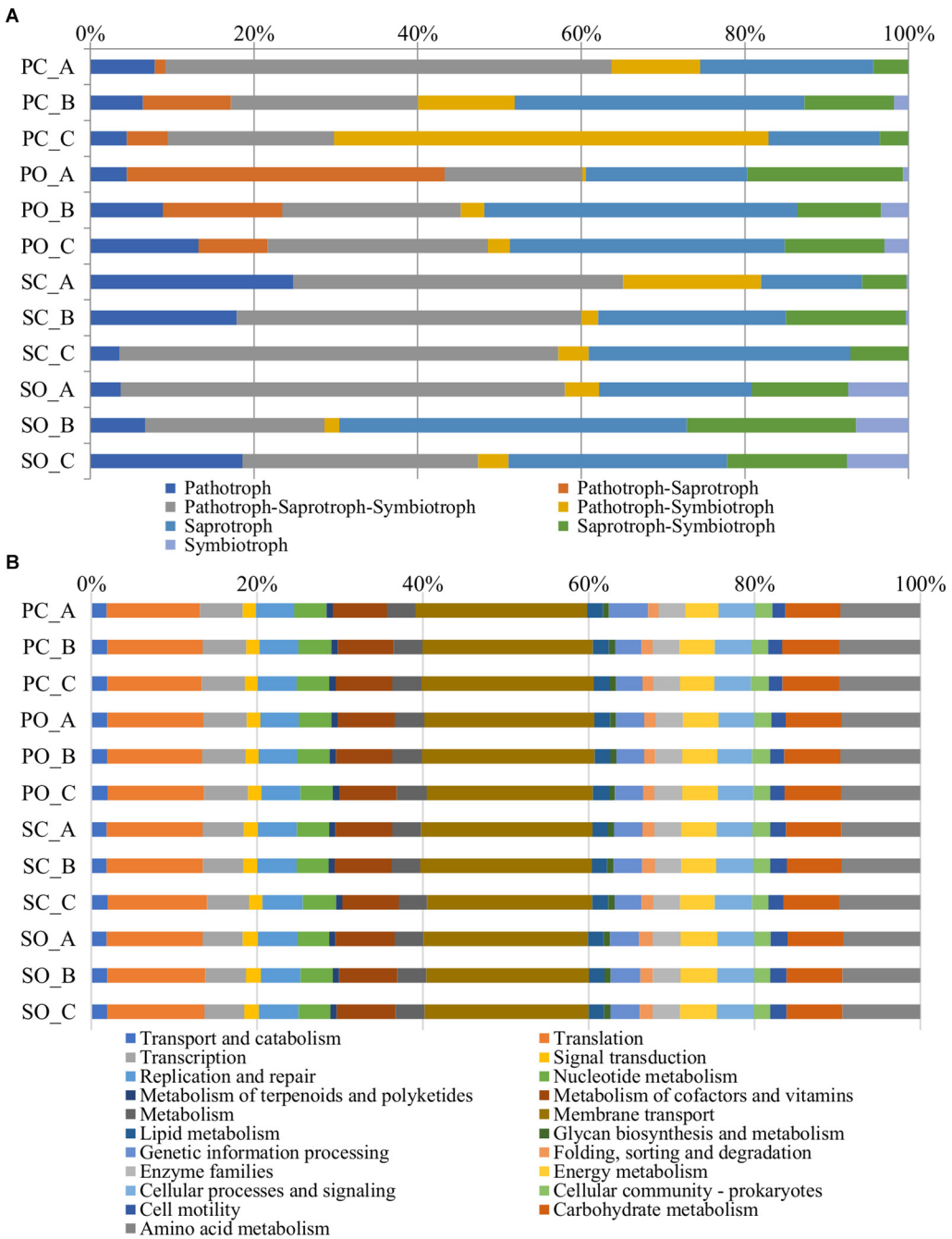

FIGURE 6 | Metagenome predicted functions classified using FUNGuild for fungi **(A)** and the Kyoto Encyclopedia of Genes and Genomes (KEGG) level 2 database in PICRUSt software for bacteria **(B)** showing the most abundant functions throughout the rhizospheric soil samples.

Planctomycetia, Spartobacteria, and Solibacteres in the organic farming system (**Figures 5C,D**).

In silico Metagenome Analysis

The functional predictions at the fungal level in both populations and both farming systems are shown in **Figure 6A**. An increase in the trophic modes defined as pathotroph–saprotroph, saprotroph-symbiotroph, and symbiotroph was observed in the "Pigarro" population under the organic farming system. The same population in the conventional farming system experienced an increase in the pathotroph–saprotroph–symbiotroph and pathotroph–symbiotroph trophic modes. In the "SinPre" population under the organic farming system, the trophic modes defined as pathotroph–saprotroph–symbiotroph,

saprotroph, saprotroph–symbiotroph, and symbiotroph were more abundant, while the latter was practically non-existent in the conventional farming system.

In detail, the trophic mode pathotroph comprised 32 genera of plant pathogens. In detail, 26 were detected in "Pigarro," five specific to the conventional farming system (*Chalastospora*, *Curvularia*, *Lectera*, *Rhizophydium*, and *Volutella*) and 10 specific to the organic farming system (*Cercospora*, *Kochiomyces*, *Dactylaria*, *Entyloma*, *Macrophomina*, *Sporisorium*, *Ramularia*, *Sclerotinia*, *Thanatephorus*, and *Gjaerumia*), while the remaining 11 were shared between farming systems (*Dendryphion*, *Farysia*, *Ilyonectria*, *Olpidium*, *Anthracocystis*, *Clonostachys*, *Drechslera*, *Gibberella*, *Monographella*, *Powellomyces*, and *Puccinia*). Similarly, in the "SinPre" population, 32 genera of fungal plant pathogens were present, three specific to the conventional farming system (*Olpidium*, *Lectera*, and *Thanatephorus*), 13 related to the organic farming system (*Protomyces*, *Dendryphion*, *Rhizophydium*, *Exserohilum*, *Macrophomina*, *Sphacelotheca*, *Entyloma*, *Microstroma*, *Glomosporium*, *Sporisorium*, *Taphrina*, *Ilyonectria*, and *Ramularia*), and eight shared between farming systems (*Monographella*, *Puccinia*, *Powellomyces*, *Clonostachys*, *Gibberella*, *Curvularia*, *Anthracocystis*, and *Drechslera*). Both populations shared seven plant pathogens despite the farming system: *Monographella*, *Puccinia*, *Powellomyces*, *Clonostachys*, *Gibberella*, *Anthracocystis*, and *Drechslera*. Another important observation from the dataset was the increase in abundance and diversity of arbuscular mycorrhiza in the organic farming system.

A total of 6,886 functional orthologs were predicted to be present in the analyzed bacterial communities, corresponding to 21 level 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways (**Figure 6B**). The results showed nine pathways for metabolism, four for genetic information processing and unclassified, three pathways for cellular processes, and two for environmental information processing. The average relative abundance of the metabolism function was 33.95%, followed by genetic information processing (21.13%), environmental information processing (20.37%), cellular processes (5.34%), and unassigned with 19.22%. The most abundant KEGG pathway belonged to membrane transport, followed by translation, amino acid metabolism, carbohydrate metabolism, and metabolism of cofactors and vitamins.

DISCUSSION

The rhizosphere is a specialized region where interactions between plant roots and the surrounding soil-associated microorganisms take place. Rhizospheric soil differs from bulk soil not only due to the direct effect of these microorganisms but also because root growth modifies the composition of the soil. Roots can release rhizodeposits, a wide range of substances containing carbon (e.g., root cells, mucilage, volatiles, and exudates), selecting and enhancing groups of microorganisms (Harkes et al., 2019), which, in addition to modifying some soil characteristics, can play a role in the plant health status.

This work intends to characterize the structural compositions of fungal and bacterial communities present in the rhizospheric soil associated with two maize populations cultivated in different farming systems. Although it is difficult to draw robust conclusions, significant interactions between the microbial diversity, farming systems, and genotype were obtained. Moreover, our results are in line with previous studies (Granzow et al., 2017; Ginnan et al., 2018; Berlanas et al., 2019) including those on maize crop (Galazka and Grzadziel, 2018; Brisson et al., 2019; Kong et al., 2020), shedding some light into the potential beneficial impacts of maize landraces and organic cultivation techniques into the rhizosphere microbial communities and network assembly.

Impact of Maize Populations and Farming System on the Associated Rhizosphere Mycobiota

The microbial community analysis revealed significant differences in the relative abundance of OTUs between maize populations, evidencing that plant genotype has a significant effect on the structure of the rhizosphere-associated microbiota. In our dataset, a higher portion of the differentially selected microbial community by genotypes was discovered compared with previous studies (Brisson et al., 2019). Indeed, 30.1% of the fungal community and 12.6% of the bacterial community were significantly associated with a specific population.

The novel landrace "SinPre" was derived from 12 populations, while the traditional landrace "Pigarro" encompassed less diversity. We expected that this intraspecific diversity would be translated into more heterogeneous rhizosphere mycobiota, but this was not the case. The rhizosphere mycobiota associated with the "Pigarro" and "SinPre" populations were similar, but significantly influenced by the farming systems. A higher heterogenicity was found in the rhizosphere mycobiota under the organic farming system, in line with previous works (Hartmann et al., 2015; **Figure 3C**). Also, high fertilization regimes are known to select and enrich fungi populations (Enebe and Babalola, 2020), aiding to explain the previously described diversity.

The fungal community was dominated by the Ascomycota phylum, which agrees with previous studies on the maize rhizosphere (Galazka and Grzadziel, 2018). The impact of the farming system in fungal diversity associated with the "Pigarro" rhizosphere translated into a higher dominance of Ascomycota in the conventional system, replaced in the organic system by Zygomycota. Our results follow the study by Galazka and Grzadziel (2018), where the abundance of the order Hypocreales (Ascomycota) was associated with a conventional farming system. Moreover, Hypocreales, Sordariales, and Helotiales were among the most abundant classes in our dataset, similar to the results described by Klaubauf et al. (2010) on the fungal diversity in arable soils. On the contrary, our results did not support the previously described relationship between the order Sordariales and the conventional farming system since, in our dataset, it was more abundant in the organic farming system.

This study also showed that the farming system was a significant factor structuring the maize-associated fungal community. This influence was less pronounced in the associated bacterial community. A similar trend was described

by Galazka and Grzadziel (2018) in a study combining different cultivation practices, reinforcing the impact that these practices have on the microbial community and soil function. Our results showed that rhizospheric soil harbored a distinct mycobiota according to the farming system. Dothideomycetes, Orbiliomycetes, Sordariomycetes, and Tremellomycetes were the main classes within the Ascomycota phylum that contributed to differentiate the fungal communities associated with the conventional agricultural system. The Dothideomycetes class includes several endophytic and plant saprobic taxa, as well as crop-related pathogens. Both *Alternaria* sp. and *Cladosporium* sp. are examples of the latter that were associated with the conventional farming in our dataset. The class Orbiliomycetes includes most of the known nematode-trapping fungi constituting an important part of the subsoil ecosystem, among which *Arthrobotrys* sp. was associated in this study with conventional farming. *Fusarium* was the most abundant genus of the class Sordariomycetes and has been commonly associated with the maize rhizosphere. This genus includes saprotrophic and plant pathogenic species causing significant economic losses in cereals, especially in maize (McMullen et al., 2012; Salgado et al., 2015). Also, *Fusarium* can produce phytotoxins that inhibit the growth of infected plants and can act as virulence factors (Salgado et al., 2015). Tremellomycetes belong to Basidiomycota, the second most abundant phylum of our dataset, including *Cryptococcus* sp., known for the ability to aid plants to extract nutrients from the soil (Wolf et al., 2014).

Classes Eurotiomycetes, Mortierellomycetes, Saccharomycetes, Ustilaginomycetes, Glomeromycetes, and Pucciniomycetes shaped the rhizospheric mycobiota associated with maize cropped in the organic system. Interestingly, the latter two were more abundant in "SinPre" than in the "Pigarro" maize population. Eurotiomycetes and Saccharomycetes belong to Ascomycota, including two of the most important and problematic genera in the cultivation of maize, *Penicillium* and *Aspergillus*, present in our dataset. *Penicillium* species are ubiquitous in the soil wherever organic material is available (Duniere et al., 2017), while *Aspergillus* sp., in spite being involved in lignin degradation and (or) melanin synthesis (Levasseur et al., 2008), crucial to improving the amount of organic matter available in the soil, can also produce the mycotoxin deoxynivalenol (DON), which is harmful to humans and livestock. Ustilaginomycetes and Pucciniomycetes belong to Basidiomycota, the second most abundant phylum comprising some genera potentially pathogenic to maize plants, namely, *Ustilago* sp., where it includes the species *Ustilago maydis*, known for the ability to cause tumors in maize by redirecting vegetative and floral development (Walbot and Skibbe, 2009). In the class Pucciniomycetes, the genus *Puccinia* includes species responsible for the foliar disease of maize (Medina and López, 2007). Within the class Mortierellomycetes (phylum Zygomycota), the genus *Morteriella* was present in our dataset and was correlated with the organic farming system. In terms of its potential ecological role, this genus has been implicated in preventing soil degradation, improvement of soil health, and stimulating the production of plant growth hormones (Li et al., 2009; Zhang et al., 2011). Finally, the class Glomeromycete (phylum Glomeromycota) included an important functional group of organisms, the arbuscular mycorrhizae, which, according to the bibliography, can establish beneficial symbiosis with maize in various geographic distributions and different environments (Miransari et al., 2009).

Our dataset comprised 12 genera belonging to this class in the rhizosphere of both crop populations ("Pigarro" and "SinPre") mostly associated with the organic farming system and lacking in most samples derived from the conventional farming system. Among the plant-associated mycobiota, these fungi are the most widespread (Desirò et al., 2014), and approximately 80% of the existing plant families have the potential to form this type of association (Trappe, 1987). Among the different alternatives toward sustainable agriculture, increasing arbuscular mycorrhizae is an important strategy with beneficial effects for the plant nutritional status by increasing plant nutrient uptake, in particular phosphorus (Smith and Jakobsen, 2013), improving the phytosanitary status (Ahmed et al., 2013; Hohmann and Messmer, 2017), expanding root range, improving tolerance to biotic and abiotic stresses, and inducing plant defense response (Oehl and Sieverding, 2004; Ismail et al., 2013). From our results, the organic farming system promotes these symbioses in the associated rhizosphere of these two Portuguese landraces, aiding the resilience of the crop without the insertion of synthetic fertilizers. *Dentiscutata*, *Gigaspora*, and *Paraglomus* were the most representative genera in the mycorrhizae community in our dataset and, along with *Acaulospora*, *Cetraspora*, *Diversispora*, *Glomus*, and *Scutellospora*, were restricted to the rhizosphere in the organic farming system; *Septoglomus* was genotype-specific in "SinPre." *Glomus* belongs to the important group of arbuscular mycorrhizal (AM) fungi involved in the exchange with the plant of carbon, phosphorus, and other physiologically significant particles. As mentioned above, this interaction of mycorrhizae with other soil microorganisms promotes beneficial cooperation enhancing the competition against phytopathogenic microorganisms. Relating the rhizosphere fungal communities with their trophic mode, as expected, the abundance of taxa with saprotrophic and pathotroph modes was higher in the organic farming system than in the conventional farming system.

Impact of Maize Populations and Farming System on the Associated Rhizosphere Bacterial Communities

There was also a clear population–farming system interaction concerning bacterial communities. A higher heterogenicity was found in the rhizosphere bacteriota under the organic farming system, in line with previous works (Hartmann et al., 2015; Lupatini et al., 2017; Fernandez et al., 2020; Lee et al., 2020; **Figure 3D**). Differences between populations were more pronounced in the organic farming system due to the presence of members from the classes Alcaligenaceae, Caulobacteraceae, Cellvibrionaceae, Chitinophagaceae, Intrasporangiaceae, Hyphomicrobiaceae, Opitutaceae, Planctomycetaceae, Rhodospirillaceae, and Sphingomonadaceae in the "SinPre" population, while Acidobacteriaceae, Bradyrhizobiaceae, Caldicoprobacteraceae, Comamonadaceae, Cystobacterineae,

Hyphomicrobiaceae, Micromonosporaceae, Nocardioidaceae, Nocardiopsaceae, Planococcaceae, Pseudonocardiaceae, Rhizobiaceae, Rhodanobacteraceae, Rhodospirillaceae, Solirubrobacteraceae, and Steroidobacteraceae were present in "Pigarro." These results are in line with the recent studies of Beirinckx et al. (2020), confirming the existence of a core bacteriome in the maize rhizosphere composed of Hyphomicrobiacea, Streptomycetaceae, Comamonadaceae, Cytophagaceae, Oxalobacteraceae, Rhizobacteraceae, Xanthomonadaceae, and Caulobacteraceae. Also, several taxa from these families were recently associated with the maize rhizosphere soils of various farming systems and growth stages.

The family Oxalobacteraceae was specific to the conventional farming system and has been associated with nitrogen fixation activities. However, in this study, two of the most abundant genera of this family were *Ralstonia*, a well-known plant pathogen responsible for wilting in numerous plants, and *Janthinobacterium*, previously reported in maize residues and stalks and the rhizospheric soil of maize. The importance of the latter resides in the effective reduction of maize stalk colonization by *Fusarium* spp. (Cobo-Díaz et al., 2019).

Genera from the families Xanthomonadaceae (*Luteimonas* sp.) and Comamonadaceae (*Methylibium* sp.) were associated with the conventional farming system in our dataset, which have been previously used in the bioremediation of hydrocarbon-contaminated soil (Kane et al., 2007; Mu et al., 2016). The latter can also induce disease resistance through the production of antimicrobial compounds (Lagos et al., 2015). In line with this, it is important to highlight that we identified in our dataset families that include genera with reported biocontrol activity, such as Sphingobacteriaceae (*Pedobacter* sp.), Pseudomonadaceae (*Pseudomonas* sp.), Sphingomonadaceae (*Sphingobium* sp.), Xhantomonadaceae (*Luteibacter* sp.), Cytophagaceae (*Dyadobacter* sp.), and Rhizobiaceae (*Rhizobium* sp.) (Cobo-Díaz et al., 2019).

The main bacterial classes that contributed to differentiate the communities associated with farming systems were Bacilli and Verrucomicrobiae in the conventional farming system and Alphaproteobacteria, Anaerolineae, Fimbriimonadia, Mollicutes, Nitrospira, Opitutae, Planctomycetia, Spartobacteria, and Solibacteres in the organic farming system. Several bacterial genera of these classes were recently associated with maize rhizosphere-associated microbiota. At the genus level, *Bacillus*, *Erwinia*, *Pseudomonas*, *Stenotrophomonas*, *Achromobacter*, *Lysinibacillus*, and *Paenibacillus* were reported by Pereira et al. (2011) as the most common taxa in the maize rhizosphere. These genera, along with *Burkholderia*, were described as having plant growth-promoting rhizobacteria common in the maize-associated rhizosphere (Yang et al., 2017). Also, the antagonist potential of the genus *Bacillus* against maize pathogens has been addressed (Figueroa-López et al., 2016; Cheng et al., 2019; Hazarika et al., 2019; Liu et al., 2020). In terms of putative functions, *Burkholderia* has been linked with antifungal activity (Stopnisek et al., 2016), while some strains were described to produce ACC deaminase and siderophores important for maize growth promotion (Byrt et al., 2011). On the other hand, *Erwinia* is the causal agent of the bacterial wilt in a large range of hosts and the bacterial stalk rot disease of maize. Finally, the genus *Kaistobacter* is common in soils with atrazine (a compound present in some herbicides) (Lin et al., 2018), while *Rhodoplanes* can increase the fertility of soils (Sun et al., 2015).

CONCLUSION

In the current context of climate change, environmental degradation, and misuse of natural resources, the transition from conventional to organic farming requires the optimization of cultivars and management, with the rhizosphere microbiota playing an important role. Since maize is one of the main crops in the world, the adoption of more sustainable practices will contribute decisively to this strategy. In this study, the analysis of the microbial community revealed significant differences among maize populations, showing that the plant genotype has a significant effect on the structure of the microbiota associated with the rhizosphere. Additionally, the farming system had a statistically significant impact on rhizosphere-associated microbiota, and several taxa were found to be specific to the agricultural system. The presence of arbuscular mycorrhizae (Glomeromycota), known for the potential to establish a beneficial symbiosis with maize, was mainly detected in the microbiota of the "SinPre" population in the organic farming system, being very rare under the conventional system. The diversity of the rhizosphere-associated microbiota in the organic farming system was significantly higher than that in the conventional system in both varieties, shedding some light into the potential beneficial impacts of maize landraces and organic cultivation techniques into the rhizosphere microbial communities and network assembly. The role of some species as plant growth-promoting rhizobacteria and with the ability to improve the tolerance of crops to stress conditions (biotic and abiotic) has been previously described, including in some of the genera detected in this study, namely, *Achromobacter*, *Burkholderia*, *Lysinibacillus*, *Paenibacillus*, and *Stenotrophomonas*. The role of these organisms in the sustainability and production of maize will be evaluated in future studies.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA675280.

AUTHOR CONTRIBUTIONS

AA, CJ, DP, and DS helped in the investigation. AA, JC, CJ, and DP did the formal analysis. JC and DP helped in funding acquisition, project administration, resources, and supervision. AA and JC contributed to visualization and writing

of the original draft. AA, JC, MM, and DP contributed to the conceptualization, reviewed, edited, and wrote the final 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.2021.636009/full#supplementary-material

Supplementary Figure 1 | LEfSe was used to identify the most differentially abundant taxa among population genotypes in the rhizosphere of maize. Bar graph showing LDA scores for fungi **(A,B)** and bacteria **(C,D)** are represented. The length of the bar column represents the LDA score. The figure shows the microbial taxa with significant differences between the "Pigarro" (red) and "SinPre" (green). Only taxa meeting an LDA significant threshold > 2 are shown.

Supplementary Figure 2 | Heat-map of the relative abundance with distance measure using Euclidean, at the level class for fungal **(A)** and bacterial **(B)** community. The relative abundance is scaled by a color gradient bar.

Supplementary Table 1 | Soil characterization of the trial locations in august 2019.

Supplementary Table 2 | Characterization of maize populations using HUNTERS descriptors in conventional and organic farming systems.

Supplementary Table 3 | Abundance of fungal genera detected in each sample and the respective taxonomic assignment.

Supplementary Table 4 | Abundance of bacterial genera detected in each sample and the respective taxonomic assignment.

Supplementary Table 5 | Fungal and bacterial core rhizosphere microbiota associated with maize.

Supplementary Table 6 | Fungal and bacterial genera specific to the rhizosphere microbiota in each population.

Supplementary Table 7 | Fungal and bacterial genera specific to the rhizosphere microbiota of maize in distinct farming system.

Supplementary Table 8 | Genera relative abundance significantly different between "Pigarro" and "SinPre" populations ($P < 0.05$).

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Evaluation of Established Methods for DNA Extraction and Primer Pairs Targeting 16S rRNA Gene for Bacterial Microbiota Profiling of Olive Xylem Sap

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Next-generation sequencing has revolutionized our ability to investigate the microbiota composition of diverse and complex environments. However, a number of factors can affect the accuracy of microbial community assessment, such as the DNA extraction method, the hypervariable region of 16S rRNA gene targeted, or the PCR primers used for amplification. The aim of this study was to assess the influence of commercially available DNA extraction kits and different primer pairs to provide a non-biased vision of the composition of bacterial communities present in olive xylem sap. For that purpose, branches from "Picual" and "Arbequina" olive cultivars were used for xylem sap extraction using a Scholander chamber device. The DNA extraction protocol significantly affected xylem sap bacterial community assessment. That resulted in significant differences in alpha (Richness) and beta diversity (UniFrac distances) metrics among DNA extraction protocols, with the 12 DNA extraction kits evaluated being clustered in four groups behaving differently. Although the core number of taxa detected by all DNA extraction kits included four phyla, seven classes, 12 orders, 16 or 21 families, and 12 or 14 genera when using the Greengenes or Silva database for taxonomic assignment, respectively, some taxa, particularly those identified at low frequency, were detected by some DNA extraction kits only. The most accurate depiction of a bacterial mock community artificially inoculated on sap samples was generated when using the PowerPlant DNA extraction kit, the combination of 799F/1193R primers amplifying the hypervariable V5–V7 region, and the Silva 132 database for taxonomic assignment. The DESeq2 analysis displayed significant differences among genera abundance between the different PCR primer pairs tested. Thus, *Enterobacter*, *Granulicatella*, *Prevotella*, and *Brevibacterium* presented a significant higher abundance in all PCR protocols when compared with primer pair 799F/1193R, while the opposite was true for *Pseudomonas* and *Pectobacterium*. The methodological approach followed in this study can be useful

to optimize plant-associated microbiome analysis, especially when exploring new plant niches. Some of the DNA extraction kits and PCR primers selected in this study will contribute to better characterize bacterial communities inhabiting the xylem sap of olives or other woody crop species.

Keywords: DNA extraction kits, NGS, 16S rRNA gene, mock community, microbiome, xylem

INTRODUCTION

Olive (*Olea europaea* subsp. *europaea*) is a primary element in the agricultural economy of most countries in the Mediterranean Basin, where about 5 million hectares of olive orchards are grown only in European countries. More than half of the cultivated olives worldwide are in Spain, accounting for 70–75% of world production of olive oil and more than one third for table olives (EUROSTAT)[1]. During the last years, the health of the olive groves is being seriously threatened, as a consequence of a notable increase, both in extent and in severity, of diseases caused by diverse pathogens, which are capable of adversely affecting its growth and production. Among olive diseases, those caused by the vascular plant pathogenic bacterium *Xylella fastidiosa* and the soilborne fungus *Verticillium dahliae* are, without a doubt, the two major global threats to olive production worldwide (Jiménez-Díaz et al., 2011; Saponari et al., 2018; Landa et al., 2019; Anguita-Maeso et al., 2020).

Research on plant-associated microorganisms or plant microbiome has gained importance in the last decade as a key component in the health and productivity of the plant (Berg et al., 2014). Thus, recent studies have shown that certain endophytic bacteria are capable of modifying the development of diseases in plants, promoting their growth and protecting them against insects and pathogens (Müller et al., 2015). In addition, they could confer other important benefits for plants, such as greater resistance to stress conditions, alteration in physiological properties, and production of phytohormones and other compounds of biotechnological interest (Porras-Alfaro and Bayman, 2011; Hacquard and Schadt, 2015; Santoyo et al., 2016). Within the plant tissues, xylem vessels are considered ideal niches for microorganisms by providing an effective internal pathway for distribution throughout the plant and a continuous source of nutrients (McCully, 2001). However, information on the nature and role of the xylem microbiome and its contribution to plant health and crop productivity is still scarce (Anguita-Maeso et al., 2020).

For olive trees, most microbiota studies have focused on determining the microbial composition of its rhizosphere (Mercado-Blanco et al., 2004; Aranda et al., 2011; Berg et al., 2016; Gómez-Lama Cabanás et al., 2018; Lei et al., 2019; Fernández-González et al., 2020) and to which extent these microbial communities can act as potential antagonists of olive pathogens such as *V. dahliae*. Other studies have focused on the effect of abiotic factors (edaphic, climatic, and agronomic) on the olive soil- and rhizosphere-associated microbiota (Montes-Borrego et al., 2013; Landa et al., 2014; Caliz et al., 2015). More scarce are the works in which the endosphere olive microbiota has been investigated. In those studies, diverse methodological approaches based on next-generation sequencing (NGS) technologies were used to analyze the microbiota composition (Kennedy et al., 2014; Müller et al., 2015; Fausto et al., 2018; Sofo et al., 2019; Anguita-Maeso et al., 2020). Moreover, it has been demonstrated that the method used for DNA extraction can lead to dramatic differences in microbial output composition (Henderson et al., 2013; Brooks et al., 2015), which makes validation of DNA extraction methods with a mock microbial community essential to ensure an accurate representation of the microbial communities in the samples under study.

Most of the referred studies analyzing xylem microbiota have been based on amplicon sequencing using "universal" primers targeting the 16S rRNA gene in bacteria, as it is the most cost-effective and facile tool to provide valuable phylogenetic information for the comparison of bacterial diversity in large numbers of samples. However, the lack of standardization procedures among plant microbiota studies can make the comparison of results among them difficult (Stulberg et al., 2016). Both choice of the hypervariable region of 16S and the primer pair have been shown to influence the description of microbial diversity (Claesson et al., 2010; Fouhy et al., 2015; Teng et al., 2018). Thus, care should be taken in choosing appropriate primer pairs, as limited taxa coverage or overrepresentation or underrepresentation of taxa in a specific environment due to biases in primer amplification could occur that could lead to unreliable results (Claesson et al., 2010; Wasimuddin et al., 2020).

An additional problem when working with plant tissues is the co-amplification of undesirable or non-target sequences from organellar origin (e.g., mitochondria and/or chloroplast DNA) that may represent a major source of "contamination" due to the homology between bacterial 16S rDNA, chloroplast DNA, and mitochondrial DNA. This leads to significant challenges in the selection of appropriate primer pairs to address the study of plant–microbe interactions (Ghyselinck et al., 2013). Several methodologies have been proposed to reduce co-amplification of plant organellar sequences such as a reduction of co-extraction of organellar DNA based on differences in methylation density (Feehery et al., 2013), blocking primers and suicide polymerase endonuclease restriction (SuPER) (Green and Minz, 2005), and the use of specific mismatch primers during PCR amplification (Beckers et al., 2016). Among them, the preferred or most used approach is the use of specific mismatch primers, which amplify bacterial 16S rDNA sequences while simultaneously avoiding the amplification of organellar DNA sequences. Thus, several

[1]https://ec.europa.eu/eurostat

primer pairs have been developed with that purpose, revealing different performances depending of the study (e.g., Chelius and Triplett, 2001; Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016; Dos Santos et al., 2017). However, the experimental performance of these mismatch primers and their efficacy in reducing co-amplification of non-target DNA in different plant species or plant compartments that may differ on organellar input have not been evaluated enough. Consequently, it is essential to evaluate the amplification efficiency and robustness of selected primer pairs in plant–bacteria interaction studies to assess their behavior in different host plants and specific plant compartments, especially on those rarely addressed such as the xylem tissue.

To our knowledge, no study has systematically evaluated the effects of different DNA extraction methods and the choice of primer pairs to conduct studies on xylem sap bacterial microbiota, which is a key information that is still missing. Consequently, the objectives of this work have been: (1) to compare several standard DNA extraction kits and primer pairs targeting different hypervariable regions of the 16S rRNA gene and described as suitable to avoid co-amplification of plant organellar rRNA gene sequences for their efficacy in the description of the structure and diversity of xylem sap bacterial microbiota when coupled with NGS and bioinformatic tools; (2) to assess whether one of the selected DNA extraction methods combined with different primer pairs could provide an accurate representation of bacterial communities using a mock microbial community standard; and (3) to demonstrate the utility of the selected protocol for assessing differences in xylem bacterial composition of two of the most widely grown olive cultivars in Spain.

MATERIALS AND METHODS

Xylem Sap Collection

Xylem sap extraction from olive branches was performed using a Scholander pressure chamber pressurized with compressed nitrogen and coupled with an external 60-cm-long super chamber following the Bollard process and as described by Anguita-Maeso et al. (2020). Shortly, xylem sap was extracted from 30-cm-long, 2-year-old branches of adult olive trees, which were approximately 1 cm in diameter in their thicker part and which were debarked at the external part to avoid contamination by phloem fluids. Pressure was increased progressively up to a maximum of 35 bar (3.5 MPa), with the first few drops of sap being discarded; then the xylem sap was collected into 5-ml Eppendorf tubes kept on ice for 20–30 min until a minimum volume of 4.0 ml per branch was obtained. Xylem sap was kept at −80°C prior to DNA extraction. All the processes described above took place under sterile conditions into a flow hood chamber.

Extraction of Microbial DNA From Xylem Sap

For the initial experiment to evaluate different microbial DNA extraction protocols, a total of 15 olive branches were used from four 8-year-old olive trees of cv. "Picual." The xylem sap extracted from all branches was combined into a composite sample, vortexed to homogenize it, and then split into 3-ml aliquots. Samples were then centrifuged at 12,000 × *g* for 20 min at 4°C. The supernatant was removed into a new tube, and the pellet and the supernatant were stored at −20°C separately.

Xylem sap pellets were used for total DNA extraction using 11 commercial microbial DNA extraction kits following the protocol as indicated by the manufacturer or with slight modifications (**Table 1**). Additionally, the cetyltrimethylammonium bromide (CTAB) protocol (2% hexadecyltrimethylammonium bromide, 0.1 M Tris–HCl pH 8, 20 mM EDTA, and 1.4 M NaCl) was included. Two replicates of xylem sap samples were used for each protocol. Before starting each protocol, the pellet was resuspended in the corresponding extraction buffer of each protocol by vortexing briefly. DNA was eluted in a final volume of 50 μl of ultrapure, filtered-sterilized distilled water. For each protocol and replicated sample, the following parameters were determined: (1) the yield and purity of the DNA (absorbance 260/280 nm ratio) by using a NanoDrop®156 ND-1000 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, United States), (2) the PCR yield using PCR primers 967F/1193R and agarose gel electrophoresis (see below), (3) time of processing, and (4) cost (**Table 1**).

Validation of a DNA Extraction Protocol and PCR Primers

Microbial composition profiling techniques powered by NGS can suffer from significant bias at different steps from bacterial sampling to bioinformatic analysis workflows. A mock standard microbial community was used to validate which of the four primer pair combinations could provide the most accurate representation of the microbial communities present in the xylem sap and to which extent the selection of the reference database for taxonomic assignment could also influence results. The ZymoBIOMICS microbial standard (Zymo Research) was used as a mock standard microbial community. This is a well-defined, accurately characterized mock community consisting of three easy-to-lyse bacteria, five tough-to-lyse bacteria, and two tough-to-lyse yeasts. Its theoretical bacterial composition according to the manufacturer based on 16S rRNA gene abundance is *Listeria monocytogenes*, 14.1%; *Pseudomonas aeruginosa*, 4.2%; *Bacillus subtilis*, 17.4%; *Escherichia coli*, 10.1%; *Salmonella enterica*, 10.4%; *Lactobacillus fermentum*, 18.4%; *Enterococcus faecalis*, 9.9%; and *Staphylococcus aureus*, 15.5%.

The PowerPlant DNA extraction method was selected to verify its efficiency at extracting accurate representative quantities of DNA from both Gram-positive and Gram-negative bacteria present in the mock microbial community standard (MC). Mock sap samples were prepared using 3 ml of bacterial-free xylem sap (previously filtered through a 0.22-μm filter and showing no amplification of 16S rRNA gene with PCR1, see below) to which we added 50 μl of ZymoBIOMICS Microbial Community Standard Cells (Zymo Research Corp., Irvine, CA, United States). Mock sap samples were then processed as indicated above for the

TABLE 1 | Characteristics of the DNA extraction protocols used in the study.

ID Protocol	Protocol[a]	Trademark	DNA yield (ng/μl)	Absorbance 260/280	Manufacturer's instructions procedure	Amplification[b] 16S	Price to 50 preps[c] (€)	Extraction time (min)
PowerPlant	DNeasy PowerPlant Pro Kit	Qiagen	5.9 ± 1.4	1.7	Yes	+++	4.0	40
PowerSoil	DNeasy PowerLyzer PowerSoil kit	Qiagen	2.7 ± 0.1	1.7	Yes	++	7.3	50
MoBioSoil	PowerSoil® DNA Isolation Kit	Mo Bio	5.4 ± 2.8	1.3	Yes	+	5.3	55
PureLink	PureLink™ Microbiome DNA Purification Kit	Invitrogen	8.5 ± 3.4	1.4	Yes	+	5.4	50
NorgenMicrobiomeV1	Microbiome DNA Isolation kit	Norgen	1.6 ± 0.3	1.3	Yes	++	4.0	65
NorgenMicrobiomeV2	Microbiome DNA Isolation kit	Norgen	16.7 ± 1.5	2.0	Yes, using Binding Buffer B instead of Binding Buffer I	+++	4.0	65
QuickPick	QuickPick™ SML Plant DNA	Bio-Nobile	16.6 ± 0.1	2.5	Yes	+	2.3	70
CTAB	CTAB[c]		1.0 ± 0.5	1.8	Yes	++	1.0	105
NucleoSpinPL1	NucleoSpin® Plant II	Macherey-Nagel	3.1 ± 1.4	1.9	Yes, using PL1 lysis buffer	+	3.2	80
NucleoSpinPL2	NucleoSpin® Plant II	Macherey-Nagel	1.1 ± 0.7	1.1	Yes, using PL2 lysis buffer	+	3.2	95
CanvaxSoil	HigherPurity™ Soil DNA Isolation Kit	Canvax Biotech	5.9 ± 3.7	1.4	Yes	+++	5.6	70
CanvaxTissue	HigherPurity™ Tissue DNA Purification Kit	Canvax Biotech	2.6 ± 0.4	2.3	Yes	++	2.4	95

[a] *Commercial kit name. CTAB, cetyltrimethylammonium bromide.*
[b] *Relative amplification as measured by the intensity of the amplified product after agarose gel electrophoresis visualization: (+++) = very good, (++) = good, (+) = weak.*
[c] *Times that the cost for each kit is more expensive than the CTAB cost for extracting 50 samples.*
[b] *Sample preparation time not including sap extraction.*

PowerPlant DNA extraction protocol. After DNA extraction, the four PCR protocols described below were evaluated. There were four replicates per combination of PCR protocol.

Sampling Xylem From Olive Cultivars and Extracting DNA

Olive orchards from an experimental plot located at the Institute for Sustainable Agriculture from Spanish National Research Council (IAS-CSIC) facilities in Córdoba (southern Spain) were used to test the differences in xylem bacterial composition between two of the most widely grown olive cultivars in Spain. The orchard was established in September of 2014, with 2-year-old olive trees of cultivar "Picual" and "Arbequina" propagated at "Plantas Continental S.A." nursery (Ribero de Posadas, Córdoba, Spain). Both genotypes were planted in the orchard with a random block design and received similar growing practices until sampling.

Seven trees of each cultivar were sampled in May 2017, and xylem sap was extracted as described above. The average sap volume extracted ranged from 3.5 to 4 ml per sampled branch. In this case and to facilitate DNA extraction, the sap samples were immediately filtered through a 0.22-μm Millipore filter, and the filters retaining all microbial cells contained in the sap and the filtered sap were stored independently at −20°C or −80°C, respectively. DNA extraction was performed using the PowerPlant protocol. First, the filtered cells were resuspended in the extraction buffer by vortexing briefly, and then the microbial suspension was processed following the manufacturer's instructions (**Table 1**). To analyze bacterial communities, DNA samples were amplified using the four PCR protocols described below.

16S rRNA Gene Amplification

Four PCR protocols using five primer pairs targeting different hypervariable regions of the 16S rRNA gene were compared to evaluate their performance in metabarcoding studies of xylem sap bacterial communities. Those primer pairs have been described in previous studies as appropriate to avoid co-amplification of plant chloroplast and mitochondrial DNA. For that, two primer pairs were used in two direct PCR protocols, and additionally, three primer pairs were used in two nested PCR protocols to evaluate if an increase in amplification yield could be obtained by the nested PCR approach (**Table 2**):

PCR1-(799F/1391R) uses primers 799F and 1391R that amplify a 600-bp fragment of the V5–V8 region of the bacterial 16S rRNA gene (Chelius and Triplett, 2001; Walker and Pace, 2007; Beckers et al., 2016), and its amplification product is used as a template for the second round of nested PCRs described below.

PCR2-(967F/1193R) uses primers 967F and 1193R that amplify the V6–V7 region of the bacterial 16S gene (Dos Santos et al., 2017). This PCR was initially selected to test the different

TABLE 2 | PCR protocols used in the study, with primer sequences, hypervariable region of 16S rRNA gene amplified, and expected product size.

PCR	Primer pairs	Primer forward	Primer reverse	Region	Size (pb)	References
PCR-1	799F + 1391R	AACMGGATTAGATACCCKG	GACGGGCGGTGWGTRCA	V5–V8	600	Walker and Pace, 2007; Beckers et al., 2016; Dos Santos et al., 2017
PCR-2	967F + 1193R	CAACGCGAAGAACCTTACC	ACGTCATCCCCACCTTCC	V6–V7	230	Bodenhausen et al., 2013; Dos Santos et al., 2017
PCR-3	799F + 1193R	AACMGGATTAGATACCCKG	ACGTCATCCCCACCTTCC	V5–V7	400	Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016
N1PCR1*	799F + 1193R	AACMGGATTAGATACCCKG	ACGTCATCCCCACCTTCC	V5–V7	400	Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016
N2PCR1*	967F + 1391R	CAACGCGAAGAACCTTACC	GACGGGCGGTGWGTRCA	V6–V8	430	Walker and Pace, 2007; Callahan et al., 2016; Dos Santos et al., 2017

**N1PCR1 and N2PCR1 were nested PCRs that used as DNA template the amplification product obtained with primers 799F + 1193R (PCR1).*

DNA extraction protocols, and it was also included to compare its performance with the other three PCR protocols described below.

PCR3-(799F/1193R) uses primers 799F and 1193R that amplify the V5–V7 region of the bacterial 16S rRNA gene (Chelius and Triplett, 2001; Bodenhausen et al., 2013; Beckers et al., 2016).

N1PCR1-(799F/1391R + 799F/1193R) uses primers 799F and 1391R in the first round of PCR (i.e., PCR1) and primers 799F and 1193R in the second round of PCR.

N2PCR1-(799F/1391R + 967F/1391R) uses primers 799F and 1391R in the first round of PCR (i.e., PCR1) and primers 967F and 1391R that amplify the V6–V8 region of the bacterial 16S rRNA gene in the second round of PCR (Sogin et al., 2006; Walker and Pace, 2007; Beckers et al., 2016).

All PCRs were carried out in 25-μl reaction volumes containing 0.05 units of MyTaq^{TM} DNA Polymerase (Bioline Laboratories, London, United Kingdom), 1× MyTaq^{TM} Mix (Bioline) and forward and reverse primers at a concentration of 0.3 μM each, and 3 μl of template DNA. PCR protocol consisted of an initial denaturalization step at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 53°C for 45 s, 72°C for 1 min, and a final elongation step at 72°C for 8 min. For the nested PCR approach, 1 μl of the amplification product of the first reaction was used as the template for the second PCR that was performed with the same conditions as those of the first PCR.

Library Preparation and Sequencing

PCR amplicons were cleaned up before adaptor addition using the Ampure XP magnetic bead system (Beckmann Coulter, MA, United States) according to manufacturer's recommendation. Dual barcode indices and sequencing adaptors were attached to each amplicon using the Illumina Nextera XT Index kit (Illumina, Inc., San Diego, CA, United States) following the manufacturer's protocol, followed by a further Ampure XP cleanup step. Purified amplicons were quantified using the Quant-iT^{TM} PicoGreen^{TM} dsDNA Assay Kit (Thermo Fisher Scientific) and a Tecan Safire microplate reader (Tecan Group, Männedorf, Switzerland). Equimolecular amounts from each individual sample in 10 mM of Tris were combined, and the pooled library was additionally purified with two rounds of Ampure XP cleanup step. The library was sequenced by the Genomics Unit at "Fundación Parque Científico de Madrid" (Madrid, Spain) using the Illumina MiSeq platform (Nano-V2; PE 2x 250 bp). The ZymoBIOMICS microbial standard (Zymo Research Corp., Irvine, CA, United States) and water (no template DNA) were used as internal positive and negative controls, respectively, for library construction and sequencing. Raw sequence data have been deposited in the Sequence Read Archive (SRA) database at the NCBI under BioProject accession number PRJNA684121.

Data Processing and Bioinformatic Analysis

16S rRNA gene sequences were analyzed and classified using the Quantitative Insights into Microbial Ecology bioinformatic pipeline, QIIME2 (version 2019.10[2]; Caporaso et al., 2010; Bolyen et al., 2018) with default parameters unless otherwise noted. Demultiplexed sequences were imported as CASAVA format. Sequence quality control, denoising, and chimeric filtering were performed with DADA2 pipeline (Callahan et al., 2016). Taxonomy affiliation was identified by operational taxonomic units (OTUs) at 99% similarity using VSEARCH consensus taxonomy classifier (Rognes et al., 2016) based on Greengenes_13_8_99 (DeSantis et al., 2006; McDonald et al., 2011) and Silva_132_99 (Quast et al., 2013; Yilmaz et al., 2014) reference databases. Singletons were discarded for downstream analysis.

Alpha diversity and beta diversity as well as alpha rarefaction curves were conducted rarefying all samples to the minimum number of reads found. Sequencing depth was of 2,276 and 2,489 for the evaluation of the DNA extraction protocols and the microbiota of the olive tree cultivars, respectively. Rarefaction curves and alpha diversity indexes (Shannon and Richness or number of observed OTUs) were performed using the OTU frequency matrixes at the OTU level with the online tool MicrobiomeAnalyst (Chong et al., 2020)[3]. The Kruskal–Wallis test ($P < 0.05$) with false discovery rate (FDR) correction (Benjamini and Hochberg, 1995) was used to determine the effects of the factors in the study on alpha diversity. To analyze the association of PCR protocols and olive genotypes with these alpha diversity matrices, we applied general linear modeling (GLM)

[2]https://view.qiime2.org/

[3]https://www.microbiomeanalyst.ca

using the lme4 package in R (Bates et al., 2015), with a two-factor factorial design, being the PCR protocols [967F-1193R (PCR2), $n = 14$; 799F-1193R (PCR3), $n = 14$; 799F-1391R + 799F-1193R (N1PCR1), $n = 14$; 799F-1391R + 967F-1391R (N2PCR1), $n = 14$] and the olive genotype ("Arbequina"; $n = 28$; "Picual," $n = 28$) the two main factors. Venn diagrams were generated using the "Venn diagram" online tool[4] and were used to identify shared (core microbiota) or unique taxa according to the DNA extraction protocol or the PCR primers used.

For beta diversity analysis, we performed multivariate hierarchical clustering analysis (using Bray–Curtis index to measure distance and the Ward clustering algorithm), as well as non-supervised principal coordinate analysis (PCoA) using Bray–Curtis distance (a non-phylogenetic metric) (Beals, 1984), and the weighted UniFrac distances (a phylogenetic metric) (Lozupone and Knight, 2005) were performed using the OTU frequency matrixes at the OTU level with QIIME2 to test for similarities among the bacterial communities according to the DNA extraction protocol or among the PCR protocols and between the olive genotypes. In addition, the PERMANOVA test ($P < 0.05$) was used to determine the effects of those factors.

The theoretical relative abundance of the ZymoBIOMICS Microbial Community Standard was compared with the estimated relative abundances of the identified OTUs at the genus level that were obtained for each PCR and reference database (Silva or Greengenes) using Spearman's correlation analysis (McGovern et al., 2018). Similarity was considered as significant if P value < 0.05 and as a trend if $0.05 \geq P$ value ≤ 0.1.

Finally, to analyze in further detail the differences in microbiota composition at the genus level among the different PCR protocols, a negative binomial model approach based on the DESeq2 package in R (Love et al., 2014) was used. Wald tests were performed, and only genera remaining significant ($P < 0.01$) were retained.

RESULTS

Effect of DNA Extraction Protocols on Xylem Sap Bacterial Community Assessment

In the bacterial community analyses of the 12 different DNA extraction protocols tested, a total of 108,236 high-quality 16S rRNA gene paired-end sequences with an average of 4,510 sequences per DNA protocol were retained after discarding poor-quality sequences. From those, approximately 56% could be classified into bacterial OTUs with a mean of 2,460 and 2,468 bacterial sequences in Greengenes_13.8 or Silva_132 databases, respectively (**Table 3**). However, the number of plant organellar rRNA gene sequences amplified varied according to the DNA extraction kit, with the NorgenMicrobiomeV2 and PureLink DNA extraction kits showing the highest proportion of organellar reads (≥70%) and both NucleoSpin and the PowerLyzer DNA extraction kits showing the lowest proportion (≤4%), independently of the reference database used for taxonomic assignment (**Table 3**). A total of 209 or 248 OTUs, with an average number of 58 or 60 OTUs, were identified as bacteria when using the Greengenes_13.8 or Silva_132 databases, respectively (**Table 3**), with the higher number of OTUs being identified when using the NucleoSpin and PowerLyzer DNA extraction kits (i.e., identified OTUs ranged between 84 and 93) (**Table 3**).

Rarefaction curves of observed OTUs (Richness) indicated a good sequencing coverage among all DNA extraction protocols, with no differences between both reference databases (**Supplementary Figure 1**). DNA extraction protocol significantly affected xylem sap bacterial community assessment. Thus, Richness alpha diversity index showed significant differences ($P < 0.044$) among the different DNA extraction methods, whereas no significant differences ($P > 0.091$) were found for the Shannon index regardless of the taxonomy database used (**Figure 1**). On the other hand, hierarchical clustering analysis and PCoA of Bray–Curtis index and weighted UniFrac distances using OTU frequencies differentiated xylem bacterial communities in four clusters according to the DNA extraction method regardless of the database used for taxonomy assignment (**Figure 2A**). Thus, most DNA extraction kits from four different brands clustered together (Group 4), whereas the CTAB protocol (Group 1), both Canvax kits (Group 2), and the two NorgenMicrobiome protocols (Group 3) clustered independently from each other, although Group 1 and Group 2 clustered closer and apart from the other DNA extraction kits (**Figure 2A**). Similarly, PCoA of Bray–Curtis and weighted UniFrac distances differentiated xylem bacterial communities according to the DNA extraction kits in the four groups (**Figure 2B**) regardless of the database used. PERMANOVA indicated a significant clustering due to the DNA extraction protocol used (*pseudo-F* < 12.386; $P = 0.001$). This clustering, derived from beta diversity analyses, of the 12 DNA extraction kits in four independent groups was used to summarize the influence of DNA extraction kits on assessing the composition of bacterial communities in olive xylem sap.

A total of 13 or 16 phyla, 29 or 34 classes, 53 or 65 orders, 118 or 124 families, and 209 or 248 genera were identified when using the Greengenes or Silva database, respectively (**Supplementary Figure 2**). When comparing results from both databases, the core number of taxa detected by all the DNA extraction kits was four phyla, seven classes, and 12 orders, whereas 16 or 21 families and 12 or 14 genera were detected when using the Greengenes or Silva database, respectively. The lowest numbers of phyla and classes were identified when using the Canvax DNA extraction kits (Group 2), whereas the lowest numbers of orders, families, and genera were identified with the CTAB DNA extraction protocol (Group 1). In all cases, the highest numbers of total and unique phyla, orders, classes, families, and genera were identified for the DNA kits included in Group 4. Finally, the DNA extraction kits included in Groups 3 and 4 shared the highest number of bacterial taxa (**Supplementary Figure 2**).

Four phyla including Proteobacteria, Actinobacteria, Firmicutes, and Bacteroidetes were the most abundant and were identified by all DNA extraction kits, representing 51.8%, 26.2%, 9.7%, and 9.6% of the total, respectively (**Figure 3** and **Supplementary Figure 2**). In addition,

[4]http://bioinformatics.psb.ugent.be/webtools/Venn/

TABLE 3 | Number of reads and operational taxonomic units (OTUs) derived from NGS analysis of xylem sap microbiota from DNA samples extracted with 12 extraction protocols, amplified with the 967F + 1193R primer pair (PCR2), and taxonomic assignment using the Greengenes 13-8 and Silva 132 reference databases.

Reads/OTU numbers		Norgen MicrobiomeV1	Norgen MicrobiomeV2	Pure Link	Quick Pick	Power Soil	Power Lyzer	Power Plant	Nucleo Nucleo	Nucleo SpinPL2	Canvax Soil	Canvax Tissue	CTAB*
Number of reads (Mean/sample)		4,586	5,888	3,931	4,977	4,724	4,502	3,265	3,828	4,731	5,777	4,090	3,821
Greengenes	Unassigned Reads (%)	15	11	8	12	13	8	11	46	21	5	13	6
	Plant (chloroplast + mitochondria) reads (%)	33	70	79	18	18	3	28	2	4	59	36	14
	Bacterial reads (%)	52	19	13	70	69	89	61	52	75	36	51	80
	OTUs at the Phylum level	7	6	4	7	7	8	7	8	7	6	5	8
	OTUs at the Class level	14	7	6	12	9	14	13	12	14	10	7	12
	OTUs at the Order level	25	10	12	24	16	24	21	26	28	18	12	19
	OTUs at the Family level	42	17	19	48	34	49	41	56	58	42	30	35
	OTUs at the Genus level	51	24	21	66	41	75	54	81	77	55	32	45
	Total Assigned OTUs	56	24	21	73	50	90	63	91	88	62	35	47
	Unassigned Reads (%)	15	11	8	11	13	7	11	46	21	5	13	6
Silva	Plant (chloroplast + mitochondria) reads (%)	33	70	79	18	18	3	28	2	4	59	36	14
	Bacterial reads (%)	52	19	13	71	69	90	61	52	75	36	51	80
	OTUs at the Phylum level	8	6	4	8	8	10	8	9	9	6	6	9
	OTUs at the Class level	16	7	6	14	11	17	15	14	19	10	9	14
	OTUs at the Order level	31	15	14	30	20	30	28	32	37	21	16	23
	OTUs at the Family level	47	21	19	49	35	52	42	52	60	39	30	39
	OTUs at the Genus level	60	28	21	67	45	80	58	78	82	54	33	49
	Total Assigned OTUs	64	29	21	74	49	93	65	84	91	61	35	52

CTAB, cetyltrimethylammonium bromide.

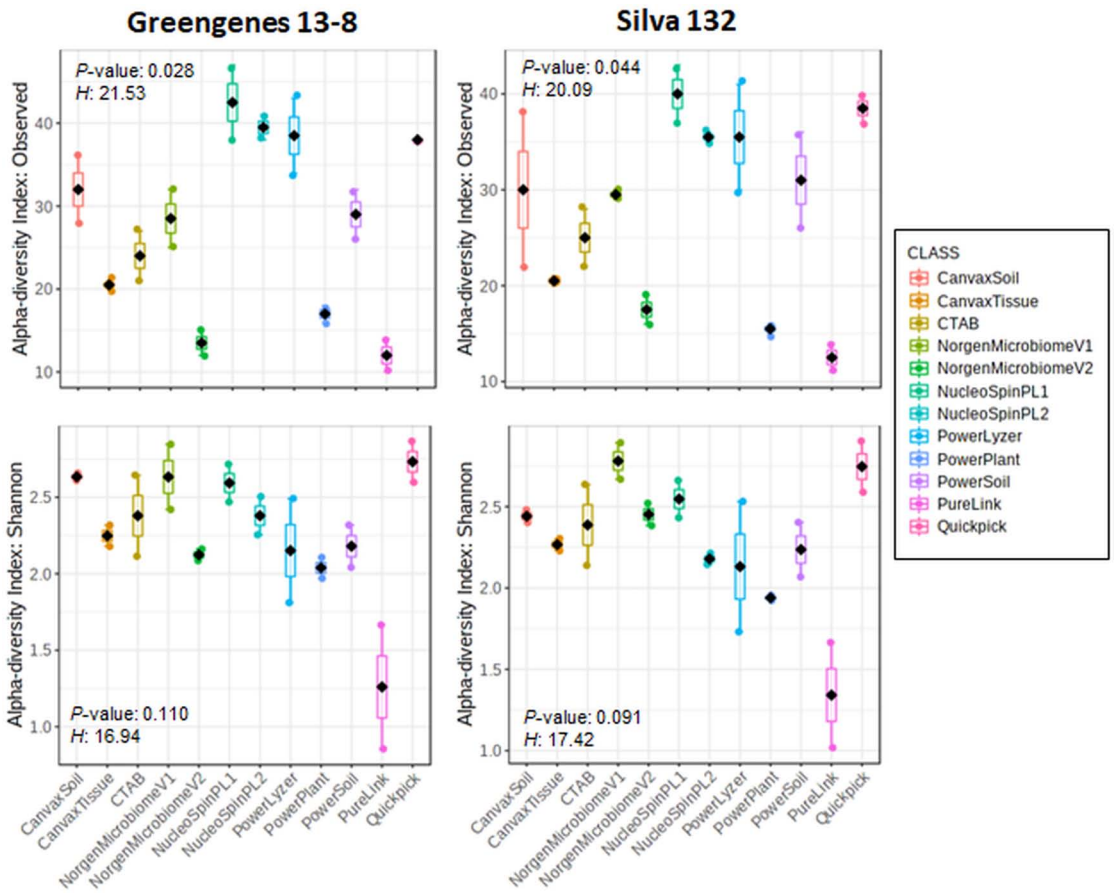


FIGURE 1 | Boxplots of Richness and Shannon alpha diversity indices of olive xylem bacterial communities at operational taxonomic unit (OTU) taxonomic level determined by different DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Boxes represent the interquartile range, while the black dots inside the box define the median, and whiskers represent the lowest and highest values. *P* value was calculated using Kruskal–Wallis test.

three minority phyla (<0.11% abundance), Nitrospirae, Fibrobacteres, and Chloroflexi, were only identified when using the NorgenMicrobiome DNA extraction kits (Group 3), whereas three other phyla, Saccharibacteria, Gemmatimonadetes, and Verrucomicrobia, showed a low frequency (0.2%) and were only identified when using the different DNA extraction kits from Group 4 (**Supplementary Figure 2**). Seven bacterial classes were detected by all DNA extraction kits, with Actinobacteria, Alphaproteobacteria, Betaproteobacteria, Bacilli, and Gammaproteobacteria being the most abundant, representing 26.1%, 22.6%, 19.7%, 9.0%, and 8.8% of the total, respectively (**Figure 3** and **Supplementary Figure 2**). At the family level, 16 or 21 families, depending on the reference database used, comprised the core bacterial microbiota of all DNA extraction kits, being Propionibacteriaceae, Bradyrhizobiaceae, Comamonadaceae, and Chitinophagaceae the most abundant (mean frequency ranged between 5.9 and 18%) (**Figure 3** and **Supplementary Figure 2**). Finally, at the genus level, 12 or 14 genera, depending of the reference database (**Supplementary Figure 2**), comprised the core bacterial microbiota, being *Propionibacterium*, and unidentified Comamonadaceae, *Sediminibacterium*, and unidentified Rhizobiales, and unidentified Methylophilaceae, and unidentified Bradyrhizobiaceae, *Novosphingobium*, *Staphylococcus*, *Bradyrhizobium*, and *Flavobacterium* the most abundant genera (mean frequencies ranged from 18.8 to 3.0%) by all DNA extraction kits when using the Greengenes database; whereas with the Silva database, *Propionibacterium*, *Bradyrhizobium*, an unidentified Comamonadaceae, an unidentified Chitinophagaceae, an unidentified Methylophilaceae, *Novosphingobium*, *Bosea*, *Flavobacterium*, *Staphylococcus*, and *Pseudomonas* were the most abundant genera (mean frequencies ranged from 18.7 to 2.8%). Interestingly, up to 114 genera could be detected uniquely by the DNA extraction kits within Group 4.

Validation of a DNA Extraction Method, Primer Pair Performance, and Taxonomy Reference Databases

In this study, we used a mock microbial community standard to determine if a selected DNA extraction kit (the PowerPlant from Qiagen) within Group 4 (the one providing the highest values of alpha diversity) provides an accurate representation of

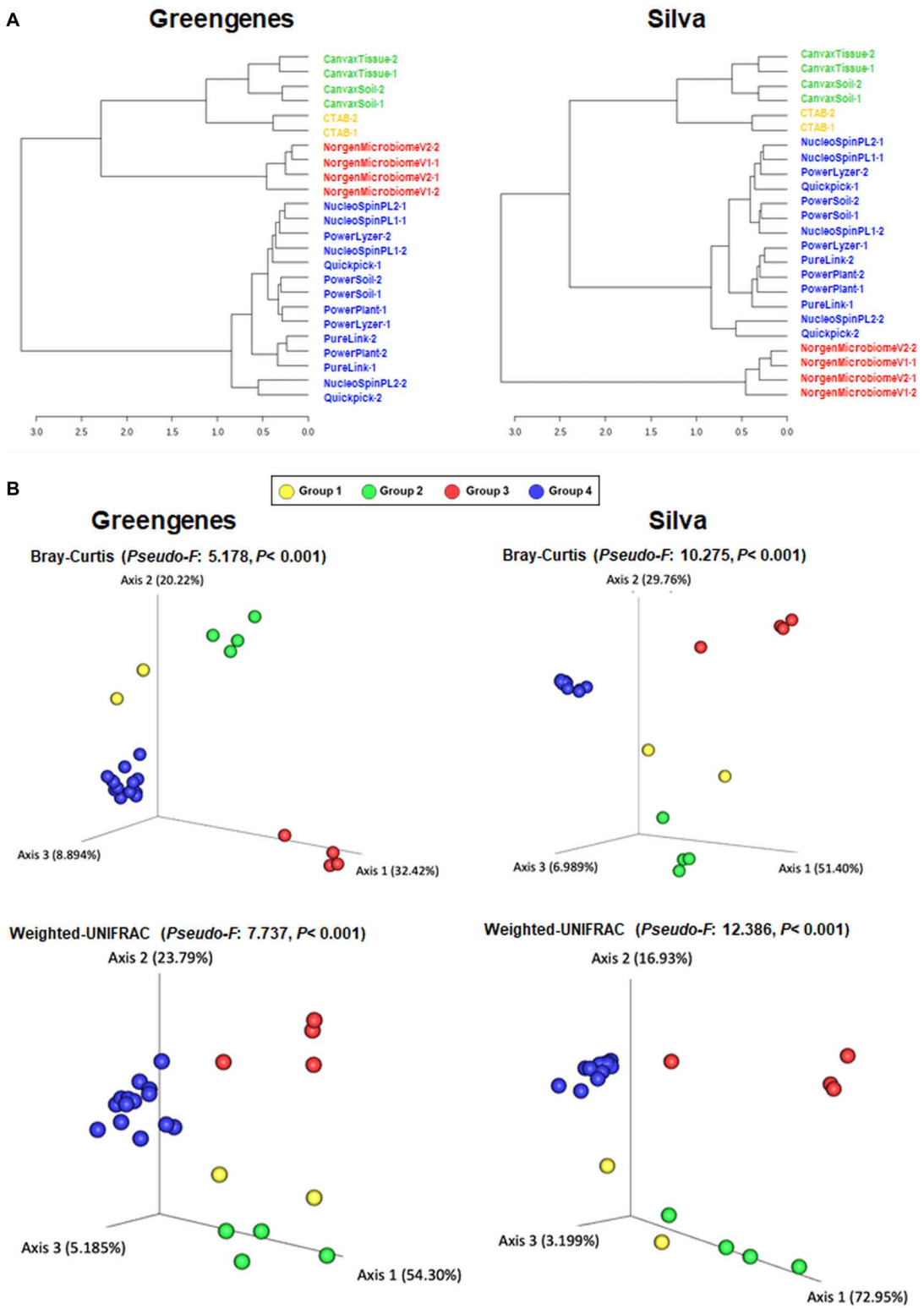


FIGURE 2 | Hierarchical clustering dendrogram analysis using Ward method and Bray–Curtis distance **(A)** And principal coordinate analysis (PCoA) of weighted UniFrac and Bray–Curtis distances **(B)** Of olive xylem bacterial communities obtained by using different DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Colored dots represent the four clusters obtained in the hierarchical clustering analysis. PERMANOVA (999 permutations; $P < 0.05$) was performed to test significant differences according to DNA extraction kits.

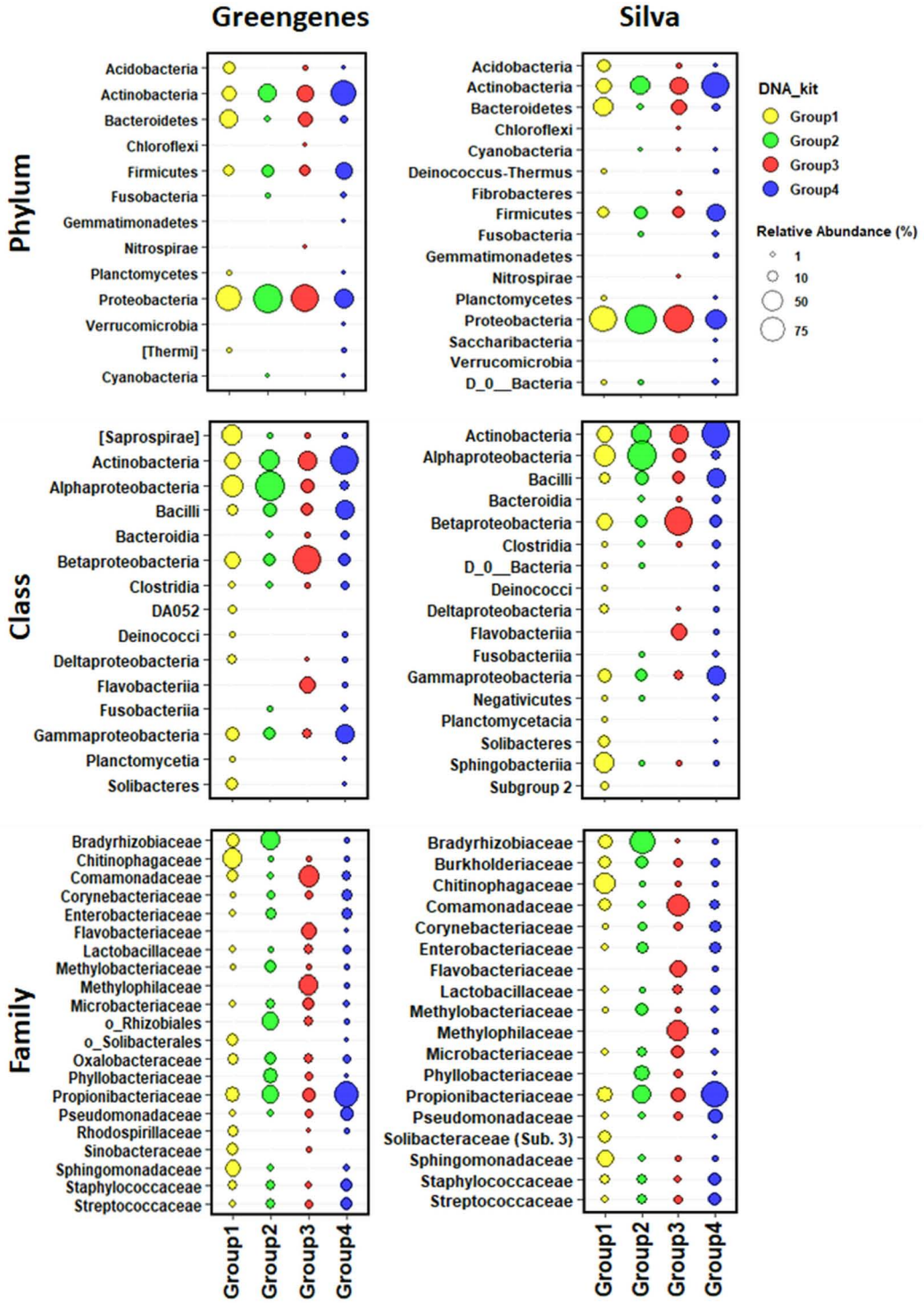


FIGURE 3 | Taxonomic bubble plot of olive xylem bacteria relative abundance at phylum, class, and family level present in four groups of DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Circle sizes represent the relative abundance, and colors indicate DNA extraction kit groups shown in **Figure 2**. Only abundances greater than 1% are shown.

the identified microbial communities and if this can be affected by the PCR protocol and the reference database used for taxonomic assignment (Greengenes 13-8 and Silva 132).

We found a very small background amplification of other OTUs or genera other than the eight expected to be present in the ZYMO mock community. Background amplification was

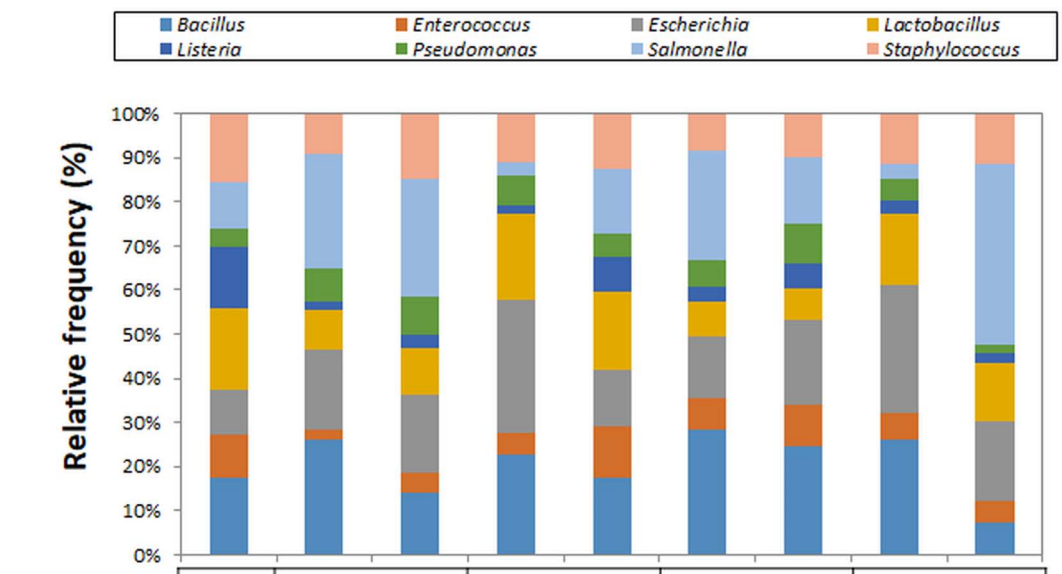
observed on all samples and PCRs when Silva database was used and represented about 9.8–32.6% of reads. Among the background bacteria amplified, unidentified OTUs belonging to the Class Bacilli, the Order Lactobacillales, and the genus *Granulicatella* were detected, although most of the "contaminant" reads were assigned only as Bacteria and could not be assigned taxonomically to any phylum. On the other hand, when using Greengenes database, background amplification was detected only on PCR2 and N1PCR1 but represented less than 2% of reads, with only the genus *Granulicatella* being detected.

All eight expected bacterial genera were detected by the four PCR protocols used regardless of the reference database used (**Figure 4**). However, there was a large effect on accuracy of genus relative abundance estimation depending on the PCR and the reference database used. Thus, only when using primer pair 799F/1193R (PCR3) and when performing the taxonomic assignment with the Silva 132 database was a significant correlation (Spearman coefficient 0.714, $P = 0.046$) found between the theoretical and the estimated bacterial community composition (**Figure 4**). Consequently, we decided to select the Silva database for taxonomic assignment for further analysis.

Effect of Primer Pairs on Xylem Sap Bacterial Community Assessment of Two Olive Cultivars

For the bacterial community analyses of the 14 samples from "Picual" and "Arbequina" olive trees, after screening our data for poor-quality sequences and removing chimeras and unassigned reads, we recovered a total of 86,489, 54,143, 44,444, and 33,261 high-quality paired-end sequences with the PCR2, PCR3, N1PCR1, and N2PCR1 protocols, respectively; with an average of 6,178, 3,867, 3,175, and 2,376 sequences per PCR, respectively (**Table 4**). The number of unassigned reads was lower (<3%) for the two direct PCRs (PCR2 and PCR3) as compared to the two nested PCRs (>6.5%). However, and only for primers 967F-1193R (PCR2), a high percentage of reads were of plant origin, whereas no such amplification occurred with the other PCR protocols (**Table 4**).

For Richness alpha diversity index, we found a significant effect for the PCR protocol ($P < 0.001$) and no effect for olive genotype ($P = 0.537$). For Shannon diversity index, no significant effect was found for any of the factors ($P > 0.554$) (**Figure 5**). In general, both nested PCR approaches showed lower values of alpha diversity indexes (**Figure 5**). To determine whether the choice of PCR approach (primer pair) influenced microbial community composition, we calculated two beta diversity metrics (Bray–Curtis and weighted UniFrac) and included the PCR protocol, olive genotype, and the interaction PCR protocol × olive genotype as explanatory variables in PERMANOVA models. In these analyses, microbial beta diversity estimates were significantly influenced by the PCR protocol (Bray–Curtis: pseudo-$F = 22.12$, $P < 0.001$; weighted-UniFrac: pseudo-$F = 11.96$, $P < 0.001$), as well by the interaction PCR protocol × genotype (Bray–Curtis: pseudo-$F = 10.22$, $P < 0.001$; weighted-UniFrac: pseudo-$F = 5.93$, $P < 0.001$), but not by



	ZMCS	PCR2		PCR3		N1PCR1		N2PCR1	
	Theoret.	gg	silva	gg	silva	gg	silva	gg	silva
Spearman's Rho	1	0.333	0.143	0.286	**0.714**	0.357	0.024	0.31	0.333
P-value	NA	0.420	0.734	0.493	**0.046**	0.385	0.955	0.456	0.420

FIGURE 4 | Relative abundance of ZymoBIOMICS Microbial Community Standard (ZMCS) composition according to the PCR primer pairs tested and the database used for taxonomic assignment [Greengenes_13-8 (gg) and Silva_132 (silva)]. Comparison with the theoretical abundances of ZMCS was based on Spearman correlation coefficient.

TABLE 4 | Number of reads and OTUs derived from NGS analysis of xylem sap microbiota from DNA samples extracted with the PowerPlant protocol, amplified with four PCR protocols, and taxonomic assignment using the Silva 132 reference database.

Reads/OTUs	PCR-2	PCR-3	N1PCR1*	N2PCR1*
	967F-1193R	799F-1193R	799F-1193R	967F-1391R
Number of reads (Mean/sample)	6178	3867	3175	2376
Unassigned Reads (%)	2.06	2.89	12.09	6.58
Plant (chloroplast + mitochondria) reads (%)	56.75	0	0	0
Bacterial reads (%)	41.19	97.11	87.91	93.42
OTUs at the Phylum level	13	10	8	7
OTUs at the Class level	27	17	13	13
OTUs at the Order level	52	47	39	39
OTUs at the Family level	94	93	68	70
OTUs at the Genus level	154	162	100	107
Total Assigned OTUs	233	242	141	156

N1PCR1 and N2PCR1 were nested PCRs that used as DNA template the amplification product obtained with primers 799F + 1193R (PCR1) (Table 2**).*
NGS, next-generation sequencing; OTU, operational taxonomic unit.

the olive genotype (Bray–Curtis: pseudo-F = 0.53, P = 0.90; weighted-UniFrac: pseudo-F = 1.61, P = 0.118) on microbial beta diversity estimates (**Figure 6**). N1PCR1, which is a nested PCR that uses in the second round of PCR the same primers as those used in PCR3, clustered and grouped together and closer to samples amplified with this later PCR both in the hierarchical cluster analysis (**Figure 6A**) and in the PCoA using Bray–Curtis distance (**Figure 6B**). According to the phylogenetic distances among the bacterial communities amplified by each PCR protocol, primers 967F-1193R (PCR2) showed a more distinct bacterial community composition as compared to PCR3, N1PCR1, and N2PCR1 that tended to overlap (**Figure 6B**).

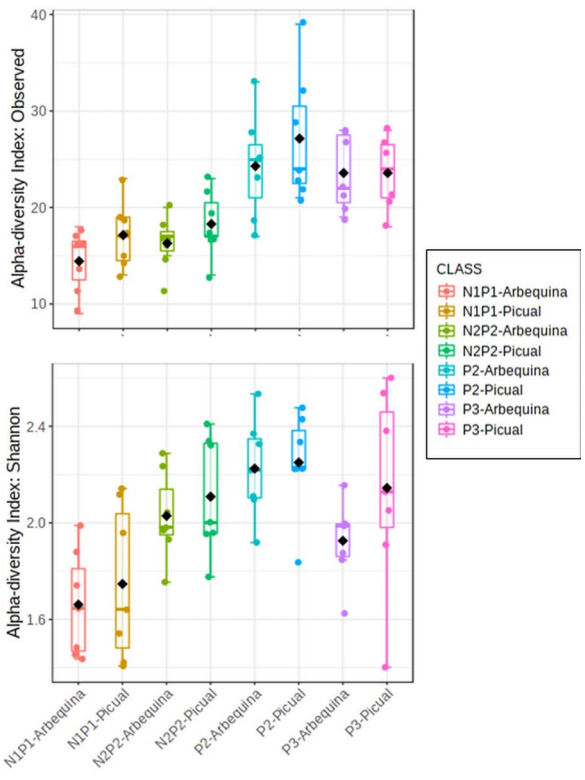


FIGURE 5 | Boxplots of Richness and Shannon alpha diversity indices of xylem bacterial communities from "Picual" and "Arbequina" olive cultivars at operational taxonomic unit (OTU) taxonomic level determined by using different PCR primer pairs. Boxes represent the interquartile range, while the black dots inside the box define the median, and whiskers represent the lowest and highest values. *P* value was calculated using general linear modeling (GLM).

The differences on alpha and beta diversity indexes found among PCR protocols were due to differences on both the number of bacterial taxa identified and their abundances. In fact, a total of 8–13 phyla, 13–27 classes, 39–52 orders, 68–94 families, 100–162 genera, and 141–242 species were identified depending of the PCR protocol (**Table 4** and **Supplementary Figure 3**). The core number of taxa detected by all the PCR protocols included six phyla, nine classes, 28 orders, 48 families, and 50 genera. The lowest numbers of all bacterial taxa were obtained for both nested PCR protocols, whereas the highest numbers of phyla, classes, and orders were detected with primers 967F-1193R (PCR2), and those of genera and species were obtained with primers 799F-1193R (PCR3). In all cases, the highest numbers of unique and shared taxa occurred for both direct PCR protocols (PCR2 and PCR3) (**Table 4** and **Supplementary Figure 3**).

In general, the bacterial taxa with higher abundance were detected by all the four PCR protocols (**Figure 7** and **Supplementary Figure 3**). At phylum level, Actinobacteria, Proteobacteria, Firmicutes, and Deinococcus-Thermus represented 51.9%, 29.0%, 16.8%, and 1.2%, respectively. Six bacterial classes were detected by all PCR protocols, with Actinobacteria, Gammaproteobacteria, Bacilli, Alphaproteobacteria, Clostridia, and Deinococci being the most abundant, representing 51.6%, 23.4%, 15.6%, 5.5%, 1.2%, and 1.2%, respectively (**Figure 7** and **Supplementary Figure 3**). Fourteen families were detected by all PCR protocols, with Propionibacteriaceae, Staphylococcaceae, Pseudomonadaceae, Burkholderiaceae, Enterobacteriaceae, Sphingomonadaceae, Corynebacteriaceae, Microbacteriaceae being the most abundant

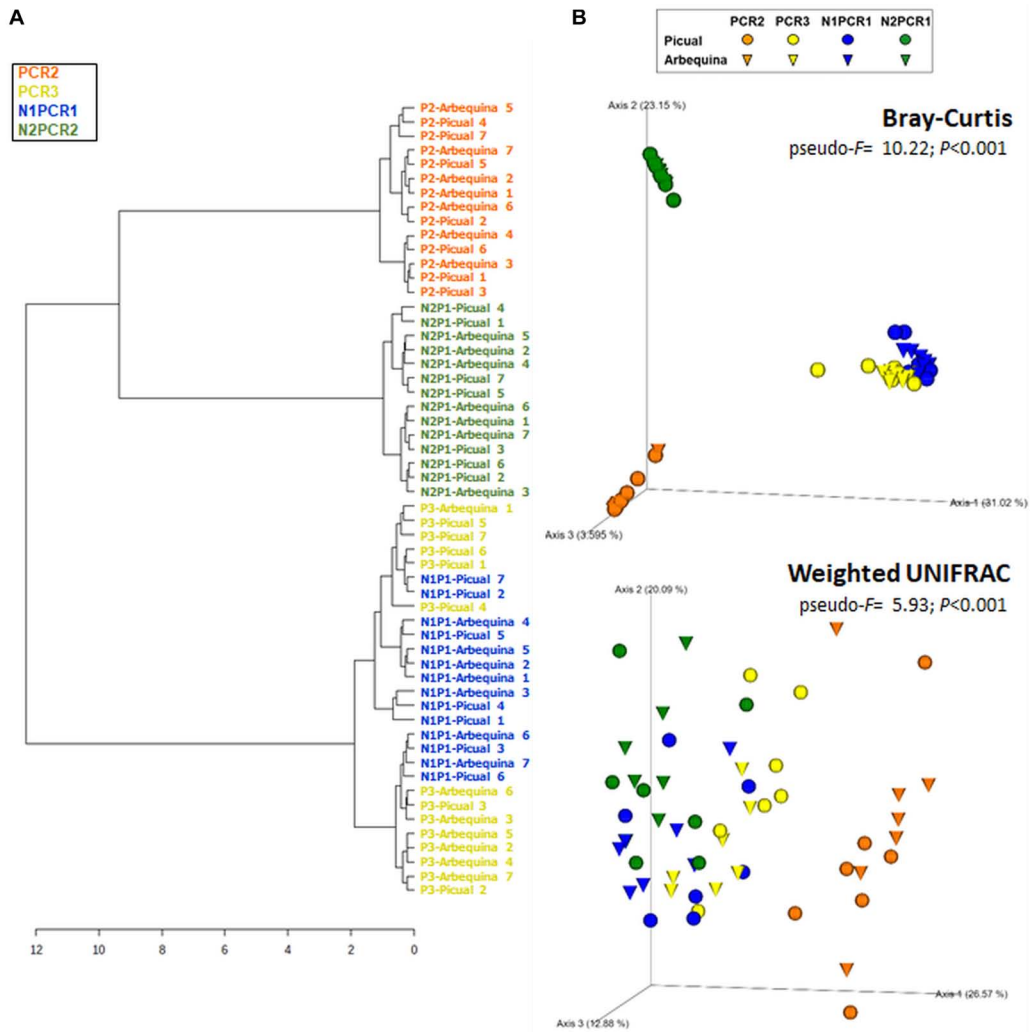


FIGURE 6 | Hierarchical clustering dendrogram analysis using Ward method and Bray–Curtis distance **(A)** And principal coordinate analysis (PCoA) of weighted UniFrac and Bray–Curtis distances **(B)** Of xylem bacterial communities from "Picual" and "Arbequina" olive cultivars obtained by using different PCR primer pairs. PERMANOVA (999 permutations; $P < 0.05$) was performed to test significant differences according to PCR primers used and olive genotypes.

(mean frequency ranged between 42.4 and 3.8%, in that order) (**Figure 7** and **Supplementary Figure 3**).

Finally, at the genus level, 50 genera comprised the core bacteria, where the highest numbers of genera were detected by direct PCR primers (154 in PCR2 and 162 in PCR3), while the lowest numbers were obtained for both nested PCR protocols (100 in N1PCR1 and 107 in N2PCR1). Direct PCR protocols showed the same unique number of genera (51 each one), while N2PCR1 displayed nine unique genera. No unique genera were found in N1PCR1 (**Table 4** and **Supplementary Figure 3**). Differential abundance analysis using DESeq2 displayed significant differences among genera between each of the PCR primer pairs tested when compared to the selected PCR3 protocol. Thus, DESeq2 identified a significant and high enrichment (Log2 Fold Change >5) of *Faecalibacterium*, *Prevotella*, *Geodermatophilus*, and *Frigoribacterium* in PCR2, *Rhizobium*, *Enterobacter*, *Granulicatella*, and *Brevibacterium* in N1PCR1, and *Brevundimonas*, *Staphylococcus*, *Prevotella*, and *Dermacoccus* in N2PCR2 (**Figure 8**). Interestingly, *Enterobacter*, *Granulicatella*, *Prevotella*, and *Brevibacterium* presented a significant higher abundance in all PCR protocols when comparing to PCR3, while *Pseudomonas* and *Pectobacterium* displayed an opposite behavior (**Figure 8**).

DISCUSSION

Although assessment and optimization of different DNA extraction protocols should be one of the most important initial steps when developing a protocol for analysis of microbial communities in a new plant niche due to its potential significant influence on the structure and diversity of the recovered community profile (Rubin et al., 2014), for all the studies assessing olive microbiota, this assessment is lacking.

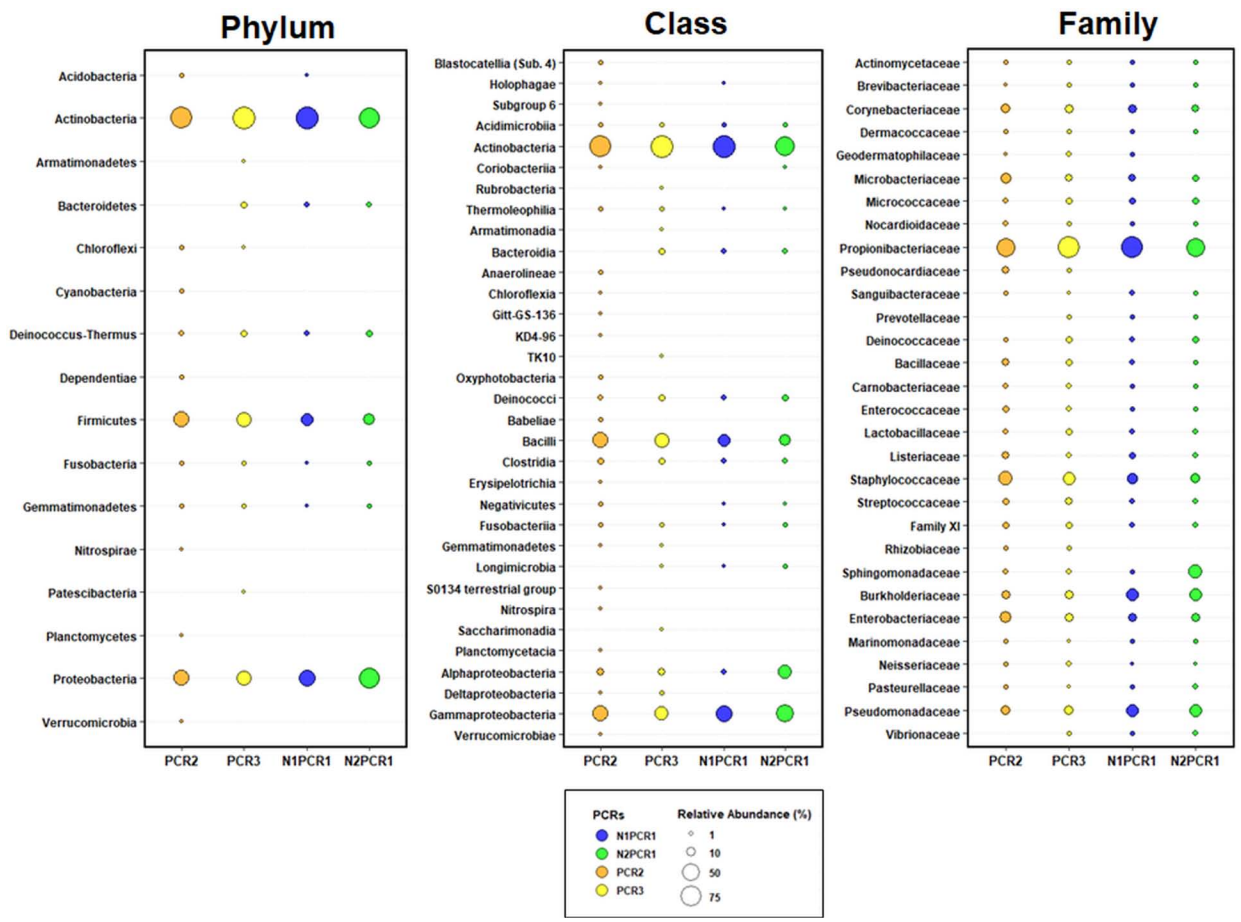


FIGURE 7 | Taxonomic bubble plot of bacterial relative abundance at phylum, class, and family level present in different PCR primer pair combinations. Circle sizes represent the relative abundance, and colors indicate PCR primer pairs used. All phyla and classes were shown, while only the most abundant bacteria (98% of reads) at family level were represented. Horizontal lines indicate the taxonomy lineage from each bacterial family.

Thus, although several studies have described olive endophytic microorganisms either after extraction from xylem woody chips (e.g., Keykhasaber et al., 2017; Anguita-Maeso et al., 2020; Giampetruzzi et al., 2020; Vergine et al., 2020) or from xylem sap when using a pressure chamber (Fausto et al., 2018; Sofo et al., 2019; Anguita-Maeso et al., 2020), none of those studies have examined the influence of DNA extraction kits or choice of primer pairs on microbiota characterization, which might be essential to avoid possible bias on the results.

Nowadays, a wide range of commercial and ready-to-use DNA extraction kits are available from global life science companies where cell lysis, washing, and DNA capture are considered general steps among all of them. Aside from these similarities in the protocols, the commercial kits have some differences within these steps that included a distinct cell lysis procedure based on chemical lysis and/or mechanical cell disruption with bead beating and on the DNA capture, which is based on either a silica matrix in the presence of a high concentration of salt solution or the use of magnetic beads (Burbach et al., 2016). Additionally, the bead beating type (e.g., glass, garnet, ceramic, etc.) used in cell disruption must be considered in the DNA extraction procedure (Knudsen et al., 2016). In general, the vast majority of commercial DNA extraction kits include chemical and mechanical lysis methods together with silica membrane-based columns for DNA capture (e.g., DNeasy PowerPlant Pro, DNeasy PowerLyzer PowerSoil, or PowerSoil® DNA Isolation). The 11 commercial kits tested in our study included all of the abovementioned variables.

Several studies have shown the effects of using different commercial kits for DNA extraction in NGS studies (e.g., Henderson et al., 2013; Kennedy et al., 2014; Brooks et al., 2015; Fouhy et al., 2015; Teng et al., 2018). However, according to our knowledge, this is the first study that compares such a high number of kits. Interestingly, we found that seven DNA extraction kits from five trademarks (Group 4) differing in the lysis procedure, the inclusion or not of a bead beating step, and the capture of DNA (magnetic beads or membrane-based) provided a similar description of the xylem bacterial communities (**Figure 2**). The significant differences shown by the CTAB procedure (Group 1) and the Canvax (Group 2) and Norgen Biotech (Group 3) kits may be explained by differences in the lysis and purification buffer used (CTAB)

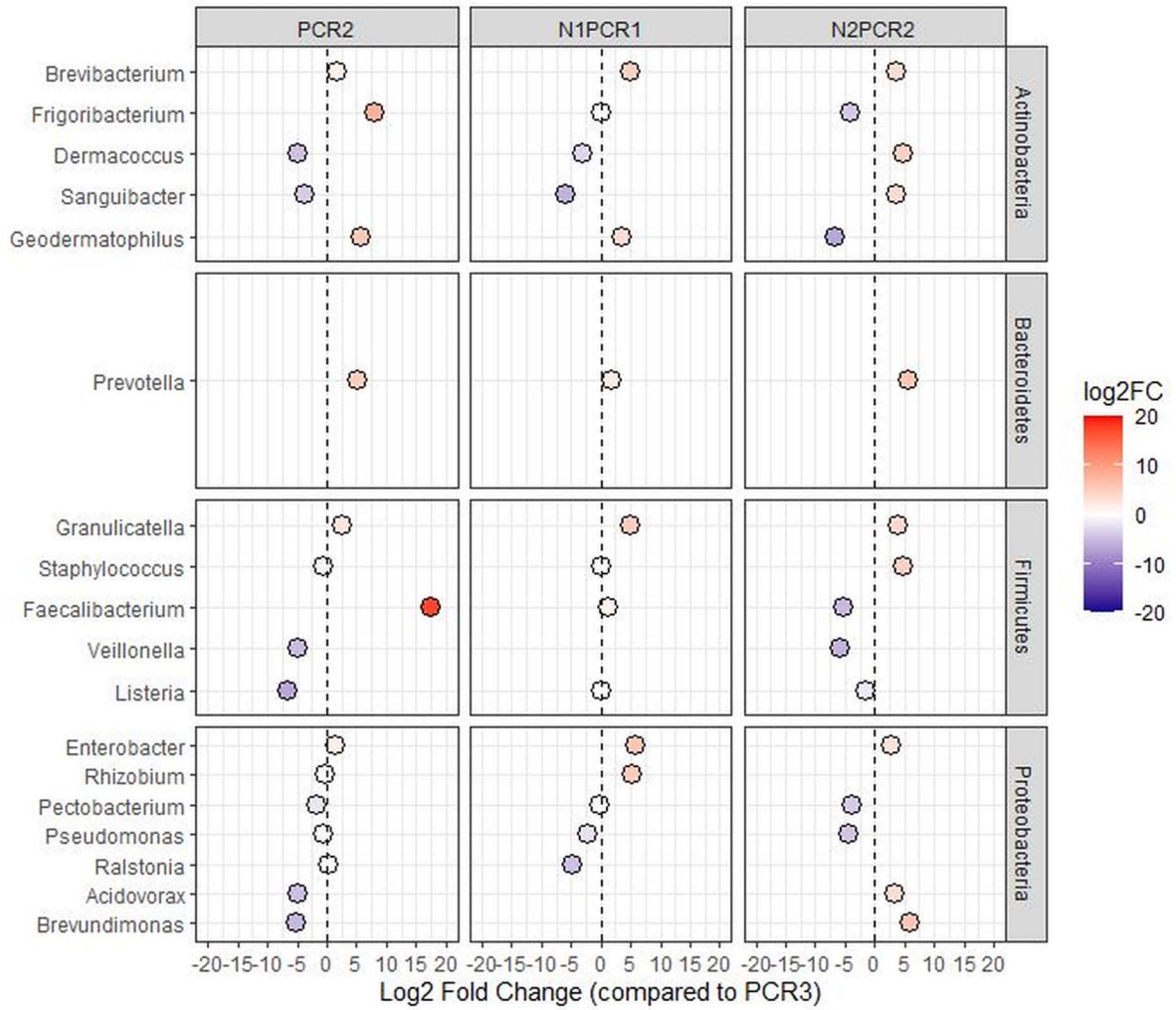


FIGURE 8 | DESeq2 analysis of differentially enriched bacterial genera when using different PCR protocols. PCR2, N1PCR1, and N1PCR1 were compared against PCR3. The color scale bar indicates log2 fold change. Only significant genera ($P < 0.01$) are shown.

or the inclusion of a preheating treatment (Groups 2 and 3) that can affect differentially bacterial cell wall lysis (Teng et al., 2018). After performing each DNA extraction protocol, we showed that the different DNA extraction methods led to various levels of DNA quantity and quality. However, higher DNA yields were not necessarily correlated with a better amplification efficiency (**Table 1**). From the 12 DNA kits assayed, we found PowerPlant as the most suitable DNA extraction kit for the characterization of olive xylem sap microbiota due to the quality and concentration of DNA obtained, it is a short time-consuming procedure, the market price is within the lowest, and the high percentage (92%) of sequences assigned to bacteria. These results are in line with other works that support its usefulness for subsequent microbiota analysis (Corcoll et al., 2017). However, PowerSoil kit also showed good characteristics as those shown by the PowerPlant kit, although the time for extraction is a little bit longer. Consequently, it could also be considered useful for the analysis of olive microbiota, especially on studies in which other plant niches such as the rhizosphere may also be explored. In fact, this kit has gained special interest as the standard technique for extracting microbial DNA from environmental samples, including two of the largest microbiome initiatives, the Earth Microbiome Project and the Human Microbiome Project (Rubin et al., 2014).

Since bacterial 16S rRNA gene sequences present a high homology with chloroplast and mitochondrial rRNA genes (Sakai et al., 2004), the discrimination between host plant DNA, organellar rDNA, and microbial 16S rDNA suppose an enormous challenge for the application of PCR-based methods in the study of plant microbiota (Beckers et al., 2016; Jackrel et al., 2017). Therefore, we determined the effect of primer pairs on xylem sap bacterial community characterization when using a mock- and an indigenous-microbial community extracted from two olive cultivars. The primers selected for our study had been reported to reduce co-amplification or

organellar sequences being widely and commonly used for the analysis of plant-associated bacterial communities using Illumina MiSeq sequencing. The ZymoBIOMICS Microbial Community Standard, designed to assess bias and errors in DNA extraction protocols and to improve the quality and reproducibility of metagenomics analyses, was used to determine the efficacy of four primer pairs targeting different hypervariable regions of 16S rRNA gene for giving an accurate representation of the xylem microbial communities. This validation procedure has been used by other authors (e.g., McGovern et al., 2018; Neuberger-Castillo et al., 2020). The ZymoBIOMICS Microbial Community Standard offers a well-defined composition and an accurately characterized mock community consisting of three Gram-negative and five Gram-positive bacteria, easy and tough to lyse, respectively, and two tough-to-lyse yeasts with varying sizes and cell wall composition. This wide range of organisms with different properties enables characterization, optimization, and validation of different lysis methods and PCR amplification protocols, and it can guide construction of entire workflows being used as a routine quality control. This mock microbial DNA community standard allowed the identification of significant differences among the different primer pairs used in the study, with PCR3 (799F/1193R) as the most accurate primer pair in combination with the Silva 132 database for taxonomic assignment when comparing results with the theoretical composition of ZymoBIOMICS (**Figure 3**). A significant influence of the choice of primer pairs using mock communities has also been shown in other studies (Fouhy et al., 2015; Teng et al., 2018). These results emphasize the need for testing several primers to use standardized approaches to analyze microbial communities, especially when exploring new plant niches.

When using natural olive samples from "Arbequina" and "Picual" cultivars and comparing the four primer pairs, results indicated that primer pair 799F-1193R recovered the highest number of bacterial OTUs (242) and displayed a low co-amplification rate of organellar rRNA gene, although for several of the samples, a removal of unspecific bands by agarose gel purification was needed prior to library sequencing. The Actinobacteria, Firmicutes, and Proteobacteria phyla have been described as the most abundant in olive xylem sap by other authors (Sofo et al., 2019; Anguita-Maeso et al., 2020). In our study, although the same most abundant phyla were detected by all PCRs, some phyla were detected exclusively by some PCR primers. For instance, PCR2 was the only one detecting Dependentiae, Verrucomicrobia, Nitrospirae, Planctomycetes, and Cyanobacteria, whereas the Armatimonadetes and Patescibacteria phyla were exclusively detected by PCR3. At lower taxonomic level, 48 families formed the core microbiota among all PCR primer pairs tested. Among these, the families Propionibacteriaceae, Staphylococcaceae, Sphingomonadaceae, Burkholderiaceae, Enterobacteriaceae, and Pseudomonadaceae were the most predominant, agreeing with the results obtained in olive by other authors (Müller et al., 2015; Fausto et al., 2018; Sofo et al., 2019; Anguita-Maeso et al., 2020; Giampetruzzi et al., 2020). However, the relative abundance of each family varied according to the PCR primer pairs used. Thus, PCR3 and N1PCR1 detected higher abundance of Propionibacteriaceae in comparison with PCR2 and N2PCR1. On the other hand, the families Staphylococcaceae and Enterobacteriaceae were detected at higher frequencies with PCR2, whereas the relative amount of Sphingomonadaceae was higher when using N2PCR1. Similarly, the families Burkholderiaceae and Pseudomonadaceae presented higher relative abundance when using nested PCR primers instead of direct PCRs. These differences obtained in bacterial community structure depending on the target primer set used may be due to the different hypervariable regions of 16S rRNA gene targeted (**Table 2**) or a disparity of matching efficiencies between primers for some microbial groups as has been found in other studies (Cruaud et al., 2014). The differences obtained in the nested PCRs as compared to the direct PCR approaches can be due to the introduction of a bias during amplification for the most abundant bacterial families, as it has been recently shown (Yu et al., 2015). Consequently, this approach should be used only when the amount of DNA is too low to be amplified by standard PCR to avoid potential biases measuring the bacterial community structure.

The tendency to find a low number of taxa in nested PCRs when compared to direct PCR protocols was also observed at the genus level. Although 50 genera composed the core bacterial microbiota, we found different unique genera depending on the PCR protocol. Interestingly, a high number of unique genera were found in PCR2 and PCR3 (51 each one), whereas N2PCR1 showed nine exclusive genera and no one was found unique in N1PCR1. Differential abundance analysis showed distinct enrichment of some genera based on the PCR primer pairs used. In such a way, when comparing PCR3 against the other three PCR protocols, we observed a significant enrichment of *Faecalibacterium*, *Prevotella*, *Geodermatophilus*, and *Frigoribacterium* in PCR2; *Rhizobium*, *Enterobacter*, *Granulicatella*, and *Brevibacterium* in N1PCR1, and *Brevundimonas*, *Staphylococcus*, *Prevotella*, and *Dermacoccus* in N2PCR2. Within this comparison, *Faecalibacterium*, *Enterobacter*, and *Brevundimonas* in PCR2, N1PCR1, and N2PCR2, respectively, displayed the greatest values of enrichment. These genera have been detected in other plant niches previously such as in rhizosphere or phyllosphere (Teixeira et al., 2010; Compant et al., 2019). Among them, *Brevundimonas* has been already described to confer fitness advantages to host plants due to its potential to act as a soil bioremediator and plant growth promoter (Kumar and Gera, 2014; Singh et al., 2016), although its use as a biological control agent against plant diseases is compromised due to the human pathogenic activity presented by some members of this genus (Ryan and Pembroke, 2018). On the other hand, we observed differences in "Picual" and "Arbequina" olive genotypes where the phyla *Acidobacteria* and *Gemmatimonadetes* were only present in "Picual" cultivar. However, an in-depth study targeting a wide range of olive cultivars is needed to better understand the effect of olive genotype in shaping the xylem microbiome.

Our study demonstrates significant and noticeable differences among DNA extraction kits and PCR primers that influence

the interpretation of the bacterial community composition of olive xylem sap. Overall, our findings provide new insights and an integrated assessment of both the benefits and drawbacks of several commercially available DNA extraction kits and offer guidance to other researchers in the choice of best-suited kits, considering cell lysis efficacy, DNA yield, microbial diversity recovered, processing time, and cost-effectiveness. Also, this study highlights the crucial choice of a good primer set to provide a non-biased vision of the true composition of the analyzed microbial community avoiding the co-amplification of plant organellar rRNA genes. Our results offered the road map to design an optimized strategy for selecting the most suitable PCR primer pair for assessing bacterial communities based on the use of an artificial commercially available mock community together with a precise and accurate bioinformatic workflow that can be followed when optimizing protocols for accurate depiction of the bacterial communities present in xylem vessels or other plant niches.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, PRJNA684121.

AUTHOR CONTRIBUTIONS

CH, MA-M, and BL conceived the research, performed statistical and bioinformatic analyses, interpreted the results, and wrote the manuscript. CH and MA-M prepared the materials and equipment and performed the experiments. MM and JN-C contributed to reviewing the manuscript and interpreted the results. All authors viewed the draft 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/fpls.2021.640829/full#supplementary-material

Supplementary Figure 1 | Richness rarefaction curves at OTU taxonomic level obtained when using different DNA extraction kits and after taxonomic assignments with the Greengenes_13-8 and Silva_132 databases. Error bars show the standard deviation.

Supplementary Figure 2 | Prevalence Venn diagram showing the unique and shared bacterial taxa at phylum, class, order, family, and genera level using the four clustered DNA extraction kits obtained shown in **Figure 2**. For each taxa, the venn diagram is shown using the Greengenes 13-8 and Silva_132 databases. Tables show the bacterial taxonomy interaction within each reference database.

Supplementary Figure 3 | Prevalence Venn diagram showing the unique and shared bacterial taxa obtained at phylum, class, order, family, and genera level using the four PCR protocols. Tables show the bacterial taxonomy interaction within each PCR protocol.

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frontiers in Plant Science

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Microbiome-Assisted Breeding to Understand Cultivar-Dependent Assembly in *Cucurbita pepo*

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Recently, it was shown that long-term plant breeding does not only shape plant characteristics but also impacts plant-associated microbiota substantially. This requires a microbiome-integrative breeding approach, which was not yet shown. Here we investigate this for the Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) by analyzing the microbiome of six genotypes (the complete pedigree of a three-way cross-hybrid, consisting of three inbred lines and one open pollinating cultivar) in the seed and rhizosphere as well as the progeny seeds. Using high-throughput amplicon sequencing targeting the 16S rRNA and the ITS1 genes, the bacterial and fungal microbiomes were accessed. Seeds were found to generally carry a significantly lower microbial diversity compared to the rhizosphere and soil as well as a different microbial composition, with an especially high fraction of *Enterobacteriaceae* (40–83%). Additionally, potential plant-beneficial bacterial taxa, including *Bacillaceae*, *Burkholderiaceae*, and *Pseudomonadaceae*, were found to be enriched in progeny seeds. Between genotypes, more substantial changes can be observed for seed microbiomes compared to the rhizosphere. Moreover, rhizosphere communities were assembled for the most part from soil. Interestingly, bacterial signatures are mainly linked from seed to seed, while fungal communities are shaped by the soil and rhizosphere. Our findings provide a deep look into the rhizosphere and seed microbiome assembly of pumpkin-associated communities and represent the first steps into microbiome-driven breeding for plant-beneficial microbes.

Keywords: pumpkin, *Cucurbitaceae*, plant–microbe interactions, 16S rRNA gene amplicons, ITS sequencing, microbiome transmission

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INTRODUCTION

Microbes play a key role in plant development and health throughout the whole life cycle (Mendes et al., 2011; Philippot et al., 2013; Vandenkoornhuyse et al., 2015). Therefore, it is important to identify all influencing factors as well as sources of the plant microbiota (Berg et al., 2016; Cordovez et al., 2019). The impact of plant genotype and soil quality on the diversity of the rhizosphere microbiome was studied for decades now (Smalla et al., 2001; Berg and Smalla, 2009; Lundberg et al., 2012). The influence of breeding on the microbiome, however, was just recently identified (Peiffer and Ley, 2013; Bouffaud et al., 2014; Cardinale et al., 2015). Another recent discovery

was shown for the seed microbiome; plant-associated microorganisms, including plant-beneficial microbes, are transferred *via* seed into the next generation (Johnston-Monje et al., 2016; Adam et al., 2018; Bergna et al., 2018). Interestingly, genotype-specific microbial communities are even more pronounced in seeds (Rybakova et al., 2017; Adam et al., 2018; Chen et al., 2020). Taken together, the knowledge of transferring core microbiomes from one generation to the other (Berg and Raaijmakers, 2018), which can be influenced by targeted breeding (Mendes et al., 2019), opens new possibilities for innovative plant breeding and protection strategies (Cordovez et al., 2019). They are urgently needed to ensure food security despite climate change, biodiversity loss, and emerging pathogens, but not yet exploited.

The Styrian oil pumpkin (*Cucurbita pepo* L. subsp. *pepo* var. *styriaca* Greb.) represents a relatively new *Cucurbitaceae* derived from the Austro-Hungarian Empire in the nineteenth century. In contrast to other *C. pepo* varieties, the Styrian oil pumpkin lacks lignification of the seed coat, which makes it suitable for oil extraction. The dark-green oil made from the seeds is traditionally consumed in Austria and found its way into international gourmet cuisines. The oil is rich in polyunsaturated fatty acids and contains vitamins, polyphenols, minerals, and phytosterols (Fruhwirth and Hermetter, 2007). The unique pumpkin cultivar is, with an acreage of over 35,000 ha, one of the mainly grown crops in southern Austria, and additional growing areas are established in Africa, China, and Eastern Europe (Estyria, 2018). However, the lack of lignification of the seed coat also leads to a high susceptibility to various fungal and bacterial diseases during seed germination (Heinisch and Ruthenberg, 1950). The seeding of untreated seeds frequently ends in huge losses due to seed infections; therefore, commercial seeds are treated with chemical strippers such as copper-based or synthetic fungicides. The Styrian oil pumpkin is a good model to study beneficial plant–microbe interactions during breeding because the breeding history is short and well known, seed and pumpkin microbiomes were already studied, and main pathogens causing dumping off, fruit rot, viruses, and leaf diseases are identified (Winkler et al., 2008; Grube et al., 2011; Bedlan, 2012; Pachner et al., 2015; Adam et al., 2018). Moreover, novel disease-resistant cultivars and potential key antagonists are important to reduce the dependence of pumpkin grown on chemicals.

Our study aims to disentangle the effect of genotypes on bacterial and fungal communities in seeds and in the rhizosphere of oilseed pumpkin. Therefore, we studied six genotypes (the complete pedigree of a three-way cross-hybrid, consisting of three inbred lines and one open pollinating cultivar) by analyzing 16S rRNA and ITS gene amplicon libraries. We assessed differences between genotypes in seed and rhizosphere and compared seeds sown and their progeny seeds to observe microbiome shifts induced by propagation. By tracking microbial transmission from seeds, soil, and rhizosphere of the different genotypes, the impact of breeding on seed colonization is evaluated, and new insights from a breeder's and microbiologist's perspective, respectively, are generated.

MATERIALS AND METHODS

Pumpkin Genotypes

For this study, the mainly used cultivar in organic farming ("GL Classic") and the most prevalent cultivar in conventional farming ("GL Rustikal") as well as its pedigree components were chosen. Seeds of inbred lines A, B, and D as well as cross-hybrids 'Gl. Diamant' (A × B), 'GL Rustikal' (AB × D), and the open pollinating cultivar 'GL Classic' were provided by Saatzucht Gleisdorf GmbH. Production of the seeds used in the experiments was done on two field sites in Gleisdorf (province of Styria, Austria) in 2014 (**Table 1**). Post-harvest processing in seed production was performed according to the standard procedures of the Saatzucht Gleisdorf GmbH breeding station by washing the seeds with water directly after harvest and drying them at a temperature of maximum 40°C down to a moisture content of 8%.

Field Experiments and Sampling Strategy

Seeds were planted in a field at the breeding station of Saatzucht Gleisdorf GmbH (47°06'55.1″ N 15°42'28.9″ E). A total of 40 seeds per genotype were coated with 0.3 g of the fungicide Maxim® XL (Syngenta) and split into four replicates. The soil of the field sites is described as gleyed loose brown earth, loamy silt, and cover loams on a quaternary terrace deficient in lime, with a pH value of 6.5. Rhizosphere samples were taken from four randomly chosen plants per replicate at 1 month after sowing (phenological growth stage inflorescence emergence, BBCH: 52). The soil around a plant was loosened with a spade, and the root system was exposed. Parts of the primary and secondary roots as well as of fine roots were pooled to one rhizosphere sample per plant. Additionally, four soil samples were also taken from the field site at random locations in the free place between the replicate plots with approximately 80 cm distance to the plants at the same depth where the main parts of the root system was located at that stage (5 to 15 cm). Five to 7 g of each rhizosphere and soil replicate was suspended in 50 ml 0.85% NaCl and homogenized by a 3-min bag mixer (stomacher) treatment; then, 4 ml of the homogenized solution was pelleted for 20 min at 4°C and 13,500 *g*.

Additionally, a total of 40 seeds of each genotype (original seeds sown) were washed five times and soaked in 25 ml sterile deionized water for 4 h at 100 rpm. Seeds were divided into four replicates (10 seeds each) and grounded with a pestle in 10 ml 0.85% NaCl in a sterile bag (Nasco Whirl-Pak®). A total of 3 ml suspension was pelleted as described above. The same procedure was performed with seeds harvested in the course of the field experiment (progeny seeds). Briefly, pumpkin fruits were washed and opened with a sterile knife. The seeds were extracted carefully using clean gloves, washed with water, and dried separately per fruit. Then, seeds of at least four fruits per replicate plot were pooled to a sample of 10 seeds per plot and processed as described above. A total of 76 samples [four replicates per sample type (seed, progeny seeds, and rhizosphere) and genotype ($N = 6$); four soil samples] were prepared for further analysis.

TABLE 1 | Characteristics of *Cucurbita pepo* genotypes selected for the microbiome analysis.

Denomination	Category	Pedigree	Field site origin of seeds
Line A	Inbred line	–	47°06′46.0″ N, 15°41′57.9″ E (Pfarrhoffeld)
Line B	Inbred line	–	47°06′46.0″ N, 15°41′57.9″ E (Pfarrhoffeld)
Line D	Inbred line	–	47°06′46.0″ N, 15°41′57.9″ E (Pfarrhoffeld)
Gl. Diamant	Single cross-hybrid	Line A × line B	47°07′03.6″ N, 15°42′22.8″ E (Teichacker)
GL Rustikal	Three-way cross-hybrid	Gl. Diamant × line D	47°07′03.6″ N, 15°42′22.8″ E (Teichacker)
GL Classic	Open-pollinated cultivar	–	47°06′46.0″ N, 15°41′57.9″ E (Pfarrhoffeld)

DNA Extraction and Amplicon Sequencing

Total DNA was extracted from all samples using FastDNA™ SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA, United States) with a slightly modified protocol. DNA samples were quality-checked using Nanodrop 2000 (Thermo Fisher Scientific, Wilmington, DE, United States) and stored at −20°C for further PCR reactions.

Using a targeted amplicon strategy, the bacterial and fungal microbiomes were assessed. The 515f/806r primer pair (515f: 5′-GTGYCAGCMGCCGCGGTAA-3′; 806r: 5′-GGACTACNVGGGTWTCTAAT-3′) targeting the 16S rRNA gene V4 hypervariable region (Caporaso et al., 2011; Parada et al., 2016) and the ITS1f/ITS2 primer pair (ITS1f: 5′-CTTGGTCATTTAGAGGAAGTAA-3′; ITS2r: 5′-GCTGCGTTCTTCATCGATGC-3′) targeting the ITS1 region (White et al., 1990) were used. All PCR reactions were performed in triplicate. The 16S rRNA gene amplification was performed in 30 cycles at 96°C denaturation for 60 s, 78°C peptide nucleic acid (PNA) annealing for 5 s, 54°C primer annealing for 60 s, and 74°C elongation for 60 s. Peptide nucleic acid PCR clamps were used to block the amplification of plastids and mitochondrial 16S rRNA gene sequences. For blocking of pumpkin DNA in ITS amplification, a customized PCR clamp was designed. ITS amplicons were performed in a two-step PCR approach. The first PCR step was performed using ITS primer with an attached linker (to attach barcodes). Following a 5-min initial denaturation at 30 cycles of 95°C denaturation for 30 s, 78°C PNA annealing for 5 s, 58°C primer annealing for 35 s, and 72°C elongation for 40 s were performed. The second PCR step was 15 cycles of 95°C for 30 s, 53°C for 30 s, and 72°C for 30 s using the first PCR as template and attaching individual barcode sequences for each sample. The PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, United States) and pooled in a 16S and ITS pool with equimolar concentrations. The barcoded Illumina libraries were sent for paired-end Illumina MiSeq sequencing (2 × 300 bp, GATC Biotech, Berlin, Germany). The obtained 16S rRNA and ITS amplicon raw reads were deposited at the European Nucleotide Archive under project number PRJEB41779.

Bioinformatic Data Processing

Quality-checked sequences were demultiplexed using cutadapt (Martin, 2011). The DADA2 algorithm in QIIME2 was used to generate representative sequences and a feature table (Callahan et al., 2016; Bolyen et al., 2019). Taxonomic classification was performed using vsearch algorithm and SILVA v132 and UNITE v8 databases as bacterial and fungal references (Kõljalg et al., 2013; Quast et al., 2013; Rideout et al., 2014; Nilsson et al., 2019). Non-target sequences (chloroplasts, mitochondria, and Archaea) were removed prior to further statistical analyses. The sourcetracker2 software was used to identify community linkage between the sample types of each genotype (Knights et al., 2011).

Statistical Analysis

R version 1.2.1335 (Allaire, 2012; R Core Team, 2013) was used to perform statistical analysis and create graphs unless stated otherwise. Feature table and taxonomic information were exported and further analyzed using the Phyloseq package (McMurdie and Holmes, 2013). Prior to the alpha and beta diversity analysis, each dataset was normalized to the lowest number of read counts by randomly selecting subsets of sequences (N = 485 and 300 for bacterial and fungal datasets, respectively). The alpha diversity was calculated using Shannon diversity index. Prior to analysis of variance (ANOVA), a normality test was performed using Shapiro test. If the data were not normally distributed, the non-parametric Kruskal–Wallis test was used instead of ANOVA. The beta diversity analysis was assessed using normalized Bray–Curtis dissimilarity matrix and then subjected to permutational analysis of variance (PERMANOVA, 999 permutations). Principal coordinate analysis plots were generated to visualize the clustering of bacterial and fungal communities according to sample type and genotype.

RESULTS

Bacterial and Fungal Diversity in Seeds, Soil, and Rhizosphere

After quality filtering and removal of non-target taxa (chloroplast, mitochondrial DNA, and archaeal sequences), a total of 14,259,303 and 591,998 high-quality reads were retained from bacterial and fungal datasets, respectively (**Supplementary Table 1**). By applying the DADA2 algorithm, the bacterial and fungal reads were clustered into 32,221 and 780 amplicon sequence variants, respectively. Analyzing the alpha diversity of seed, rhizosphere, soil, and progeny seed samples, overall major differences were observed. Soil generally carried the highest Shannon diversity index [mean H' = 6.0 (bacteria) and H' = 3.3 (fungi)], followed by rhizosphere samples [mean

H' = 5.3 (bacteria) and H' = 3.0 (fungi)]. Sown seed and progeny seed samples carried the lowest diversity [mean H' = 1.7 and 2.4 (bacteria) as well as H' = 2.0 and 2.1 (fungi)]. Overall significant differences ($p < 0.001$) were found for all sample types in bacterial diversity measures. Fungal diversities were different between belowground (rhizosphere and soil) and seed samples, however not within those groups (**Figures 1A,C**). Moreover, beta diversity analysis showed a clear clustering between seed samples, soil, and rhizosphere from both community datasets. PERMANOVA analysis demonstrated that sample type significantly affected bacterial and fungal community structure (p = 0.001). This factor explained 35 and 27% of bacterial and fungal community variation, respectively (**Figures 1B,D**).

After feature classification, the most abundant features (> 1% relative abundance) were visualized to reduce the complexity (**Figure 2**). Overall, a clear difference in obtained bacterial and fungal microbiomes was observed for all sample types. In general, *Proteobacteria* (67.9%) was the most predominant bacterial phylum, followed by *Firmicutes* (12.4%) and *Bacteroidetes* (6.5%). While seeds and progeny seeds were mainly colonized by *Enterobacteriaceae* (82.8 and 40.5%, respectively), the main observed families in the rhizosphere were *Burkholderiaceae* (16.4%), *Rhizobiaceae* (7.5%), and *Flavobacteriaceae* (6.5%). In the soil, generally, a higher diversity was observed, with numerous bacterial families (n = 25) distributed evenly in a range between 1 and 4% of total relative abundance. Taxa with a higher abundance were "unidentified Subgroup_6" (12.9%) and *Pyrinomonadaceae* (4.6%). A total of 43.6% of observed taxa were below 1% relative abundance and therefore classified as other (**Figure 2A**).

In the fungal dataset, *Ascomycota* and *Basidiomycota* were the dominant fungal phyla and accounted for 73.1 and 21.9% of total community, respectively. Seeds were mainly colonized by the families *Tremellaceae* (36.2%) and *Nectriaceae* (12.2%) and an unidentified *Sordariomycetes* taxon (15.1%). Progeny seeds were mainly colonized by unidentified *Sordariomycetes* taxa (28.1%), *Nectriaceae* (24%), and unidentified *Pleosporales* taxa (11.3%). The rhizosphere was mainly colonized by *Nectriaceae* (28%), unidentified *Sordariomycetes* (14.3%), and *Hypocreaceae* (6.8%). The soil showed a higher taxonomic diversity and was mainly colonized by an unidentified *Pleosporales* taxa (15.7%), *Nectriaceae* (15.4%), *Psathyrellaceae* (13.3%), and *Mortierellaceae* (7.7%) (**Figure 2B**).

Genotype-Specific Differences in Seeds, Rhizosphere, and Progeny Seeds

The genotype affected bacterial and fungal beta diversity, but it was not statistically significant for the alpha diversity in seeds sown. In general, GL Rustikal [(A × B) × D] carried a higher bacterial alpha diversity (H' = 2.1) compared to other genotypes (H' = 1.5–1.8). On the other hand, line B carried a higher fungal alpha diversity (H' = 2.4) compared to other genotypes (H' = 1.5–2.3). However, the differences were only statistically significant for bacteria (p = 0.039 and p = 0.221 for bacterial and fungal datasets, respectively). Beta diversity showed a significant

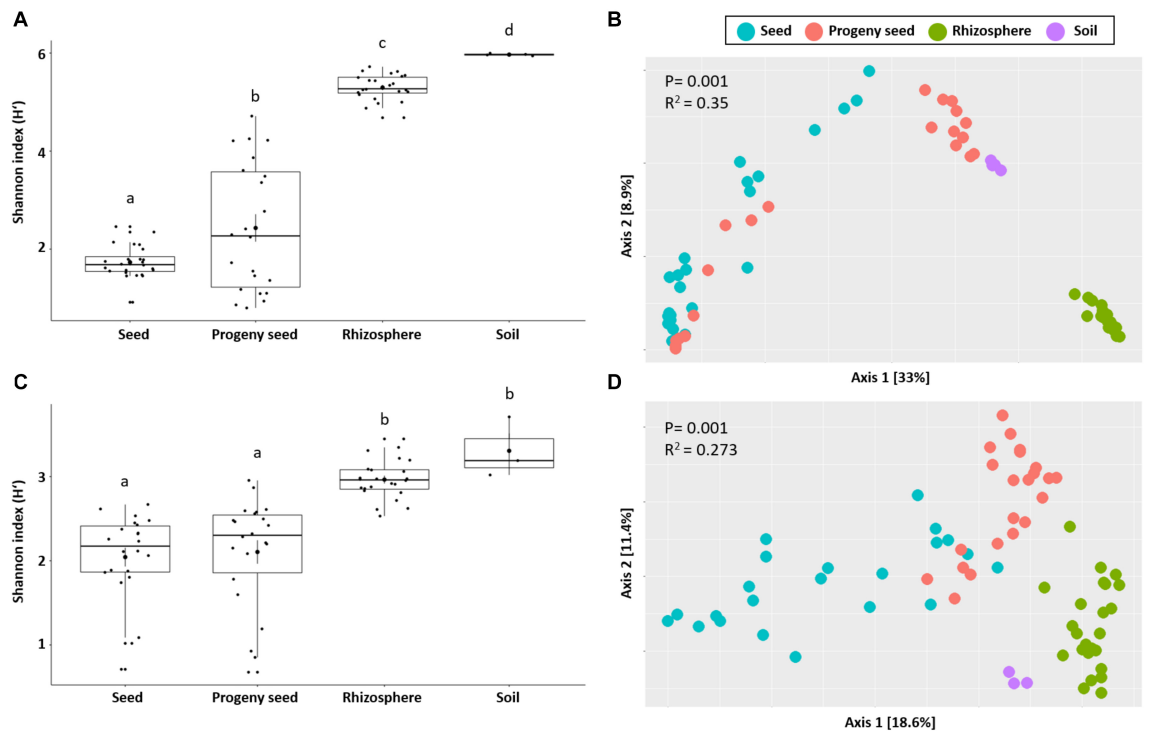

FIGURE 1 | Observed bacterial **(A,B)** and fungal **(C,D)** diversity in samples. Shannon index (alpha diversity) as well as beta diversity obtained by Bray–Curtis distance matrix is shown. Significant differences are indicated by different letters as well as given p-values.

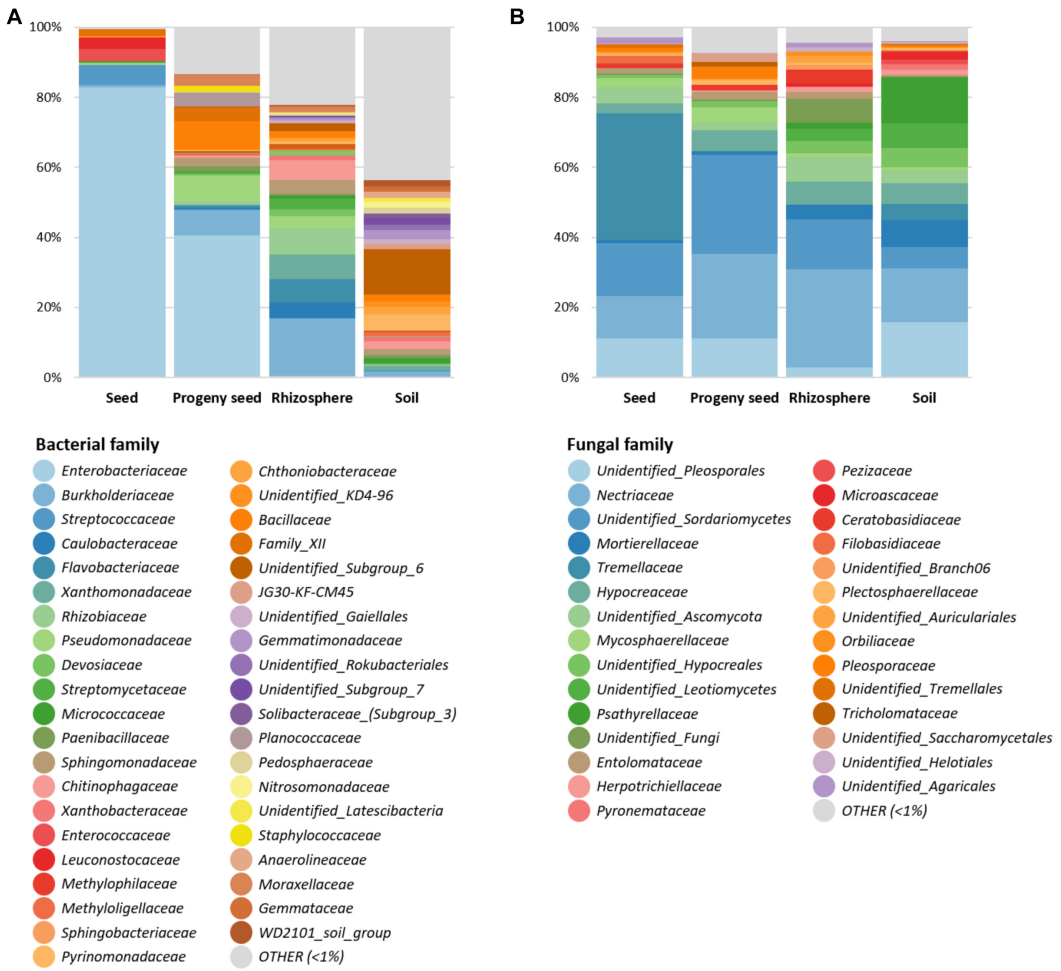

FIGURE 2 | Relative taxonomic composition of four sample types (seed, progeny seed, rhizosphere, and soil). Bacterial **(A)** and fungal **(B)** taxa on family level with abundance higher than 1% are shown individually. Low-abundance taxa are summarized as "OTHER."

clustering of samples in both the bacterial and fungal datasets (p = 0.001) (**Supplementary Figure 1**). The genotype effect explained 72 and 43% of variance between samples for bacteria and fungi, respectively. In addition, a separate beta diversity analysis indicated that the field origin of sown seeds also affected the bacterial and fungal community structures (p = 0.001). The field origin explained 17 and 19% of variance between samples for bacteria and fungi, respectively (**Supplementary Figure 2**). The soil analysis of those fields showed a slight variation in soil characteristics (**Supplementary Table 2**). The genotype effect was less pronounced for progeny seeds. No significant differences were found for alpha and beta diversity for both bacterial and fungal communities in progeny seed ($p > 0.05$). However, the genotype still explained 22 and 29% of diversity between samples for bacteria and fungi, respectively (**Supplementary Figure 3**).

The observed differences in diversity are also reflected in the found taxonomic compositions of the seeds and progeny seeds. In seeds sown, despite a generally high occurrence of *Enterobacteriaceae* as previously described, GL Rustikal [(A × B) × D] carried relatively higher proportions of *Streptococcaceae* (17.7%) and *Leuconostocaceae* (18.5%) in comparison to the other genotypes (0–9.2 and < 1.1%, respectively). Moreover, Gl. Diamant (A × B) carried a relatively higher abundance of *Enterococcaceae* (7.5%) compared to others (< 1.2%). The progeny seeds overall showed a higher taxonomic diversity. They were also colonized by a high abundance of *Enterobacteriaceae* except for line A, which showed mainly *Bacillaceae* (43.6%), *Family XII* (42%), and *Burkholderiaceae* (7.3%). Additionally, a higher proportion of *Pseudomonadaceae* was found in line B (13.6%) and Gl. Diamant (A × B) (19.4%) in comparison to others (< 6.2%). The families *Planococcaceae*, *Burkholderiaceae*, *Sphingomonadaceae*, and *Bacillaceae* were further also found in higher abundances in multiple genotypes (**Figures 3A,C**).

An analysis of the fungal taxonomic composition of sown seeds indicated that line A and GL Classic harbored a relatively higher *Tremellaceae* fraction (63 and 53%, respectively) in comparison to other pedigree lines (1.7–42.1%). In contrast, these two genotypes harbored a lower unidentified *Sordariomycetes* fraction (8.5 and 7.1%) in comparison to other genotypes

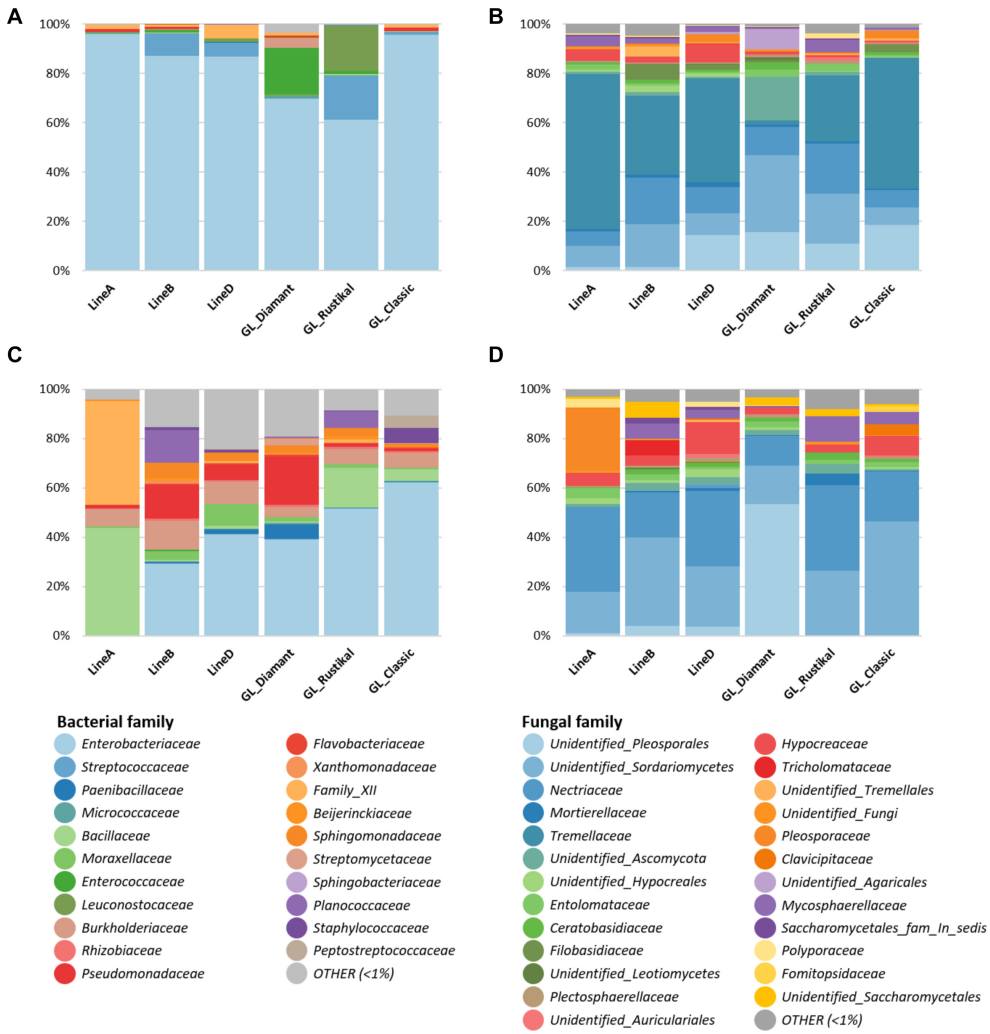

FIGURE 3 | Relative taxonomic composition of seeds from different genotypes [sown seeds **(A,B)** and progeny seeds **(C,D)**]. Bacterial **(A,C)** and fungal **(B,D)** taxa above 1% relative abundance on family level are shown. Low-abundance taxa (< 1%) are summarized as "OTHER."

(8.9–31%), of which the highest proportion of unidentified *Sordariomycetes* was detected in Gl. Diamant (A × B). Fungal families such as *Pleosporales* and *Nectriaceae* were further of higher abundance in all genotypes. In the fungal community of progeny seeds, Gl. Diamant (A × B) again showed a distinguishable microbial composition from the other cultivars, with a high proportion of unidentified *Pleosporales* (53.5%). Progeny seeds from GL Classic harbored a relatively higher unidentified *Sordariomycetes* (46.4%) in comparison to others (13.5–35.9%). Line A showed a relatively high proportion of *Pleosporaceae* (25.4%), which was not found in other genotypes. Additionally, *Nectriaceae* and *Hypocreaceae* were fungal taxa found in higher abundance in all progeny seeds (**Figures 3B,D**).

The rhizosphere microbiome of the different genotypes was, in contrast to the seeds, relatively uniform. The alpha bacterial and fungal diversities were not significantly different between genotypes (p = 0.566 and p = 0.679, respectively). Although the genotype explained 23% of both bacterial and fungal community variation, this factor did not significantly affect the bacterial and fungal beta diversity (p = 0.271 and p = 0.466, respectively). Moreover, a relatively similar bacterial and fungal taxonomic composition was observed in the rhizosphere samples, and only minor genotype effects are visible (**Supplementary Figure 4**).

Microbiome Assembly Along the Pedigree and in the Rhizosphere and Progeny Seeds

Using the sourcetracker2 software, microbiome similarities were further analyzed along the pedigree. Both the sown seeds and progeny seeds were analyzed (**Figure 4A**). In the sown seeds, high bacterial fractions (33–93%) were shared between the hybrid lines and their parental components. In contrast, fungal traces were shared to a lesser extent (7–19%). Interestingly, the assembly of the microbiome of GL Rustikal was more influenced by line D (86 and 19% for bacterial and fungal traces, respectively) as

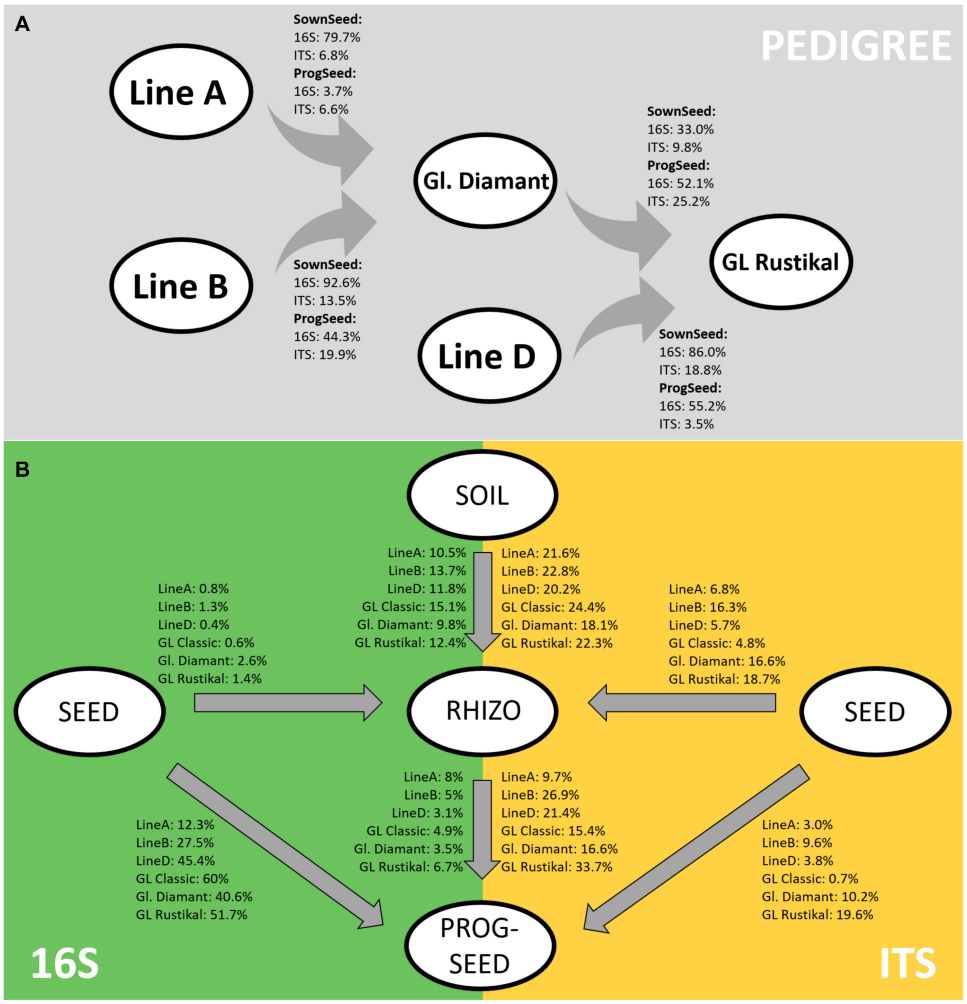

FIGURE 4 | Tracking of bacterial and fungal microbiomes across the pedigree in sown seeds and progeny seeds **(A)** as well as from sown seeds and soil to rhizosphere and progeny seeds **(B)** using sourcetracker2. Ident bacterial (16S) and fungal (ITS) traces are indicated as percentage.

by Gl. Diamant (33 and 10% for bacterial and fungal traces, respectively). Similarly, in the progeny seeds, higher fractions were generally shared in the bacterial microbiome, compared to the fungal fraction, even though fungi were shared by up to 25%. One exception was observed with line A, which did not share much with Gl. Diamant (3.7 and 6.6% for the bacterial and fungal fraction, respectively).

Moreover, the link of microbial communities between sown seeds to the rhizosphere and their progeny seeds was tracked. The highest percentage of bacterial linkage was found from sown seeds to progeny seeds, while fungi were mostly linked between soil and rhizosphere as well as rhizosphere and progeny seeds (**Figure 4B**). Rhizosphere microbiomes, even though the sampling time was at the inflorescence emergence stage, were mainly influenced by the soil (mean identity bacteria, 12.2%; mean identity fungi, 21.6%), while only minor fungal traits from the seed were found in the rhizosphere (mean bacteria, 1.2%; mean fungi, 11.5%). Interestingly, major differences of microbial community linkage between genotypes were observed. The main cultivar of organic Styrian oil pumpkin farming, GL Classic, showed the highest link of bacterial microbiome (60%) from sown seed to progeny seed, while almost no fungal traits were linked (0.7%). Especially the two cross-hybrids Gl. Diamant (A × B) and GL Rustikal [(A × B) × D] showed, in comparison to other genotypes, a higher linkage of fungal traits from sown seed to progeny seed [10.2% (Gl. Diamant) and 19.6% (GL Rustikal)]. Moreover, both showed, in addition to line B, a higher linkage of the fungal microbiome in sown seeds and the rhizosphere [16.6% (Gl. Diamant), 18.7% (GL Rustikal), and 16.3% (line B)]. Interestingly, the weakest link for bacterial and fungal strains from soil to rhizosphere (9.8 and 18.1%) was recorded for Gl. Diamant and the strongest link (15.1 and 24.4%) for GL Classic.

DISCUSSION

By performing an in-depth analysis of the bacterial and fungal communities associated with pumpkin plants throughout

their life cycle and comparing multiple related genotypes, overall a strong genotype specificity of seed-associated microbial communities was observed. Besides habitat type (seed, rhizosphere, and soil), the genotype was an influential factor for differences between samples. Moreover, in accordance to previous studies, the seed microbiome, in comparison to the rhizosphere and soil microbiomes, has a lower diversity for the bacterial and fungal communities, with the highest diversity generally found in soil (Bulgarelli et al., 2012; Adam et al., 2018). In the rhizosphere, plants attract soil microbes with the release of plant root exudates, which often belong to plant-beneficial bacteria (Berendsen et al., 2012). In our study, we showed a higher abundance of *Burkholderiaceae*, *Rhizobiaceae*, and *Flavobacteriaceae* in the rhizosphere, which are bacterial families associated with plants and often harbor beneficial traits (Soltani et al., 2010; Carrión et al., 2018; Harman and Uphoff, 2019). Interestingly, in comparison to seeds, no genotype-specific differences in colonization were observed in the rhizosphere, even though the sampling time was at a stage where the plant was almost fully developed (BBCH:52). This could be due to plants being in the exact same soil or indicate that all pumpkin genotypes investigated in this study release similar root exudates; however, further investigations are needed to fully answer this question.

Genotype-specific seed microbial communities were discussed before and found for multiple plant species (Rybakova et al., 2017; Adam et al., 2018; Chen et al., 2020). Plant seeds were moreover discussed as carriers of plant-beneficial bacteria to the next generation (Bergna et al., 2018). In the course of breeding, the genetic traits of plants are selected, which possibly also change the microbiome, which is why a genotype-specific seed and rhizosphere microbiome can be observed (Adam et al., 2018; Wassermann et al., 2019). In addition, our results show that the soil in which the plants are cultivated has a severe effect on the microbiome of the next generation of seeds. This is also shown as the field origin and soil characteristics of sown seeds were shown to have a substantial influence on the observed beta diversity in our experiment. In our experiment, all seeds were planted in the same field to exclude this factor; however, additional experiments using other soil characteristics (e.g., different pH) could give a better indication of the severe soil effect. Interestingly, in accordance to Bergna et al. (2018), a high fraction of the bacterial microbiome of the sown seeds is linked to the progeny seeds. However, when looking at the fungal microbiome, a different picture was observed. Nevertheless, to fully disentangle the vertical transmission of seed-associated microbes, sequencing resolution on strain level is necessary. While only a low fraction of bacteria found in progeny seeds was linked to the soil and rhizosphere, for fungi this was the strongest linkage. This observation could be due to the fungicide treatment of seeds prior to sowing. Therefore, the assembly of fungal rhizosphere communities was mainly shaped by the soil and, to a lesser extent, by seed communities. The fact that the linkage of soil to rhizosphere was weak for Gl. Diamant and strong for GL Classic might be the first hint to a genotype-specific susceptibility to soil pathogens of GL Classic. Gl. Diamant expresses a very high tolerance to fruit rot, whereas GL Classic is the most susceptible genotype in the present study. Gl. Diamant is a genotype carrying resistance genes against ZYMV and is expressing a very high tolerance to that virus (AGES, 2020). A link between tolerance to ZYMV and some general pathogen recognition genes is discussed already (Capuozzo et al., 2017).

The generally high abundance of *Enterobacteriaceae* in seeds (sown seeds and progeny seeds) was remarkable. However, this was already reported in our previous study on pumpkin seeds (Adam et al., 2018) and found for other plants, e.g., tobacco, as well (Chen et al., 2020). Apart from *Enterobacteriaceae*, progeny seeds harbored higher abundances of *Bacillaceae*, *Pseudomonadaceae*, *Burkholderiaceae*, and *Sphingomonadaceae*. These groups, however only found in low abundances in sown seeds, are possibly enriched by the plant over the growing process due to their plant-beneficial traits (Mendes et al., 2013; Mandic-Mulec et al., 2016; Carrión et al., 2018). For instance, *Burkholderiaceae* and *Pseudomonadaceae* are known for their bioactive metabolite production, which has anti-fungal activity and influences plant growth (Kai et al., 2007; Thomashow et al., 2019). Furthermore, *Burkholderiaceae* and *Sphingomonadaceae* were found associated to plants in disease-suppressive soils (Chapelle et al., 2016). Our data indicate that plants could enrich specific microbes in their next-generation seeds that may play important roles in supporting the health, growth, and fitness of their hosts.

No differences regarding microbial diversity could be found between the group of homozygous inbred lines (lines A, B, and D), the single cross-hybrid (Gl. Diamant—heterozygous), the three-way cross-hybrid (GL Rustikal—heterozygous), and the population cultivar (GL Classic—heterozygote genome in certain loci), indicating that homozygote lines do not suffer from inbreeding depression regarding their microbial diversity. Moreover, high microbial linkages between breeding lines and hybrids were observed. The breeding lines and their hybrids shared an especially high fraction of their bacterial microbiome. The fungal microbiomes, however, were generally shared to a lesser extent. As noted previously, the fungal traits found in progeny seeds are mainly linked to the soil and rhizosphere. This could explain the weak linkage between fungal traits in sown seeds along the pedigree line since seeds originated from different fields. In contrast, the progeny seeds showed, similarly to the sown seeds, a generally lower linkage of fungal compared to bacterial traits. Since those seeds were grown in the same field, this could indicate that the transmission of fungal traits to the next generation is generally less directed, but more data are needed to fully explain this. Nevertheless, knowledge of the microbial composition of an inbred line or hybrid might be of high interest in the future, especially if more knowledge about which strains, transmitted from one generation to the next generation, in seed propagation are beneficial and which strains are potential pathogens is available. It could be worth knowing if a line tends to transmit more pathogens to the next generation or tends to attract more beneficial strains from soil than another line.

CONCLUSION

The present study of pumpkin bacterial and fungal communities in seeds, rhizosphere, and progeny seeds showed a strong genotype specificity of bacteria in contrast to fungal communities in seeds. Bacterial traits are mainly linked from sown seeds to progeny seed, while fungal communities are mainly shaped by the soil and rhizosphere, which supports the understanding of seed microbiome assembly. Due to the selection of related pumpkin genotypes, our findings contribute to the new direction of microbiome-assisted plant breeding. With a targeted approach during plant breeding, not only desired plant traits but also healthy and plant-beneficial microbial communities on the seeds could be achieved.

DATA AVAILABILITY STATEMENT

All sequencing data supporting the findings of this study was submitted to the European Nucleotide Archive (ENA) and can be found under accession no. PRJEB41779 at www.ebi.ac.uk/ena.

AUTHOR CONTRIBUTIONS

EA, MB, HM, and GB designed the study. EA, MB, and HM performed the field experiments and prepared the samples for sequencing. PK, WW, and EO analyzed the data. PK, EA, WW, and GB wrote the manuscript. PK and EA contributed equally. All authors agreed on 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/fpls.2021.642027/full#supplementary-material

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Root Exudates Alter the Expression of Diverse Metabolic, Transport, Regulatory, and Stress Response Genes in Rhizosphere *Pseudomonas*

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Plants live in association with microorganisms that positively influence plant development, vigor, and fitness in response to pathogens and abiotic stressors. The bulk of the plant microbiome is concentrated belowground at the plant root-soil interface. Plant roots secrete carbon-rich rhizodeposits containing primary and secondary low molecular weight metabolites, lysates, and mucilages. These exudates provide nutrients for soil microorganisms and modulate their affinity to host plants, but molecular details of this process are largely unresolved. We addressed this gap by focusing on the molecular dialog between eight well-characterized beneficial strains of the *Pseudomonas fluorescens* group and *Brachypodium distachyon*, a model for economically important food, feed, forage, and biomass crops of the grass family. We collected and analyzed root exudates of *B. distachyon* and demonstrated the presence of multiple carbohydrates, amino acids, organic acids, and phenolic compounds. The subsequent screening of bacteria by Biolog Phenotype MicroArrays revealed that many of these metabolites provide carbon and energy for the *Pseudomonas* strains. RNA-seq profiling of bacterial cultures amended with root exudates revealed changes in the expression of genes encoding numerous catabolic and anabolic enzymes, transporters, transcriptional regulators, stress response, and conserved hypothetical proteins. Almost half of the differentially expressed genes mapped to the variable part of the strains' pangenome, reflecting the importance of the variable gene content in the adaptation of *P. fluorescens* to the rhizosphere lifestyle. Our results collectively reveal the diversity of cellular pathways and physiological responses underlying the establishment of mutualistic interactions between these beneficial rhizobacteria and their plant hosts.

Keywords: *Pseudomonas*, *Brachypodium*, rhizosphere, root exudates, transcriptome

INTRODUCTION

Plants are meta-organisms or holobionts that rely in part on their microbiome for specific functions and traits. The ability of the plant microbiome to influence plant development, vigor, health, and fitness in response to abiotic stressors associated with global climate change is documented by numerous studies (Lugtenberg and Kamilova, 2009). There is mounting evidence that plants actively recruit beneficial microbiomes, but many aspects of this process are still very much a black box (Reinhold-Hurek et al., 2015). The foundation for the differential affinity of rhizobacteria toward host plants is built upon complex chemical cross talk between microorganisms and plant roots. Up to 40% of photosynthetically fixed carbon is released by plant roots in the form of exudates and secretions, lysates, and mucilages (Curl and Truelove, 1986; Lynch, 1990; Whipps, 1990; Badri and Vivanco, 2009). The release of these compounds is actively controlled in response to environmental stimuli, and the composition of root exudates varies greatly according to plant species and physiological condition (Lynch, 1990; Nguyen, 2003; Phillips et al., 2004; De-la-Pena et al., 2008). The presence and composition of exudates strongly impact soil microorganisms, which is consistent with the idea that plants actively select and shape their root microbiota (Zolla et al., 2013).

Primary root exudates include simple and complex sugars, amino acids, polypeptides and proteins, organic, aliphatic and fatty acids, sterols, and phenolics (Nguyen, 2003; Badri and Vivanco, 2009; Badri et al., 2009). These compounds serve as carbon and energy sources for rhizobacteria, and the presence of the intact corresponding catabolic pathways is essential for competitive colonization of roots and disease suppression (Lugtenberg et al., 2001; Kamilova et al., 2005; Lugtenberg and Kamilova, 2009). Root exudates also contain numerous signal molecules and secondary metabolites, the significance of which is only now emerging (Walker et al., 2003; Bais et al., 2005, 2006). A handful of analyses of plant-induced gene expression by transcriptional profiling *in vitro* (Mark et al., 2005) or in the rhizosphere (Silby and Levy, 2004; Ramos-Gonzalez et al., 2005; Matilla et al., 2007; Barret et al., 2009) have identified multiple genes that are differentially regulated by exposure to roots or root exudates. Bacterial pathways expressed during rhizosphere colonization control utilization of plant-derived metabolites (Simons et al., 1996, 1997; Camacho-Carvajal, 2001; Lugtenberg and Kamilova, 2009), motility and chemotaxis (de Weert et al., 2002; Lugtenberg and Kamilova, 2009), phase variation (Dekkers et al., 1998; Sanchez-Contreras et al., 2002; van den Broek et al., 2005), outer membrane integrity (de Weert et al., 2006; Lugtenberg and Kamilova, 2009), and the ability to sequester limiting resources (Raaijmakers et al., 1995) and resist environmental stresses (Sarniguet et al., 1995; Miller and Wood, 1996; van Veen et al., 1997; Schnider-Keel et al., 2001). In its spatial and temporal properties, root colonization resembles biofilm formation, and biofilm-related pathways also have been implicated in adhesion to seeds and roots and rhizosphere colonization (Espinosa-Urgel et al., 2000; Hinsa et al., 2003; Yousef-Coronado et al., 2008; Fuqua, 2010; Martinez-Gil et al., 2010; Nielsen et al., 2011; Zboralski and Filion, 2020). Finally, root exudates strongly affect the expression of diverse plant growth promotion and biocontrol genes (Vacheron et al., 2013). Over the past decade, the genomes of numerous rhizosphere strains have been sequenced and analyzed, but functional genomics studies of rhizosphere competence lag behind the availability of sequence data.

This study explored the molecular dialog between the model host plant *Brachypodium distachyon* and several well-characterized rhizosphere strains of the *Pseudomonas fluorescens* group. *Brachypodium* is a small annual grass originating in semi-arid regions of the Middle East that has emerged as a prime model for economically important food, feed, forage, and biomass crops of the grass family (Bevan et al., 2010; Schwartz et al., 2010; Brkljacic et al., 2011; Hong et al., 2011; Tyler et al., 2014). The biology, extensive collection of resources, and research tools make *B. distachyon* an attractive model to investigate interactions between plants and root-associated microbes. Pseudomonads are ubiquitous Gram-negative γ-proteobacteria that colonize eukaryotic hosts and include both commensals and economically important pathogens of plants and animals (Moore et al., 2006; Schroth et al., 2006; Yahr and Parsek, 2006). The genus *Pseudomonas* currently comprises > 100 named species that have been separated based on multilocus sequence analysis into 14 species groups (Garrido-Sanz et al., 2016; Hesse et al., 2018). The *P. fluorescens* group is the most diverse regarding both the genetic distances within it, the number of species and the large pangenome that makes up > 50% of the pangenome of the genus as a whole (Loper et al., 2012). The group also encompasses an unusually high proportion of strains that inhabit the plant rhizosphere and possess plant growth promoting and biocontrol properties. Naylor et al. (2017) profiled bacterial communities associated with root tissues and rhizosphere of 18 different plant species of the *Poaceae* family. That study identified *Pseudomonas* among taxa constituting the core grass root microbiome and demonstrated that these bacteria were enriched in C3 plants, including wheat, rye, barley, oat, and *Brachypodium*. We confirmed the capacity of *B. distachyon* Bd21 to serve as a host for rhizobacteria of the *P. fluorescens* group in preliminary greenhouse assays with biocontrol strains *P. synxantha* 2-79, *P. brassicacearum* Q8r1-96, and *P. protegens* Pf-5. Results of these experiments revealed that all strains successfully established and colonized the roots of *Brachypodium* (**Supplementary Table 1**).

In this study, we focused on eight well-studied strains of the *P. fluorescens* complex that are supported by years of studies, numerous refereed publications, and high-quality genome sequences. By profiling transcriptomes of these strains during growth in root exudates of *B. distachyon*, we revealed the diversity of cellular pathways and physiological responses that underlie the establishment of mutualistic interactions between beneficial rhizobacteria and the host plant. Our results also confirmed that root exudates contain carbohydrates, amino acids, organic acids, and phenolics that serve as carbon and energy sources for rhizobacteria. The root exudates also contained osmoprotectants that may help microorganisms to persist in the rhizosphere of drought-stressed plants. The diversity of microbial genes perturbed by root exudates reflects the importance of

the variable genome in adaptation of individual strains of *Pseudomonas* to the rhizosphere lifestyle.

MATERIALS AND METHODS

Bacterial Strains Used in the Study

The eight *Pseudomonas* strains used for this study are *P. synxantha* 2-79 (Thomashow and Weller, 1988), *P. fluorescens* SBW25 (Silby et al., 2009), *Pseudomonas* sp. R1-43-08 (Parejko et al., 2012), *P. brassicacearum* Q8r1-96 (Raaijmakers and Weller, 1998), *P. fluorescens* Q2-87 (Bangera and Thomashow, 1996), *P. chlororaphis* 30-84 (Thomashow et al., 1990), *P. fluorescens* Pf0-1 (Silby et al., 2009), and *P. protegens* Pf-5 (Howell and Stipanovic, 1980). The selected organisms have been studied extensively for their role in biological control and plant growth promotion (**Supplementary Table 2**). The strains were maintained in the laboratory as frozen stocks (−80°C) and routinely cultured in King's medium B (King et al., 1954) or 21C medium, which contained (per 1 L): 1.0 g of NH_4Cl, 3.5 g of $Na_2HPO4{\cdot}2H_2O$, 2.8 g of KH_2PO_4, 3.0 g of glucose, and 20 ml of a microelement solution (Smibert and Kreig, 1994; Halverson and Firestone, 2000).

Propagation of Plants and Collection of Root Exudates

B. distachyon Bd21 was established from seed material obtained from the USDA-ARS Plant Germplasm Introduction and Testing Research Unit (Pullman, WA, United States). *Brachypodium* seeds were imbibed for 3 days at 4°C and sown in 7 × 7 cm pots filled with Sunshine Potting Mix #4 (Sun Gro Horticulture, Agawam, MA, United States). Plants were grown in an IR-89X (Percival Scientific, Perry, IA, United States) controlled environment chamber retrofitted with 6500K and 3000K T5 54W grow lights (Spectralux) under a 20-h light, 24°C/4-h dark, 18°C cycle. Plants were watered and fertilized with Jack's professional water-soluble fertilizer (20:20:20) (JR Peters, Allentown, PA, United States). After 12 weeks and plant senescence, seeds were collected, processed, and stored under desiccant and dark conditions at room temperature.

To collect root exudates, seeds of *B. distachyon* Bd21 were surface-sterilized, pregerminated, and placed in sterile 1 L wide-mouth glass jars containing 113 g of 6-mm glass beads and 25 ml distilled water. Jars were covered with vented caps and plants were grown hydroponically in an environmental controlled growth chamber under conditions described above. After 6 days, root exudates were extracted from individual jars and their sterility was confirmed by spotting on nutrient agar. Multiple batches of root exudates were collected, filtered (0.22 μm), aliquoted in Falcon tubes (10 ml), lyophilized, and stored at –80°C.

Metabolomic Profiling of Root Exudates

Exudates were analyzed for primary metabolites at the Murdock Metabolomics Laboratory at Washington State University (Pullman, WA, United States). Freeze-dried residues were suspended in 500 μl 50% aqueous acetonitrile and clarified by centrifugation for 20 min at 21,000 × *g* and 4°C. The liquid chromatography mass spectrometry analysis was conducted with a Synapt G2-S quadrupole-ion mobility spectrometry-time of flight mass spectrometer system equipped with an acquity ultra-performance liquid chromatograph (UPLC) and an acquity photodiode array detector (all from Waters, Milford, MA, United States). The exudate metabolites were separated on a SeQuant ZIC-pHILIC HPLC column (2.1 × 100 mm, 3 μm) (Milllipore Sigma, Burlington, MA, United States) using acetonitrile with 0.1% formic acid as solvent B and water with 0.1% formic acid as solvent A at a flow rate of 400 μl min^{-1} and the following linear gradient extending over 14 min: 0 min, 80% B; 4 min, 80% B, 6 min: 10% B; 7.5 min, 10% B; 10 min, 80% B; and 14 min, 80% B. Mass spectra were collected in positive ion mode over a range of *m/z* 50–1,200 with a scan time of 0.2 s. The Q-TOF-MS source was at 3.0 kV and 120°C; the sampling cone at 40 V, desolvation temperature was 250°C; cone gas and desolvation gas flow were at 0 and 850 L h^{-1}, respectively. Leucine enkephalin was used for post-acquisition mass correction. Target compounds were visualized using selected ion chromatograms at 0.05 Da window width. The compound identification was based on comparison of chromatographic behavior and accurate masses to those of authentic standards.

For gas chromatography, derivatization was carried out using a modification of the procedure of Lee and Fiehn (2008). The freeze-dried residues were suspended in 950 μl aqueous methanol (84%, *v/v*) and clarified by centrifugation for 15 min at 21,000 × *g* at 4°C. The supernatants were spiked with 1 μg of the internal standard salicylic acid-d_6 (C/D/N Isotopes, Quebec, Canada) and dried *in vacuo*. The dry residues were suspended in 10 μl of *O*-methoxylamine hydrochloride (30 mg ml^{-1} in anhydrous pyridine, both from Millipore Sigma) and incubated while mixing (1,000 RPM) for 90 min at 30°C. Subsequently, samples were derivatized with 90 μl of MSTFA with 1% TMCS (Thermo Fisher Scientific, Waltham, MA, United States) for 30 min at 37°C. Gas chromatography-mass spectroscopy analysis was performed using a Pegasus 4D time-of-flight mass spectrometer (LECO, Saint Joseph MI) equipped with a MPS2 autosampler (Gerstel, Linthicum, MD) and a 7890A oven (Agilent Technologies, Santa Clara, CA, United States). The derivatization products were separated on a 30-m, 0.25 mm i.d., 0.25 μm d_f Rxi-5Sil column (Restek, Bellefonte, PA, United States) with an IntegraGuard precolumn using ultrapure He at a constant flow of 0.9 ml min^{-1} as carrier gas. The linear thermal gradient started with a 1-min hold at 70°C, followed by a ramp to 300°C at 10°C min^{-1}. The final temperature was held for 5 min prior to returning to initial conditions. Mass spectra were collected at 17 spectra s^{-1}. Peak identification was conducted using the Fiehn primary metabolite library (Kind et al., 2009) and an identity score cutoff of 700. Additionally, authentic standards for a number of primary metabolites were analyzed under identical conditions and the data used to compare the chromatographic behavior. Peak alignment and spectrum comparisons were carried out using the Statistical Compare feature of ChromaTOF software (LECO).

Isolation of RNA From Bacteria Cultured in Root Exudates and RNA-Seq

The strains were pregrown overnight at 25°C on 21C-glucose agar and then subcultured into 96-well microplates containing liquid 21C-glucose medium amended with *Brachypodium* exudates. The liquid medium was prepared by dissolving the lyophilized root exudate material in an appropriate volume of 21C-glucose medium to concentrate root exudates 20-fold. The growth medium was sterilized by passing it through a 0.22-μm membrane filter. The control cultures were grown under identical conditions in the absence of exudates. All treatments were inoculated at OD_{600} of 0.1 and incubated for 20 to 22 h until cultures entered late-exponential growth phase at 25°C in an atmosphere of 15% oxygen [created by a ProOx P110 oxygen controller (BioSpherix, Parish, NY, United States) with a hypoxia C-chamber]. The cells were stabilized by the addition RNAprotect reagent (QIAGEN, Germantown, MD, United States) and total RNA was purified using a RNeasy Protect Bacteria Mini Kit (QIAGEN) from three biological replicates of each strain cultured under control conditions and in exudates. The quality assessment of the extracted RNA samples was performed with a NanoDrop One^C Spectrophotometer (Thermo Fisher Scientific) and a 2100 Bioanalyzer (Agilent Technologies) and revealed A_{260}/A_{280} and A_{260}/A_{230} values of > 2.0 and a mean RNA integrity numbers (RIN) value of > 9.2.

Three biological replicates of RNA samples were shipped on dry ice to the DOE Joint Genome Institute (Walnut Creek, CA, United States), where rRNA was depleted and stranded RNA-Seq libraries were prepared, quantified by qPCR and sequenced using a HiSeq 2500 instrument (Illumina). The fastq file reads were filtered and processed with BBDuk[1] to remove reads that contained 1 or more "N" bases, had an average quality score across the read less than 10 or had a minimum length $\leq$51 bp or 33% of the full read length. Reads mapped with BBMap (see text footnote 2) to masked human, cat, dog, and mouse references at 93% identity were removed. Another category of removed sequences matched RNA spike-in, PhiX, common microbial contaminants, and ribosomal RNAs. The processed reads from each library were aligned to the reference genome using BBMap with only unique mappings allowed (BAMs/directory). If a read mapped to more than one location it was ignored. featureCounts (Liao et al., 2014) was used to generate raw gene counts, which were normalized to adjust for the length of each gene and total number of reads mapped for each library. The normalization formula used: $n = [r/(l/1{,}000)]/(t/1{,}000{,}000)$, where n = normalized read count for gene (G) for library (L); r = raw read count for gene G for library L; l = gene G length; and t = total reads mapped for library L. Raw gene counts were used to evaluate the level of correlation between biological samples using Pearson's correlation.

Bioinformatic Analysis

Count tables generated by the JGI RNA-Seq pipeline were input into DESeq2 (Love et al., 2014) to normalize and determine differential expression. Statistical significance was established through DESeq2 by using three biological replicates for control and root exudate conditions. Scatterplots were generated from the DESeq2 data table outputs using ggplot2. Genes differentially expressed between control and root exudate samples ($\log_2$ fold-changes $-2 \geq$ to ≤ 2, adjusted p value ≤ 0.05) were used in downstream analysis. The core genome and pangenome for the *Pseudomonas* strains used in this study were computed using the OthoMCL v.2.0, Species Tree Builder v.2.2.0, and Phylogenetic Pangenome Accumulation v1.4.0 apps implemented in the U.S. Department of Energy Systems Biology Knowledgebase (KBase) (Arkin et al., 2018). Additional comparisons were conducted with the PGAweb pangenome analysis pipeline (Chen et al., 2018). Differentially expressed genes were assigned to core, non-core, and singleton parts of each strain's proteome by BLASTp with an E value cutoff of e-06, identity of 40% and coverage of 60%. Functional annotation of differentially expressed genes was carried out with the Blast2GO (Conesa and Gotz, 2008) and visualized in WEGO 2.0 (Ye et al., 2018). Additional manual curation was performed using tools implemented in the Integrated Microbial Genomes (IMG) database (Markowitz et al., 2012), Pseudomonas Genome Database (Winsor et al., 2009), Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa et al., 2008), and Geneious 10.2.3 (Biomatters, Auckland, New Zealand). Metabolic functions encoded by the differentially expressed genes were mapped using iPath 3.0 (Darzi et al., 2018). Phylogenetic analyses were carried out by building multiple sequence alignments with MAFFT v7.222 (Katoh and Standley, 2013) and inferring neighbor-joining (NJ) phylogenies with Geneious Tree Builder. The resultant phylogenetic trees were visualized with iTOL (Letunic and Bork, 2016). Reproducibility of clades within the inferred NJ trees was assessed by bootstrap resampling with 1,000 replicates.

Characterization of Carbon Source Utilization With Biolog Phenotype Microarrays

The utilization of carbon sources was analyzed using Phenotype MicroArrays (Biolog, Hayward, CA, United States) as follows. The bacteria were cultured overnight on Luria-Bertani agar at 25°C, after which cells were harvested and suspended in inoculating fluid (IF-0). The transmittance of the suspension was adjusted to 42% using a Biolog turbidimeter. The cell suspension was mixed with IF−0 containing Dye Mix A (Biolog) to achieve a final transmittance of 85%. One hundred microliter aliquots of the adjusted cell suspension were inoculated into PM01 and PM02A plates, which were then incubated in an OmniLog Phenotype MicroArray System (Biolog) at 25°C for 48 h. The formation of formazan was recorded at 15 min intervals, and data were analyzed using OmniLog Parametric Analysis software v1.20.02 (Biolog). Relative growth of the studied strains was normalized to growth on D-glucose and visualized using Heatmapper (Babicki et al., 2016).

[1]https://sourceforge.net/projects/bbmap/

Data Availability

Sequences generated in this project were deposited under NCBI BioProject accession numbers PRJNA439743 through PRJNA439790.

RESULTS

Metabolomic Profiling of Root Exudates of *B. distachyon*

Metabolomics analysis of lyophilized root exudates revealed the presence of numerous plant metabolites, 86 of which were identified by matching their spectra to the LECO/Fiehn Metabolomics library (**Supplementary Table 3**). These metabolites included (i) carbohydrates and their derivatives (glucose, fructose, xylose, sucrose, trehalose, maltose, galactose, and others); (ii) sugar alcohols (β-mannosylglycerate, myo-inositol, galactinol, 2-deoxyerythritol, ribitol, threitol and cellobitol); (iii) amino acids and derivatives (glutamine, tyrosine, glutamic acid, asparagine, aspartic acid, valine, phenylalanine, isoleucine, glycine, serine, proline, leucine, tryptophan, cysteine, methionine, citrulline, and others); (iv) organic acids (aconitic, allantoic, γ-aminobutyric, azelaic, citric, fumaric, 2-furoic, D-glyceric, 3-hydroxypropionic, α-ketoadipic, malic, methylmalonic, nicotinic, quinic, succinic, threonic); and (v) assorted metabolites including heterocyclic compounds, phenolics, and biogenic amines, etc (3-hydroxypyridine, maleimide, noradrenaline, 4-hydroxy-3-methoxybenzoate, 5-methoxytryptamine, uracil, aminomalonic acid, palmitic acid, and urea). Results of the analysis also revealed that root exudates of *B. distachyon* contain hydroxyectoine and the quaternary amine (QA) glycine betaine (**Supplementary Figure 1**).

Phylogenetic and Pangenome Analyses of *Pseudomonas* Strains Used in the Study

We used a set of phylogenetic markers suggested by Mulet et al. (2010) to investigate the relatedness of the eight strains used in this study to distinct lineages recognized within the *P. fluorescens* species complex. The multilocus sequence analysis based on the concatenated sequences of the housekeeping genes *rrs* (16S rRNA), *gyrB*, *rpoB*, and *rpoD* identified R1-43-08 (along with strains 2-79 and SBW25) as a member of the *P. fluorescens* subgroup (**Figure 1**). The rest of the strains clustered closely with four additional subgroups of the *P. fluorescens* complex, namely *P. corrugata* (strains Q2-87 and Q8r1-96), *P. koreensis* (Pf0-1), *P. protegens* (Pf-5), and *P. chlororaphis* (30–84). The genomes of the eight rhizosphere *Pseudomonas* strains varied in size by 1.43 megabase (ranging from 5.65 to 7.07 Mb) and contained between 5,166 and 6,363 protein-coding genes (**Figure 2A**). The shared gene content was characterized with OrthoMCL, which uses all-against-all BLASTp followed by the Markov Cluster algorithm to identify protein groups shared between the compared genomes, as well as groups representing species-specific gene expansion families (Li et al., 2003). The pangenome analysis revealed a core comprised of approximately 3,179 orthologs that were shared among all strains and represented 50.0% to 61.5% of each predicted proteome (**Figures 2A,B**). The non-core pangenome contained genes shared by two or more (but not all) strains and contained between 1,482 and 2,080 orthologs, which corresponded to 28.7–36.3% of individual proteomes. The rest of the predicted protein-coding genes were strain-specific singletons that comprised 7.5% to 15.1% of the strain's predicted proteomes. In respect to divergence from the core genome, strain Pf-5 was found to possess the highest proportion of unique genes (n = 949) followed by 2-79 (n = 887). The entire pangenome of the *Pseudomonas* strains encompassed over 12,000 homolog and singleton gene families.

Further homolog family-based comparisons identified Q8r1-96 and R1-43-08 as the most distantly related strains, with 3349 shared homologs (**Supplementary Table 4A**). Q8r1-96 and Q2-87, which shared 4,489 homologs, were the most closely related strains. The partitioning of homolog gene families into the core, non-core, and singleton parts of the pangenome agreed with phylogenetic relationships of the strains deduced from the analysis of a selected subset of COGs (Clusters of Orthologous Groups) (**Figure 2C** and **Supplementary Tables 4B,C**). The COG-based phylogeny supported the multilocus sequence analysis and revealed that the eight *Pseudomonas* strains form three distinct clusters, the first of which contained 2-79, R1-30-84, and SBW25. The second cluster included Q8r1-96 and Q2-87, whereas the third encompassed strains 30-84, Pf-5, and Pf0-1.

Correlating the Composition of Root Exudates With Metabolic Profiles of *Pseudomonas* Strains

We used the Phenotype MicroArray PM1 and PM2 plates to profile the eight *Pseudomonas* strains for the utilization of 190 different carbon sources. Results of the analysis identified 90 compounds that supported growth and clustered by their intensities of utilization into three distinct groups (**Figure 3**). Group I was comprised of 30 highly metabolized carbon sources, which included several amino acids and intermediates of glycolysis, pyruvate metabolism, and citrate cycle. Approximately half of these compounds were catabolized by all eight strains, and included several organic acids (fumaric, citric, gluconic, malic, and pyroglutamic), amino acids (Glu, Asn, Gln, Asp, Pro, Ala, and γ-aminobutyric acid), carbohydrates (glucose, mannose, and mannitol), and the purine nucleoside inosine. Group II was composed of 44 chemically diverse carbon sources that were variably utilized by the strains. These compounds were carbohydrates, organic acids, amino acids, phenolics, and polyols, and included known compatible solutes and intermediates of metabolism of pentoses, galactose, starch, and sucrose. Group III encompassed the rest of the Phenotype MicroArray test panel and contained compounds that were not catabolized by the tested strains. Among several notable exceptions were α-hydroxyglutamic acid- γ-lactone, putrescine, and itaconic, citramalic, and succinamic acids, which supported the growth of strains 2-79, 30-84, Pf-5, and SBW25. We further matched the carbon metabolic profiles of the *Pseudomonas* strains against the list of plant-derived metabolites from the root exudates of

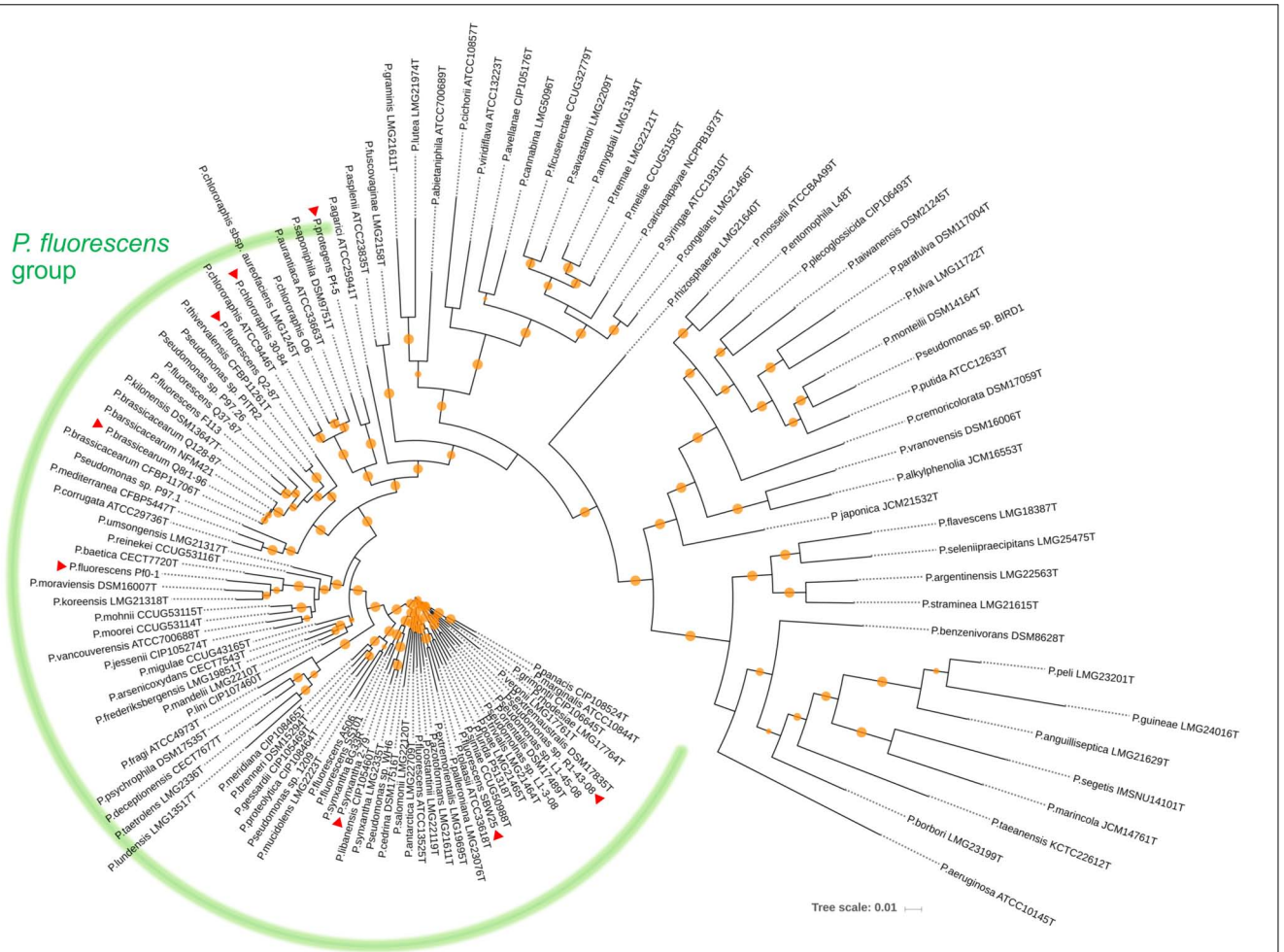

FIGURE 1 | Neighbor joining phylogeny showing the relationship of the eight strains used in this study (indicated by red triangles) to different species of the *P. fluorescens* complex. The phylogeny was established based on the concatenated sequences of the housekeeping genes *rrs* (16S rRNA), *gyrB* (subunit B of DNA gyrase), *rpoB* (β subunit of RNA polymerase), and *rpoD* (sigma 70 factor subunit of RNA polymerase). Distance matrices were calculated by the Jukes-Cantor method. Colored circles on tree nodes indicate bootstrap values (1,000 replicates) that vary between 60% (smallest circle) and 100% (largest circles).

B. distachyon Bd21. Interestingly, many carbon sources from the Phenotype MicroArray panel were also present in the root exudates of *B. distachyon* Bd21, and some of these compounds (glucose, mannose, galactose, fructose, γ-aminobutyric acid, aspartic acid, citric acid, malic acid, fumaric acid, quinic acid, alanine, glutamine, and glutamic acid) were catabolized by all strains used in this study, while others (e.g., xylose, trehalose, *m*-inositol) were actively utilized only by certain organisms (**Figure 3**). The comparison of catabolic profiles across the eight studied *Pseudomonas* strains revealed the presence of three distinct clusters. The first cluster contained strains Q8r1-96 and Q2-87, which consumed very similar sets of carbon sources, as well as strain Pf0-1. The second cluster was composed of 2-79, R1-43-08, SBW25, and 30-84, whereas the third cluster was represented by a single strain, Pf-5. The overall similarity of the catabolic profiles partially agreed with the separation of the strains into different subgroups of the *P. fluorescens* complex (see above).

Analysis of the RNA-seq Results

In order to understand the cellular responses of rhizosphere *Pseudomonas* to plant exometabolites, we analyzed the transcriptome changes in cultures grown in the presence of root exudates. Under field conditions, rhizobacteria colonize plant roots in the form of surface-attached microaerobic biofilms (Hojberg et al., 1999). To mimic these conditions, the eight *Pseudomonas* strains were grown statically at 72% air saturation in 21C-glucose medium amended with root exudates and then processed to extract total RNA (**Supplementary Figure 2**). A total of 995 million raw sequencing reads were generated from the RNA samples by using the Illumina HiSeq-2500 platform, averaging 20.7 million reads per sample. The removal of low-quality and rRNA sequences resulted in a total of 793 million filtered reads that were mapped onto the eight *Pseudomonas* genomes with a mean of 7.48 million mapped fragments per genome. The differentially abundant transcripts were identified by setting a p value of 0.05 (adjusted for multiple testing) and

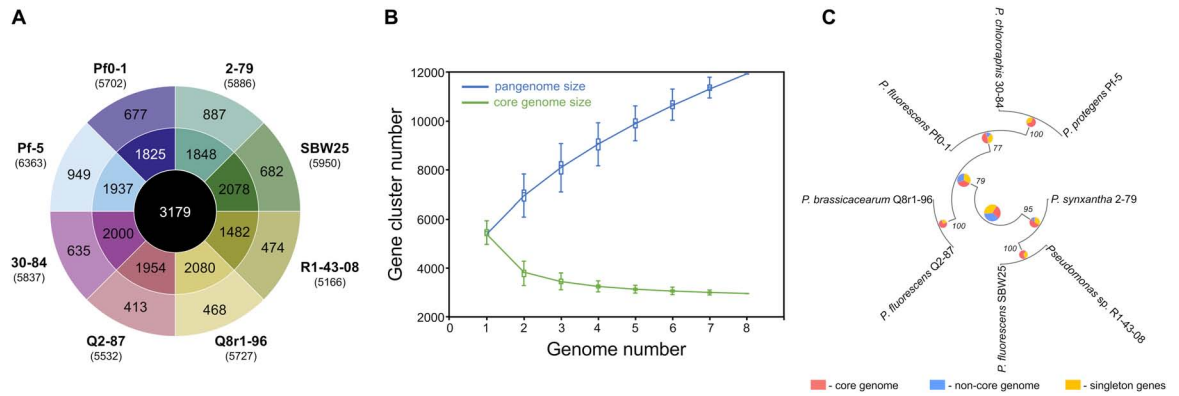

FIGURE 2 | Pangenome analysis of the studied *Pseudomonas* strains. **(A)** The innermost circle shows the number of orthologous protein families shared among all eight strains used in this study. The second circle shows orthologs present in two or more (but not all) strains, whereas the outermost circle represents strain-specific singletons. Values in brackets under strain names correspond to the total number of protein-coding genes predicted in each genome. **(B)** The gradual expansion of the pangenome (blue color) and contraction of the core genome (green color) following the sequential addition of genomes from the dataset. Box plots indicate the 25th and 75th percentiles and medians (horizontal lines) with whiskers corresponding to the 10th and 90th percentiles. The input order was randomized to avoid any bias due to the sequential addition of new genomes. The pangenome size increases steadily without reaching a plateau even after the addition of 11,939 non-redundant gene families. At the same time, the core genome converged to 3,179 genes. **(C)** The pangenome-based phylogenomic analysis of the studied strains. Here, the pangenome was defined with OrthoMCL, and orthologous gene sets were then partitioned into the core, singleton, and non-core (the remaining ortholog sets) categories. These categories were calculated for each node in the Maximum Likelihood species tree, using the set of genomes for which that node represents the ancestor. The results of the ortholog partitioning are shown in pie charts placed at tree nodes. Numbers indicate bootstrap support values. The analysis was conducted in KBase (Arkin et al., 2018).

the $\log_2$ fold-change (FC) threshold $\geq \pm$ 2.0 (**Figure 4** and **Supplementary Tables 5–12**). When compared with the control conditions, an average of 204 genes per strain were differentially expressed in the presence of root exudates, with the highest (n = 425) and lowest (n = 112) numbers observed, respectively, in SBW25 and Q2-87 (**Figure 4**). Overall, more genes were induced than repressed in response to exudates, but the actual numbers in each category varied substantially depending on the identity of the *Pseudomonas* strain. In most strains, the bulk of the differentially expressed genes was almost equally distributed between the core (mean, 48.2%) and non-core (mean, 45.8%) parts of the genome, whereas the strain-specific singleton genes constituted on average only 5.9% (**Figure 4B**). One notable exception was observed in Q8r1-96, where all differentially expressed genes belonged to the core (73.8%) and non-core (26.2%) parts of the genome. Another notable pattern was observed in R1-43-08, where the majority of genes affected by the presence of root exudate fell into the non-core category (56.3%). The highest proportion of differentially expressed singletons (11.3 and 10.4%, respectively) was identified in strains SBW25 and Pf-5.

We further explored how the identified differentially expressed genes were distributed across genomes of the eight studied rhizosphere strains. The pairwise BLASTp comparisons identified 2-79 and SBW25 as two strains that shared the highest number of genes (n = 101) induced or repressed in response to root exudates (**Table 1**). The second pair of strains with a significant number of similar differentially expressed genes (n = 86) was Q8r1-96 and Pf-5, which was followed by Pf0-1 and 30-84, which shared 56 differentially expressed genes. These patterns of shared genes were also observed when the results of the pairwise BLASTp comparisons were converted into a binary gene presence/absence matrix, which was then subjected to cluster analysis using a UPGMA algorithm based on Sorensen's dissimilarity index or examined by non-metric multidimensional scaling (NMDS) (**Figure 5**).

The differentially expressed *Pseudomonas* genes were subjected to Blast2Go analysis and Gene Ontology (GO) annotation (**Figure 6**). Metabolic process, catalytic activity, and membrane were the most common annotation terms across the three primary GO term categories (i.e., biological process, molecular function, and cellular component). A total of 1,694 GO terms was assigned to 805 upregulated genes, with the majority of the GO terms related to molecular function (682, 40.3%), followed by biological process (669, 39.5%), and cellular component (343, 20.2%). In the 539 downregulated gene category, 1,101 GO terms were assigned to biological process (420, 38.1%), molecular function (417, 37.9%), and cellular component (264, 24.0%). Within biological process, metabolic process, cellular process, localization, response to stimulus, and regulation were over-represented. Within molecular function, the largest proportion was assigned to catalytic activity, binding, and transporter activity categories. Within cellular component, the majority were assigned to membrane, membrane part, cell, and cell part categories. Across the eight strains, 37–42% of differentially expressed genes had no Gene Ontology IDs and encoded various conserved hypothetical proteins.

Functional Classification of Shared Differentially Expressed Genes

The interrogation of RNA-seq data revealed multiple cellular pathways that were differentially regulated in bacterial cultures incubated with root exudates (**Supplementary Figures 3**, **4**).

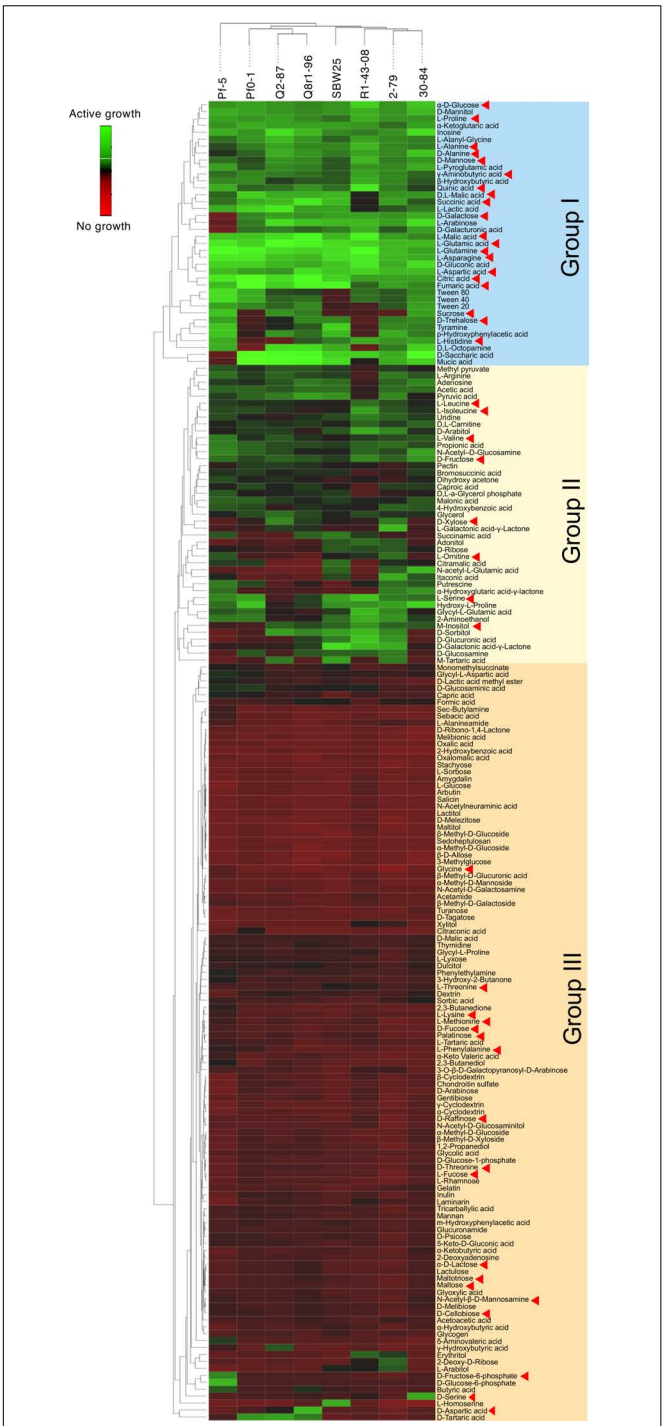

FIGURE 3 | Biolog Phenotype MicroArray profiling the eight rhizosphere *Pseudomonas* strains used in the study. The hierarchical clustering analysis was carried out using the average linkage method with Euclidean distances. Carbon sources identified by red arrowheads were also detected in the sterile root exudates of *B. distachyon* Bd21.

Although none of these differentially regulated pathways were shared by all eight strains, the cross-strain comparisons revealed several types of common and specific transcriptomic responses that were elicited by the presence of plant exometabolites (**Table 2**). The visual representation of core gene expression patterns is provided in **Supplementary Figure 5**, which shows heatmaps of expression profiles and *p*-adj values for core genes shared by the studied strains. The figure is accompanied by **Supplementary Table 13** that lists predicted functions of genes constituting the four distinct clusters observed after hierarchical clustering of gene expression values. The first category of shared differentially expressed pathways functioned in the uptake and catabolism of selected carbohydrates, quaternary ammonium compounds (QAs), and phenolics. All strains except for R1-43-08, responded to root exudates by inducing the fructose-specific phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS^{Fru}). The components of this system are encoded by a conserved operon and include the cytoplasmic polyprotein EI/HPr/$EIIA^{Fru}$ (FruB), the 1-phosphofructokinase FruK, and the fructose-specific permease EIIBC (FruA) (Chavarria et al., 2016). The PTS^{Fru} system functions by acquiring high-energy phosphates from PEP and sequentially passing them, *via* the EI/HPr/$EIIA^{Fru}$ domains of FruB, to the EIIB component of FruA. The phosphates are ultimately transferred by the EIIC transporter to fructose yielding fructose 1-phosphate, which is channeled into the central metabolic pathways through the action of the phosphofructokinase FruK.

In all strains except for Q8r1-96 and Pf-5, the exposure to root exudates resulted in the induction of two genes adjacent to the *fru* cluster that encoded a Major Facilitator Superfamily (MFS) transporter and an L-arabinonate dehydratase (**Table 2**). These genes are predicted to participate in the uptake and catabolism of L-arabinose, where L-arabinonate dehydratase plays an important role by converting L-arabinonate to 2-dehydro-3-deoxy-L-arabinonate (Rahman et al., 2017). In SBW25, R1-43-08, and Q2-87, we also observed the induction of genes encoding components of the AraFGH complex, an ATP-Binding Cassette (ABC) superfamily transporter involved in the import of arabinose into the cell (**Supplementary Tables 6, 7, 9**). Finally, all strains except SBW25 and R1-43-08 responded to the presence of exudates by upregulating a conserved gene encoding an aldose epimerase superfamily protein. Such enzymes equilibrate alpha- and beta-anomers of aldoses and ensure that stereospecific enzymes involved in the metabolism of free sugars do not act as metabolic bottlenecks (Abayakoon et al., 2018). Although some aldose epimerases have been linked to specific pathways, the *Pseudomonas* gene identified in this study could not be assigned to a particular metabolic process based on sequence analysis and genomic location.

Several *Pseudomonas* strains responded to the presence of root exudates by upregulating genes involved in the uptake and catabolism of *myo*-inositol and possibly other stereoisomers of inositol (**Table 2**). The upregulated catabolic genes encode the dehydrogenase IolG, which oxidizes *myo*-inositol to its corresponding ketone, as well as IolE, IolD, IolB, and IolC that collectively convert the 2-keto-*myo*-inositol to acetyl-CoA and the glycolysis intermediate dihydroxyacetone phosphate (Yoshida et al., 2008; Kohler et al., 2011). In R1-43-08, Q8r1-96, Q2-87, and Pf-5, the upregulated functions

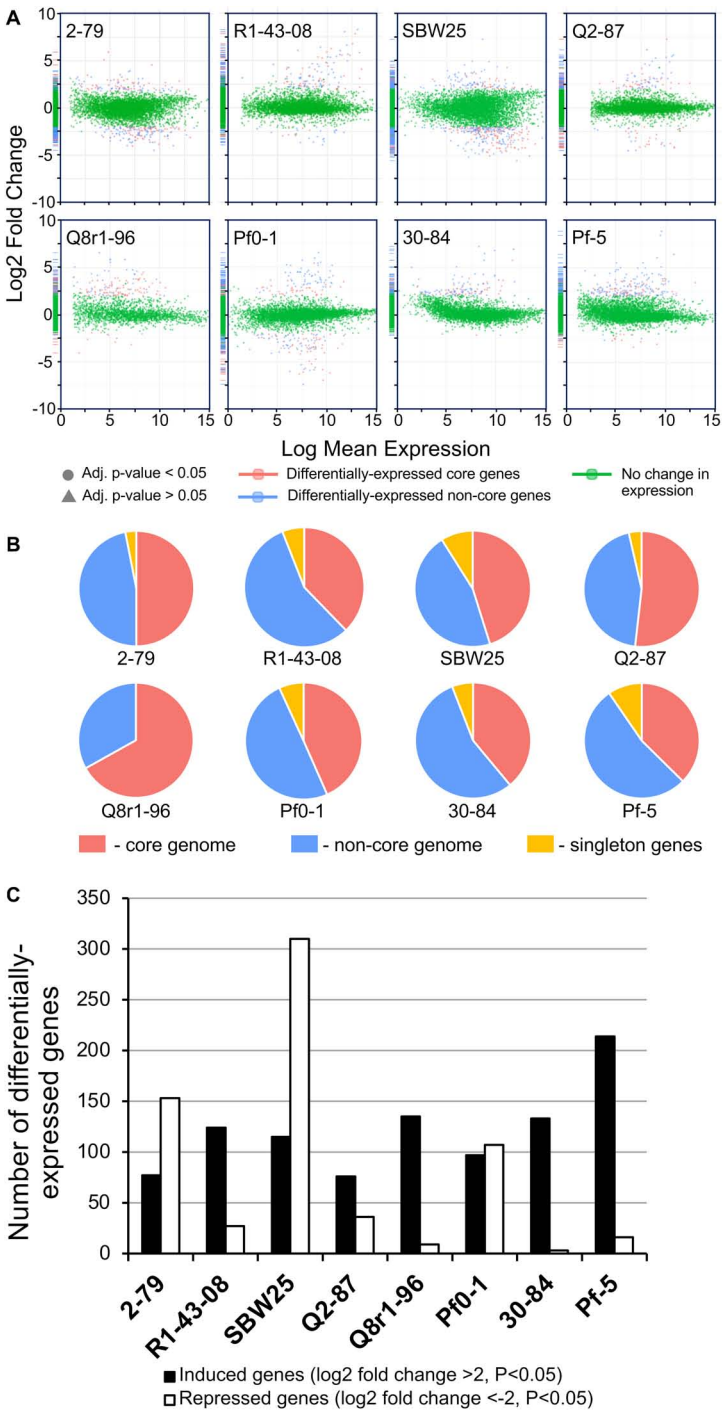

FIGURE 4 | (A) Log ratio versus abundance plots (MA-plots) showing the changes in gene expression in response to root exudates. The differentially expressed core and non-core genes are shown in red and blue, respectively. Green color indicates genes with a $\log_2$ fold-change and/or adjusted *p* values below the established threshold. **(B)** Circular diagrams depicting the distribution of differentially expressed genes among the core, non-core, and singleton proteomes of individual *Pseudomonas* strains. **(C)** The number of genes per genome that were induced and repressed by *B. distachyon* root exudates.

also involved components of the putative inositol-specific ABC transporter. The cross-genome comparisons revealed that in all studied strains except for Pf0-1, components of the *myo*-inositol utilization pathway were encoded within a well-conserved gene cluster which, in addition to catabolic and transport functions, also encodes a dedicated transcriptional repressor.

All studied strains of *Pseudomonas* carry multiple genes involved in scavenging the quaternary ammonium compounds choline, glycine betaine (GB), carnitine, choline-*O*-sulfate, and

TABLE 1 | The number of differentially expressed genes shared among the eight studied strains of rhizosphere *Pseudomonas*.

Strain	2-79	SBW25	R1-43-08	Q8r1-96	Q2-87	30-84	Pf0-1	Pf-5
2–79	260							
SBW25	101	425						
R1-43-08	30	25	151					
Q8r1-96	32	39	21	145				
Q2-87	27	28	25	31	112			
30-84	27	23	24	32	28	136		
Pf0-1	38	50	29	29	50	56	205	
Pf-5	36	41	52	86	29	55	40	230

The pairwise comparisons were conducted by BLASTp with the following cutoff parameters: E-value ≤ 1e-06, minimum percent identity ≥ 40%, and minimum percent coverage ≥ 65%. The black diagonal cells show the number of differentially expressed genes per strain. In other words, these are self comparison values.

sarcosine from the environment. Many of these genes were differentially expressed, including those encoding parts of the ABC transporter CbcXWV, which is predicted to function in the uptake of choline under water-replete conditions (**Table 2**). Among enzymes induced in the presence of root exudates were the choline dehydrogenase BetA, which converts choline to glycine betaine and a network of enzymes (i.e., the Rieske family oxygenase GbcAB, the dimethyglycine demethylase DgcAB, and the sarcosine oxidase SoxBDAG) that sequentially convert GB to glycine. In 2-79 and SBW25, this group of differentially regulated genes also included an AraC-family transcriptional activator GbdR, which perceives intercellular levels of GB and induces genes involved in the transport and catabolism of glycine betaine and detoxification of the catabolic byproducts (Hampel et al., 2014).

The last category of activated catabolic pathways included the catechol branch of the β-ketoadipate pathway for the degradation of aromatic compounds. In strains 30-84, Pf0-1, and Pf-5, growth on root exudates resulted in upregulation of catechol-1,2-dioxygenase, muconate cycloisomerase, and muconolactone isomerase, which collectively cleave the catechol ring and convert it to β-ketoadipate enol-lactone (Harwood and Parales, 1996). Finally, analysis of the *P. synxantha* 2-79 transcriptome identified an induction of *benABC* genes encoding subunits of benzoate 1,2-dioxygenase, an oxidoreductase that generates catechol from benzoate.

In addition to various catabolic pathways, the exposure to root exudates also induced several genes involved in the homeostasis of copper (**Table 2**). Four of these genes form a conserved cluster in genomes of the strains and encode the periplasmic copper-sensing two-component system CinRS, the plastocyanin/azurin-like protein CinA, and the NADPH-dependent pre-Q_0 reductase CinQ. Also, in strains Q2-87, 30-84, Pf0-1, and Pf-5, we observed upregulation of a conserved operon encoding the multicopper oxidase CopA, the periplasmic copper-binding protein CopC, the inner membrane protein CopD, and outer membrane protein CopB. In several Gram-negative bacteria, these Cop proteins are thought to have dual functions and participate both in the uptake of essential copper as well as in the sequestration of excess copper in the periplasm and outer membrane.

The analysis of shared downregulated pathways revealed that most of the strains respond to the presence of root exudates by repressing genes involved in the uptake and catabolism of sulfur compounds (**Table 2**). In strains SBW25, R1-43-08, Q8r1-96, Q2-87, Pf0-1, and Pf-5, this response involved the *ssuEADCB* operon responsible for the utilization of alkanesulfonates as sulfur sources. The *ssu* operon is highly conserved in fluorescent pseudomonads and encodes the $FMNH_2$-dependent monooxygenase SsuD and the NAD (P)H-dependent FMN reductase SsuE, which together catalyze the desulfonation of alkanesulfonates. Also, the *ssu* locus contains genes for the molybdopterin-binding protein SsuF and the alkanesulfonate-specific ABC-type transporter consisting of the sulfonate substrate-binding protein SsuA, sulfonate permease protein SsuC, and sulfonate transport ATP-binding protein SsuB. Finally, in R1-43-08, Q2-87, Pf0-1, and Pf-5, growth on root exudates coincided with repression of the *tauABCD* operon, which allows these strains to utilize taurine (2-aminoethanesulfonate) as a sulfur source. The repressed *tau* genes encoded the 2-oxoglutarate-dependent taurine dioxygenase TauD and substrate-binding, ATP-binding, and permease components of the taurine-specific ABC transporter TauABC.

Other Differentially Expressed Pathways

In addition to their effect on several shared cellular pathways, growth on root exudates resulted in the induction or repression of numerous strain-specific genes. In closely related *P. synxantha* 2-79 and *P. fluorescens* SBW25, we observed differential expression of genes involved in energy metabolism, transport of amino acids, and surface attachment (**Supplementary Tables 5, 6**). Other notable differentially expressed pathways included 2–79 gene clusters that encode enzymes for the catabolism of trehalose, a prophage, and toxin/antitoxin system, as well as the SBW25 operon predicted to control the synthesis of the capsular exopolysaccharide colonic acid. The response of *Pseudomonas* sp. R1-43-08 to root exudates also involved differential expression of different energy metabolism pathways. In addition, we observed the upregulation of genes involved in the uptake and catabolism of xylose (also upregulated in 2–79) and repression of enzymes for the biosynthesis of phenazine-1-carboxylic acid and assimilation of inorganic sulfur and L-cysteine biosynthesis (**Supplementary Table 7**).

The analysis of the Q8r1-96 transcriptome revealed perturbation of different metabolic pathways including genes encoding components of cytochrome C oxidase, transport and catabolism of sorbitol/mannitol, metabolism of butanoic acid, and biosynthesis of exopolysaccharides alginate and poly-β-1-6-*N*-acetylglucosamine (**Supplementary Table 8**). In *P. fluorescens* Q2-87, we identified differential expression of genes involved in metabolism of galactose, tryptophan, tyrosine, glycine, serine, and threonine (**Supplementary Table 9**), while in *P. chlororaphis* 30-84, growth on exudates activated the biosynthesis of molybdopterin cofactor, catabolism of galactonate and acetoin, and uptake and catabolism of putrescine (**Supplementary Table 10**). The response of *P. protegens* Pf-5 to root exudates involved upregulation of acetoin dehydrogenase, which converts acetoin to acetaldehyde and acetyl-CoA, as

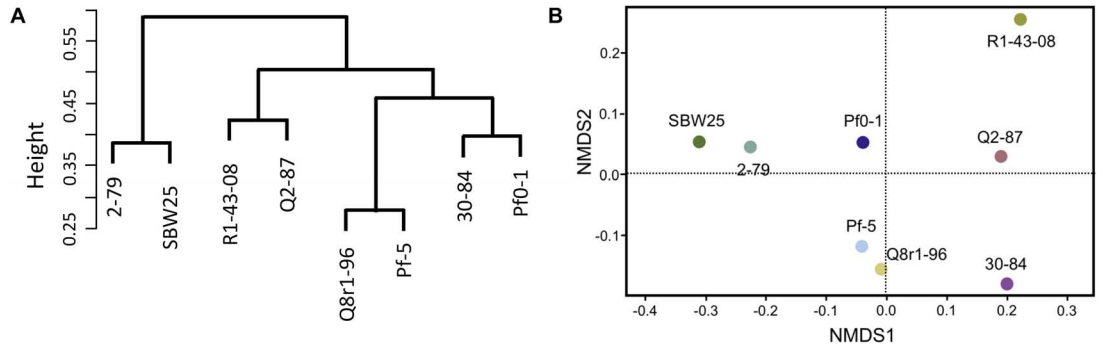

FIGURE 5 | Comparison of the eight *Pseudomonas* strains based on the content (presence/absence) of genes differentially expressed in the presence of root exudates. **(A)** UPGMA clustering based on the Sorensen's dissimilarity index. **(B)** non-metric multidimensional scaling (NMDS) analysis.

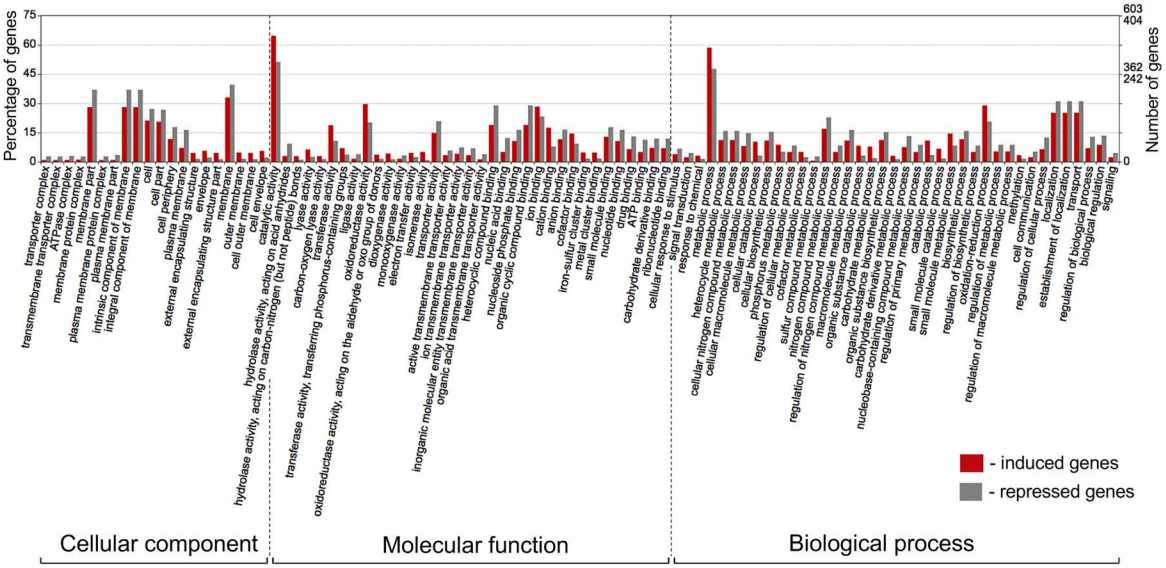

FIGURE 6 | Gene Ontology (GO) classification of *Pseudomonas* genes that were induced (red bars) or repressed (gray bars) in response to root exudates of *B. distachyon* Bd21. The terms were derived from 93 different functional groups (GO subcategories level 4). The GO terms were assigned with Blast2GO (Conesa and Gotz, 2008) and visualized in WEGO 2.0 (Ye et al., 2018). On a WEGO histogram, the percentage of 100 is defined as the total number of genes assigned a GO term. However, the subcategories do not add up to 100% because many genes fall into more than one functional class and are therefore annotated by multiple GO terms.

well as pathways for the utilization of glycolate and putrescine (**Supplementary Table 11**). Also induced were genes for the production of pyrrolnitrin and PhlG hydrolase, which modulate the metabolic loads attributed to the synthesis of 2,4-diacetylphloroglucinol. The differentially expressed genes of *P. fluorescens* Pf0-1 included, among others, operons encoding cytochrome C oxidase and enzymes for catabolism of malonic acid (**Supplementary Table 12**). Yet another interesting finding involved the induction of assorted genes acting in the homeostasis of iron and defense against reactive oxygen species (ROS). We observed activation of iron dicitrate transporters (SBW25 and 30-84), genes for the biosynthesis of siderophores ornicorrugatin (SBW25) and pyochelin (Pf-5), heme-degrading enzymes (2–79, 30–84), TonB siderophore receptors, and components of the energy-transducing inner membrane complex TonB-ExbB-ExbD (2–79 and Pf-5). The differentially expressed ROS defense pathways were represented by different catalases in strains 2–79, R1-43-08, Q8r1-96, Q2-87, Pf0-1, and Pf-5 and organic hydroperoxide resistance proteins in strains SBW25 and R1-43-08. Finally, in SBW25, Q2-87, 30–84, and Pf0-1, the addition of exudates resulted in the upregulation of peroxiredoxins that detoxify H_2O_2, peroxynitrite, and aliphatic and aromatic hydroperoxides.

DISCUSSION

Our analysis of *B. distachyon* root exudates revealed a complex mix of primary and secondary metabolites, thus supporting the view of the plant rhizosphere as a carbon-rich niche for

TABLE 2 | The distribution and predicted functions of selected differentially expressed genes[a].

Predicted function	Strain[b]							
	2–79	**SBW25**	**R1-43-08**	**Q8r1-96**	**Q2-87**	**30–84**	**Pf0-1**	**Pf-5**
Uptake and catabolism of fructose								
D-fructose PTS system, IIC component	2756598827 (2.7)	649634314 (2.3)		2597873629 (3.6)	2597850083 (2.7)	2597856046 (3.6)	637740645 (2.9)	637318202 (2.8)
1-phosphofructokinase	2756598828 (2.9)	649634313 (3.1)		2597873628 (3.9)	2597850082 (2.7)	2597856045 (3.4)	637740644 (3.1)	637318201 (2.7)
D-fructose PTS system, IIA component	2756598829 (2.6)	649634312 (3.0)		2597873627 (3.7)	2597850081 (2.6)	2597856044 (3.6)	637740643 (2.9)	637318200 (3.2)
Uptake and catabolism of arabinose								
MFS superfamily transporter	2756599521 (2.2)	649635836 (3.6)	2756590067 (4.9)		2597851595 (3.1)	2597859759 (4.2)	637743102 (2.9)	
L-arabinonate dehydratase	2756599520 (3.1)	649635835 (4.3)	2756590066 (5.5)		2597851594 (5.4)	2597859760 (4.0)	637743103 (3.5)	
Interconversion of alpha- and beta-anomers of aldoses								
Aldose epimerase superfamily protein	2756599919 (2.7)			2597878613 (4.2)	2597849545 (3.1)	2597860977 (4.2)	637742166 (3.4)	637323358 (3.5)
Uptake and catabolism of quaternary ammonium compounds								
Choline dehydrogenase BetA				2597874908 (2.3)	2597851450 (2.1)			
Transcriptional regulator GbdR	2756597125 (−3.7)	649639087 (−3.9)						
Membrane dipeptidase, *dgc* operon	2756597136 (1.9)		2756592046 (2.0)	2597878321 (3.8)	2597849833 (2.9)	2597860696 (2.6)		637323077 (3.3)
Hypothetical protein, *dgcAB* operon	2756597137 (2.4)		2756592045 (2.3)	2597878320 (3.4)	2597849834 (2.7)	2597860695 (2.3)		637323076 (3.3)
Dimethyl Gly demethylase DgcA	2756597138 (2.7)		2756592044 (2.0)	2597878317 (3.3)	2597849835 (2.7)	2597860694 (2.2)		637323075 (3.2)
Dimethyl Gly demethylase DgcB	2756597139 (2.3)			2597878318 (3.6)	2597849836 (2.6)	2597860693 (2.4)		637323074 (3.0)
Betaine demethylase, GbcA subunit	2756597143 (2.1)		2756592039 (2.3)	2597878312 (4.3)				637323070 (3.7)
Betaine demethylase, GbcB subunit				2597878311 (3.9)		2597860689 (2.0)		637323069 (3.2)
Ser hydroxymethyltransferase, *sox* operon	2756597149 (2.1)		2756592033 (2.0)	2597878308 (2.9)	2597849846 (2.7)			637323064 (3.1)
Sarcosine oxidase, γ subunit, SoxG				2597878304 (2.9)				
Sarcosine oxidase, α subunit, SoxA			2756592028 (2.1)	2597878305 (3.0)				637323061 (2.4)
Sarcosine oxidase, δ subunit, SoxD				2597878306 (2.5)				637323062 (2.2)
Sarcosine oxidase, β subunit, SoxB	2756597150 (2.4)		2756592032 (1.9)	2597878307 (2.6)				637323063 (2.3)
Betaine substrate-binding protein CbcX			2756590368 (2.3)	2597878336 (2.3)	2597850794 (3.1)		637742655 (-3.5)	
ABC transporter, ATP-binding protein CbcV				2597878338 (2.3)			637742656 (-3.5)	
Uptake and catabolism of *myo*-inositol								
5-dehydro-2-deoxygluconokinase, IolC			2756592881 (2.5)	2597876275 (4.3)		2597857598 (2.4)		637319925 (4.2)
2-keto-myo-inositol dehydratase, IolE			2756592884 (2.5)	2597876273 (4.6)		2597857602 (2.4)		637319928 (4.1)
5-deoxy-glucuronate isomerase, IolB			2756592883 (2.3)	2597876272 (4.3)		2597857600 (2.4)		637319927 (4.3)
2-keto-myo-inositol isomerase, IolL			2756592882 (2.7)			2597857599 (2.4)		637319926 (4.4)
3D-(3,5/4)-trihydroxycyclohexane-1,2-dione acylhydrolase, IolD			2756592885 (2.4)	2597876269 (4.8)		2597857603 (2.5)		637319929 (4.3)
Myo-inositol 2-dehydrogenase, IolG	2756595203 (1.9)		2756592886 (2.2)	2597876268 (4.8)		2597857604 (2.0)		637319930 (3.9)
Inositol transport substrate-binding protein			2756592888 (2.0)	2597876265 (3.8)	2597851513 (2.8)			637319932 (3.8)
Inositol transport permease protein			2756592890 (1.9)	2597876263 (3.6)	2597851515 (2.2)			637319934 (3.5)
Inositol transport ATP-binding protein			2756592889 (2.2)	2597876264 (3.8)	2597851514 (2.7)			637319933 (3.6)

(Continued)

TABLE 2 | Continued

Predicted function	Strain[b]							
	2–79	**SBW25**	**R1-43-08**	**Q8r1-96**	**Q2-87**	**30–84**	**Pf0-1**	**Pf-5**
Uptake and catabolism of fructose								
Catabolism of phenolics								
Muconate cycloisomerase						2597859089 (3.1)	637742838 (3.4)	637321199 (4.8)
Muconolactone delta-isomerase						2597859088 (2.6)	637742837 (3.5)	637321198 (4.4)
Catechol 1,2-dioxygenase						2597859087 (2.0)	637742836 (2.9)	637321197 (3.5)
AraC-type DNA-binding protein						2597859086 (2.0)		637321196 (2.2)
Benzoate 1,2-dioxygenase, α subunit	2756599329 (2.7)					2597859085 (3.4)	637742843 (3.9)	637321195 (3.8)
Benzoate 1,2-dioxygenase, β subunit	2756599330 (2.2)					2597859084 (3.3)	637742842 (4.2)	637321194 (4.8)
Benzoate 1,2-dioxygenase, reductase subunit	2756599331 (2.1)					2597859083 (3.4)	637742841 (3.9)	637321193 (4.3)
Copper homeostasis								
pre-Q_0 reductase/7-cyano-7-deazaguanine reductase CinQ	2756597439 (2.7)	649635068 (1.8)		2597874689 (3.4)				637319306 (2.5)
Cupredoxin-like copper-binding protein CinA	2756597440 (2.8)	649635067 (4.3)	2756590986 (2.03)		2597853017 (7.3)	2597857153 (5.7)	637743734 (3.5)	637319305 (3.4)
Heavy metal response regulator CinR		649635066 (1.8)		2597874687 (2.1)	2597853018 (3.2)	2597857152 (2.4)	637743735 (2.1)	637319304 (2.5)
Heavy metal sensor histidine kinase CinS		649635065 (2.0)				2597857151 (2.2)	637743736 (2.0)	637319303 (2.0)
Copper resistance protein CopA				2597877412 (5.9)	2597850492 (5.6)	2597857966 (6.9)	637743691 (5.2)	637320232 (6.4)
Copper resistance protein CopB					2597850491 (5.3)	2597857965 (6.8)	637743692 (5.5)	637320231 (6.0)
Copper resistance protein CopC					2597850490 (4.6)	2597857964 (7.2)	637743693 (5.4)	
Copper resistance protein CopD					2597850489 (4.9)	2597857963 (6.9)	637743694 (5.2)	
Conserved hypothetical protein								
Aldose epimerase superfamily protein	2756599919 (2.7)			2597878613 (4.2)	2597849545 (3.1)	2597860977 (4.2)	637742166 (3.4)	637323358 (3.5)
Uptake and catabolism of sulfonates								
FMN-dependent monooxygenase SsuE			2756592254 (−1.9)				637745334 (−3.5)	
Sulfonate substrate-binding protein SsuA		649639261 (−2.0)	2756592253 (−2.2)	2597878518 (−2.6)	2597849636 (−3.9)		637745333 (−7.0)	637323272 (−2.7)
FMN-dependent monooxygenase SsuD		649639260 (−1.9)	2756592252 (−2.3)		2597849637 (−2.8)		637745332 (−5.8)	637323271 (−2.3)
Sulfonate permease protein SsuC		649639259 (−2.4)	2756592251 (−2.1)	2597878516 (−1.9)	2597849638 (−3.2)		637745331 (−5.8)	637323270 (−2.5)
Sulfonate transport ATP-binding protein SsuB		649639258 (−2.5)	2756592250 (−2.3)		2597849639 (−2.5)		637745330 (−5.6)	637323269 (−2.7)
Molybdopterin binding protein SsuF		649639257 (−3.1)	2756592249 (−2.7)	2597878514 (−2.9)	2597849641 (−3.3)		637745339 (−6.2)	637323268 (−2.7)
Uptake and catabolism of taurine								
Taurine substrate-binding protein TauA					2597854917 (−2.0)		637740095 (−4.1)	
Taurine transport ATP-binding protein TauB			2756592398 (−1.9)				637740094 (−3.7)	637317614 (−1.9)
Taurine permease protein TauC			2756592398 (−1.9)				637740093 (−3.9)	637317613 (−2.0)
Taurine dioxygenase TauD					2597854920 (−1.8)		637740092 (−3.6)	

[a] *The shared differentially expressed genes were identified by BLASTp with the cutoff parameters of E-value ≤ 1e-06, minimum percent identity ≥ 40%, and minimum percent coverage ≥ 65%.*
[b] *Values in columns indicate JGI IMG gene IDs followed by the corresponding fold-change (FC) values (shown in brackets).*

soil microorganisms. Our results were in agreement with a recent report of 27 different sugars, amino acids, and organic acids in *Brachypodium* exudates (Kawasaki et al., 2016). We confirmed the presence of exometabolites identified in that study, along with dozens of additional analytes that were identified by matching their mass-spectra and retention indices to the LECO/Fiehn Metabolomics library (**Supplementary Table 3**). The complementation of the metabolomic analysis with profiling of the bacteria by Biolog Phenotype MicroArrays revealed that a substantial proportion of the characterized exudate constituents were catabolized by a collection of eight *Pseudomonas* strains from across the *P. fluorescens* group that is known to form associations with plant roots. The amendment of *Pseudomonas* cultures with root exudates caused changes in the expression of multiple genes encoding catabolic and anabolic enzymes, predicted transporters, transcriptional regulators, stress response, and conserved hypothetical proteins. In most strains, these differentially expressed genes were almost equally split between the core and variable genome regions, mirroring the substantial strain-to-strain variation in the genome size and gene content within the *P. fluorescens* species complex (Loper et al., 2012).

The analysis of transcriptome responses to root exudates revealed several types of cellular pathways present in the strains used in this study. The first category of such pathways was involved in the catabolism of carbohydrates such as fructose, arabinose, *myo*-inositol, xylose, trehalose, and galactose. Among these catabolic traits, the ability to utilize fructose as a carbon source is highly conserved among fluorescent pseudomonads. In contrast, growth on arabinose, *myo*-inositol, xylose, and trehalose is variably present and was traditionally used to differentiate species and biovars within the *P. fluorescens* group (Barrett et al., 1986). We speculate that such variably distributed pathways contribute to the differential affinity of pseudomonads toward host plants and/or to determine which strains flourish in response to growing roots and changing environments. Several independent studies have confirmed the importance of carbohydrate catabolism pathways for the biology of rhizosphere pseudomonads. For example, *in vivo* expression technology (IVET) profiling of *P. fluorescens* SBW25 identified xylose isomerase among genome regions essential for the colonization of sugar beet seedlings (Liu et al., 2015), whereas a genome-wide Tn-Seq screen of *Pseudomonas simiae* identified genes for the catabolism of *myo*-inositol among traits essential for the colonization of *Arabidopsis thaliana* roots (Cole et al., 2017).

The response of rhizosphere *Pseudomonas* to *Brachypodium* root exudates also involved pathways for the uptake and metabolism of amino acids. We observed differential expression of genes encoding the hydrophobic (HAAT) and polar (PAAT) amino acid uptake transporters in strains 2-79, SBW25, Q2-87, Pf0-1, and Pf-5. Other related genes encoded enzymes for the catabolism of valine and glutamic acid (2-79); metabolism of tryptophan, glycine, serine, and threonine (Q2-87); and biosynthesis of methionine (Q8r1-96). It is plausible that the abundance of amino acids in root exudates is also linked to the repression of pathways involved in the catabolism of sulfonates and taurine that was observed in several strains (**Table 2**). Although the preferred source of sulfur for *P. fluorescens* is unknown, in the closely related *P. aeruginosa*, the sulfur starvation response is triggered by the growth on any sulfur compound other than sulfate, thiocyanate, and cysteine (Hummerjohann et al., 1998). This fact, together with the presence of cysteine and cystine in the root exudates, suggest that root exudates of *Brachypodium* may serve as an important source of sulfur for rhizosphere *Pseudomonas*. These findings also agree well with the reported scarcity of inorganic sulfate in the soil, and the presence of sulfur mostly in the form of organic compounds, including amino acids, proteins, sulfate esters, and sulfonates (Autry and Fitzgerald, 1990).

Another interesting result of this study was the concerted activation of copper and iron homeostasis pathways observed in all of the *Pseudomonas* strains used in this work. In bacteria, an excess of copper is toxic and triggers oxidative stress due to the formation of free radicals, as well as disruption of protein metalation and stability of iron-sulfur clusters (Bondarczuk and Piotrowska-Seget, 2013). On the other hand, copper is an essential trace element used as a cofactor in different enzymes. Similarly, although elevated levels of iron cause redox stress, this element is also found in active energy metabolism enzymes and is crucial for bacterial growth (Andrews et al., 2003). The analysis of metal homeostasis genes identified in this study suggests that their induction was likely triggered by the deficiency of copper and iron in bacterial cultures grown in the presence of root exudates. We attribute this effect to the ability of some components of root exudates to chelate soil metals.

Despite the abundance of iron in the soil, its bioavailability is limited due to the low solubility of Fe (III) oxyhydrates at neutral pH. The non-graminaceous plants circumvent this problem by acidifying the rhizosphere and secreting flavins, phenolics, and organic acids that chelate iron. The reduction of these ferric chelates releases soluble ferrous iron taken up by root cells (Kobayashi and Nishizawa, 2012). Graminaceous plants, like *Brachypodium*, acquire iron by secreting into the soil non-protein amino acids of the mugineic acid (MA) group, which act as Fe (III)-chelating phytosiderophores. In addition to iron, low-molecular-weight organic acids and phytosiderophores bind other divalent and trivalent metals (including copper) and contribute to heavy-metal tolerance in plants (Chen et al., 2017). It is plausible that the presence of these plant exometabolites is responsible for the deficit of iron and copper observed in *Pseudomonas* cultures grown in the presence of root exudates. These results further underscore the importance of diverse and redundant metal homeostasis pathways found in genomes of the *P. fluorescens* group for the ability of these organisms to colonize and persist in the plant rhizosphere.

Recently, Klonowska et al. (2018) examined transcriptomic responses of symbiotic nitrogen-fixing bacteria to root exudates of the legume plant *Mimosa pundica*, which has an unusual ability to support both alpha- (*Rhizobium*) and beta-rhizobia (*Cupriavidus* and *Burkholderia*). Using RNA-seq, the authors characterized genes involved in the perception of root exudates in the nodulating bacteria *Burkholderia phymatum*

STM815, *Cupriavidus taiwanensis* LMG19424, and *Rhizobium mesoamericanum* STM3625. Interestingly, the analysis of differentially expressed genes revealed induction of pathways involved in the catabolism of fructose, xylose, *myo*-inositol, and protocatechuate/catechol. Also upregulated were some copper homeostasis, siderophore biosynthesis, and oxidative stress genes. Finally, the analytical profiling of *M. pundica* exudates revealed an overlap with *Brachypodium* in the types of carbohydrates, amino acids, and organic acids present. These findings suggest that differentially expressed genes shared by multiple strains of the group *P. fluorescens* are not unique to the *Brachypodium-Pseudomonas* system but represent a set of conserved cellular pathways involved in the perception of plant exometabolites by different clades of rhizosphere-dwelling *Proteobacteria*.

Most strains included in this study were originally selected based on the ability to colonize the rhizosphere and produce secondary metabolites that alleviate the plant stress response and/or inhibit soilborne pathogens. It has been suggested that plant metabolites released into the rhizosphere affect the biocontrol activity of plant-beneficial pseudomonads (de Werra et al., 2011). We provide further support to this hypothesis by demonstrating that in some strains, root exudates modulate the expression of genes for the catabolism of the plant growth-promoting metabolites acetoin and 2,3-butanediol. The exposure to exudates also affected the expression of genes for the synthesis of well-characterized antifungal compounds pyrrolnitrin, phenazine-1-carboxylic acid, and 2,4-diacetylphloroglucinol. The modulatory effects were strain-specific, suggesting significant differences in the regulatory networks involved in the perception of plant signals and regulation of the production of antibiotics and growth-promoting metabolites.

The final significant finding of this study was the induction of catabolism of quaternary amines (QAs) observed in multiple strains of the *P. fluorescens* group during growth on root exudates. This observation was supported by the detection of glycine betaine in the root secretions of *B. distachyon*. The presence of QAs in plant tissues and the capacity of these metabolites to provide stress protection and nutrients to plant pathogens and symbionts were reported before (Boncompagni et al., 1999; Chen et al., 2013; Kabbadj et al., 2017), but our study is among the first to highlight the potential importance of these metabolites for rhizosphere interactions. Pseudomonads do not synthesize QAs *de novo* but have evolved many pathways to scavenge them from eukaryotic hosts, where these metabolites are abundant due to the prominence of phosphatidylcholine in cellular membranes. Strains of *P. fluorescens* carry genes for the conversion of choline, carnitine, and glycine betaine to glycine, as well as quaternary amine transporters of the BCCT and ABC families that are also conserved in the opportunistic human pathogen *P. aeruginosa* and the plant pathogen *P. syringae* (Galvao et al., 2006; Chen et al., 2013; Wargo, 2013b).

In *P. aeruginosa*, choline catabolism genes are essential for the ability of this pathogen to persist during lung infection (Wargo, 2013a). Similarly, a *P. syringae* mutant deficient in BetT, OpuC, and CbcXWV quaternary amine transporters had reduced fitness during colonization of bean and soybean leaves under greenhouse and field conditions (Chen et al., 2013). Depending on water availability, *P. aeruginosa* and *P. syringae* catabolize exogenously supplied QAs as carbon and nitrogen sources or accumulate them as osmoprotectants (Chen et al., 2013; Wargo, 2013b). Our ongoing work in *P. synxantha* 2–79 unraveled similar physiological responses and demonstrated that QA transporters function differentially and redundantly in the uptake of quaternary amines as nutrients (Pablo and Mavrodi, unpublished). In contrast, under water stress, the QAs choline, betaine, and carnitine are accumulated preferentially for osmoprotection. Under drought stress, a 2–79 mutant devoid of all known QA transporters was less competitive in the colonization of the *Brachypodium* rhizosphere than its wild-type parental strain. Interestingly, our metabolomic profiling of root exudates also revealed proline, glutamine, and hydroxyectoine. These metabolites act as compatible solutes in different groups of microorganisms (Yancey et al., 1982; Empadinhas and da Costa, 2008), suggesting an important role of root exudates in the ability of *Pseudomonas* to persist in the rhizosphere of drought-stressed plants.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI BioProject accession numbers PRJNA439743 through PRJNA439790.

AUTHOR CONTRIBUTIONS

DM, OM, and LT conceived the research project. OM and JM collected root exudates. OM and DM cultured strains and extracted total RNA. AB and DG performed metabolomic analysis of root exudates. DM, JP, and AF analyzed RNA-seq data. LE, KH, and IP conducted Biolog analyses. DM, AF, OM, DW, and LT wrote the manuscript. All authors contributed to the manuscript revision.

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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.2021.651282/full#supplementary-material

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REVIEW
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Progress and Prospects of Mycorrhizal Fungal Diversity in Orchids

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Orchids form mycorrhizal symbioses with fungi in natural habitats that affect their seed germination, protocorm growth, and adult nutrition. An increasing number of studies indicates how orchids gain mineral nutrients and sometime even organic compounds from interactions with orchid mycorrhizal fungi (OMF). Thus, OMF exhibit a high diversity and play a key role in the life cycle of orchids. In recent years, the high-throughput molecular identification of fungi has broadly extended our understanding of OMF diversity, revealing it to be a dynamic outcome co-regulated by environmental filtering, dispersal restrictions, spatiotemporal scales, biogeographic history, as well as the distribution, selection, and phylogenetic spectrum width of host orchids. Most of the results show congruent emerging patterns. Although it is still difficult to extend them to all orchid species or geographical areas, to a certain extent they follow the "everything is everywhere, but the environment selects" rule. This review provides an extensive understanding of the diversity and ecological dynamics of orchid-fungal association. Moreover, it promotes the conservation of resources and the regeneration of rare or endangered orchids. We provide a comprehensive overview, systematically describing six fields of research on orchid-fungal diversity: the research methods of orchid-fungal interactions, the primer selection in high-throughput sequencing, the fungal diversity and specificity in orchids, the difference and adaptability of OMF in different habitats, the comparison of OMF in orchid roots and soil, and the spatiotemporal variation patterns of OMF. Further, we highlight certain shortcomings of current research methodologies and propose perspectives for future studies. This review emphasizes the need for more information on the four main ecological processes: dispersal, selection, ecological drift, and diversification, as well as their interactions, in the study of orchid-fungal interactions and OMF community structure.

Keywords: orchid mycorrhizal fungi, orchid non-mycorrhizal fungi, primer selection, fungal diversity, mycorrhizal specificity, environmental filtering, spatio-temporal variation

INTRODUCTION

Biological interactions are key for the construction and maintenance of communities (Bascompte and Jordano, 2007; Bonfante and Anca, 2009). Fungi interact with the roots of all vascular plants, 85% of them form a mutually symbiotic relationship called a mycorrhiza (van der Heijden et al., 2015) that is pivotal for plant nutrition as well as for soil biology and chemistry (Smith and Read, 2008; Brundrett and Tedersoo, 2018). As they are crucial to ecosystems, mycorrhizal fungi extensively influence plant populations and communities and are divided into four basic types: ectomycorrhizae (ECM), arbuscular mycorrhizae (AM), ericoid mycorrhizae (ErM), and orchid mycorrhizae (OM; Smith and Read, 2008; van der Heijden et al., 2015; Tedersoo et al., 2020). In this mutualistic relationship, plants receive water and mineral nutrients (particularly phosphorus) from the fungi and are protected against biotic and abiotic stresses, while host plants supply carbon from their photosynthesis (Smith and Read, 2008; Köhler et al., 2018; Tedersoo and Bahram, 2019). The strength of mycorrhizal interactions is evidenced by the evolutionary history of fungal symbionts and host plants (Selosse and Tacon, 1998; Hoeksema et al., 2018). Interestingly, OM are considered as more beneficial to orchids than fungi, which rely on other resources as saprotrophs or endophytes of non-orchid roots (Selosse and Martos, 2014). Thus, the interaction between orchids and fungi appears as interdependent asymmetry (Martos et al., 2012; McCormick and Jacquemyn, 2014; McCormick et al., 2018).

Orchids are the second largest flowering plant family after Asteraceae. In addition to being an "ideal model" group for research on the biodiversity and evolution of interactions (Selosse, 2014), this family represents a "flagship" group for the protection of endangered plants globally (Liu et al., 2015a, 2020; Zhang et al., 2017). There are nearly 30,000–35,000 species of orchids worldwide (ca.10% of angiosperms; The Plant List, 2013; Chase et al., 2015). However, more than 50% of these species are concentrated in the tropical areas of the world (Givnish et al., 2016). They reportedly colonized the epiphytic habitat approximately 35 million years ago (Givnish et al., 2015). The ratio of epiphytic to terrestrial orchids in different countries ranges from nearly 1:1 to 5:1 (Givnish et al., 2015; Zhang et al., 2015).

Fungal symbionts are essential as they provide carbon and minerals to the dust-like, reserveless orchid seeds during the early development (Dearnaley et al., 2016). The plant first develops into a spheroid organism that is achlorophyllous in most terrestrial species, called as a protocorm that later develops roots and shoots. The dependency on fungi continues until the adult stage, albeit to different degrees (Smith and Read, 2008; Waterman et al., 2011; Schweiger et al., 2018; Shefferson et al., 2018). Based on their dependence on fungal carbon, adult orchids are classified as (1) autotrophic (AT) that rely on fungi in the early developmental stage but display reduced dependence for carbon as their photosynthetic apparatus develops; (2) mycoheterotrophic (MH) that remain achlorophyllous and are completely dependent on fungal carbon throughout their life; and (3) mixotrophic (MX) orchids that can acquire carbon compounds from photosynthesis and at adulthood (Dearnaley et al., 2012). The existence of truly AT orchids is currently controversed; their isotopic difference from surrounding autotrophs for ^{13}C, ^{15}N, ^{18}O, and ^{2}H abundances suggests that they acquire some fungal biomass (Selosse and Martos, 2014; Gebauer et al., 2016; Schiebold et al., 2018). However, the net flow, considering the potential reverse flow from orchid to fungus, remains unknown in most green orchids (referred to here as AT) and the existence of C flow fungus to orchids, smaller than in the reverse direction, is anciently reported (Cameron et al., 2008).

The scientific community has been interested in orchids-fungi biological interactions, owing to pioneering research on the symbiotic germination of orchids by Noël Bernard at the end of nineteenth century (Selosse et al., 2011). The fungus is colonizing germinating seeds *via* trichomes and suspensor cells (Smith and Read, 2008) as well as root velamina and cortical cells at adult stage (Clements, 1988), further producing a large number of intracellular coiled fungal hyphae, known as pelotons and considered as a structure for exchange during their life or even at their death for C transfers to the plant (Rasmussen, 1995; Selosse, 2014; Dearnaley et al., 2016).

In-depth analysis of fungal diversity provides a better understanding of the plant-fungal interaction framework. Early culture-based methods were unable to accurately identify several isolated strains because (i) many species are uncultivable and (ii) taxonomic discrimination between morphologically similar fungal species is difficult, as exemplified by case of *Serendipita vermifera* (Cruz et al., 2011; Weiß et al., 2016; Bajpai et al., 2019). However, the development in molecular ecology through Sanger sequencing and high-throughput sequencing (HTS) technologies in the last decade has substantially improved our understanding of plant microbiota (Müller et al., 2016; Nilsson et al., 2019; Toussaint et al., 2020). Moreover, implementing these technologies to elucidate orchid-fungal interactions provided vital information about the diversity, community structure, patterns, and molecular mechanisms of this symbiosis (Martos et al., 2012; Jacquemyn et al., 2015a; Fochi et al., 2017; Miura et al., 2018; Unruh et al., 2019). Fungal partners of at least 200 genera of Orchidaceae have been identified through the analysis and assessment of orchid-associated fungi under the influence of various biotic and abiotic factors (Ma et al., 2015) such as developmental stages, habitats, or spatiotemporal scales.

To facilitate an extensive understanding of the diversity and ecological dynamics of orchid-fungal associates and to promote the resource conservation and regeneration of rare or endangered orchids with high research or commercial potential, this article summarizes six research modules: the research methods of orchid-fungal diversity, the primer selection in HTS, the fungal diversity and specificity in orchids, the difference and adaptability of orchid mycorrhizal fungi (OMF) in different habitats, the comparison of OMF in orchid roots and soil, and the spatiotemporal variation

patterns of OMF. Thus, this review provides avenues for in-depth insight into this field.

RESEARCH METHODS OF ORCHID-FUNGAL DIVERSITY

The primary research methods of orchid-fungal diversity include pure culture (dependent on isolation) and molecular identification (independent of isolation; Dearnaley et al., 2012; Kohout et al., 2013; Zettler and Corey, 2018). In fact, orchid mycorrhizal symbionts may be considered as an easy symbiotic system under experimental conditions, since both partners can be cultured aseptically in many cases (Dearnaley et al., 2012). Previous studies have demonstrated the importance of *in vitro* fungal isolation in the understanding and experimenting on OM. Moreover, the identifications of some relatively easy-to-isolate strains and biological effect detection have been reported in recent years (Meng et al., 2019a,b,c; Bell et al., 2020; Gao et al., 2020b; Zhang et al., 2020). Isolation methods mainly include isolation from whole tissue or tissue section, *in situ* seeding and trapping isolation, and single peloton isolation; of these, single peloton isolation (i.e., the isolation of pelotons from host cells by micromanipulation) is considered as the most reliable and accurate method (Zettler et al., 2005; Batty et al., 2006; Zi et al., 2014; Zettler and Corey, 2018).

Molecular identification methods mainly consist of traditional research approaches as well as sequencing *via* high-throughput platforms. Traditional methods include DNA microarrays, clone libraries, denaturing gradient gel electrophoresis, fluorescence *in situ* hybridization, and gene chips (Dearnaley, 2007; Tedersoo and Nilsson, 2016). Considering the shortcomings of these traditional methods, such as low throughput, tedious operation framework, and low accuracy, and the development and popularity of MiSeq PE300 and HiSeq PE250 platforms, the molecular identification of orchid-associated fungi has been improved, and these techniques have replaced the fastidious cloning techniques (Julou et al., 2005). HTS technologies have several advantages such as high throughput, low cost, objective reduction of microbial community structure, and trace detection of fungi (Cruz et al., 2014; Tedersoo and Nilsson, 2016; Tedersoo et al., 2018; Nilsson et al., 2019). However, they also allow the amplification of contaminants and non-OM fungi. In addition, the recent shotgun metagenomic technology using an Illumina NovaSeq/Hiseq sequencer can acquire functional gene information from all the microorganisms in a community through genomic DNA analysis (Bahram et al., 2018; Fadiji and Babalola, 2020). Together with the increasing availability of orchid and reference OM fungal genomes (Kohler et al., 2015; Zhang et al., 2016, 2017), this technology promises extraordinary progress in the study of OM.

Although isolation methods can neither acquire the uncultivable fungi, comprising a large proportion, nor determine all the taxonomic data, this method remains crucial for experiments and determining fungal functions in the symbiosis (Hyde and Soytong, 2008; Sathiyadash et al., 2012; Oliveira et al., 2014; Salazar-Cerezo et al., 2018; Raza et al., 2019). In order to effectively utilize fungi for the orchid protection and research on orchid physiology *ex situ*, it is essential to use the various types of culture media in order to isolate all the fungi that are potentially relevant for orchid growth. For this, we support single peloton isolation as the most reliable method if combined with high-efficiency molecular identification, especially if carried out at different developmental stages and in various habitats.

PRIMER SELECTION IN HTS

In fungal research, various PCR primers targeting ribosomal RNA (rRNA) locus, mainly the internal transcribed spacer (ITS), were used in the initial OM diversity studies (Selosse et al., 2004; Shefferson et al., 2005; Bonnardeaux et al., 2007). However, an accelerated rDNA sequence complicates the amplification of one of the most common OMF taxon (Taylor et al., 2002; Binder et al., 2005), the family Tulasnellaceae (see below), driving the development and optimization of OM-specific PCR primers.

Taylor and McCormick (2008) first developed specific primers ITS1-OF and ITS4-OF for studying OM diversity by amplifying the full ITS based on the sequences of Basidiomycete fungi including *Tulasnella* species. This set has been widely used for the identification of OM *via* cloning and sequencing (Xing et al., 2015, 2017, 2019; Jacquemyn et al., 2016a; Kaur et al., 2019; Rammitsu et al., 2019). The ITS4Tul primer designed by Taylor (1997) is also widely used to look for *Tulasnella* diversity along with ITS1 or ITS5 primers (Bidartondo et al., 2003; Abadie et al., 2006; Taylor and McCormick, 2008; McCormick et al., 2012, 2016). Later, an *in silico* analysis suggested that ITS3/ITS4OF and ITS86F/ITS4 primers, targeting the ITS2 sub-region of ITS, were the most ideal for orchid root samples (Waud et al., 2014), even if they not always perform well on soil samples. Moreover, because ITS2 can produce more operational taxonomic units (OTUs) and higher phylogenetic richness than the other sub-region ITS1, many researchers prefer it for fungal identification through HTS platforms (Mello et al., 2011; Tedersoo and Lindahl, 2016; Nilsson et al., 2019). In the past half-decade ITS3/ITS4OF were frequently used for identifying mycorrhizal fungal communities in orchid roots of terrestrial orchids and the surrounding soils (Jacquemyn et al., 2015a, 2017b; Esposito et al., 2016; Duffy et al., 2019). Additionally, ITS86F/ITS4 is still used for the detection of mycorrhizal partners of epiphytic orchids (Cevallos et al., 2017, 2018a; Herrera et al., 2019b; Izuddin et al., 2019; Jacquemyn et al., 2021).

Notably, ITS4OF exhibits four mismatches with 64% of Tulasnellaceae, and multiple mismatches in other assemblages of Basidiomycota and Ascomycota. Similarly, considering that ITS86F has five mismatches in 83% of Tulasnellaceae, Oja et al. (2015) tagged the modified primers ITS1ngs, ITS1Fngs and ITS4ngs, and developed the ITS4Tul2 primer for the full length of ITS. Their integrated utilization matched most of the known mycorrhizal assemblages of orchids (incl. 97% of Tulasnellaceae). Recently, a newly developed primer 5.8S-OF combined with two different reverse primers (ITS4OF and ITS4Tul) revealed good success on OMF (Vogt-Schilb et al., 2020).

Tulasnellaceae (from the order Cantharellales) are the key mycorrhizal symbionts of orchids, mainly its clades A and B. Compared to clade A, clade B is well differentiated and hardly amplified by general primers or even by Tulasnellaceae-specific primers (Girlanda et al., 2011; Lindahl et al., 2013). According to User-friendly Nordic ITS Ectomycorrhiza (UNITE) database dedicated to molecular identification of fungi,[1] approximately 3/4 of Tulasnellaceae sequences belong to clade A; however, considering strong primer and sampling biases (mainly in the Northern Hemisphere), Tulasnellaceae clade B could be underrepresented, in spite of being equally common (Oja et al., 2015). Therefore, future studies focusing on the diversity of OM should meticulously choose primers and evaluate their potential biases.

One may be aware that no primer set is perfect and consider using several sets available at the time of designing the study. Based on this and a series of works from our research team (unpublished data), it is highly recommended to sue multiple pairs of primers with low amplification overlap. They can be amalgamated and combined with a nested PCR amplification method to identify the maximum number of orchid mycorrhizal partners (Oja et al., 2015; Voyron et al., 2017; Vogt-Schilb et al., 2020). The use of three optimized primer pairs, ITS1ngs-ITS4ngs, ITS1Fngs-ITS4ngs, and ITS1-ITS4Tul2, were recommended for 454 pyrosequencing. For amplicon sequencing using MiSeq PE300 and HiSeq PE250, two primer pairs (**Supplementary Figure S1**), namely, ITS1F-ITS4 and ITS1-ITS4Tul can be recommended for the first round of amplification (Gardes and Bruns, 1993; Bidartondo et al., 2003). PCR products can be further subjected to nested PCR amplification using ITS86F-ITS4 and ITS86F-ITS4Tul primers, which capture a large diversity of mycorrhizal fungi associated with 72 varieties of tropical epiphytic orchids grown in the wild (Li et al., unpublished). For the third-generation PacBio Sequel sequencing, optimized ITS1ngs-TW14ngs, ITS1Fngs-TW14ngs, and ITS1-ITS4Tul2 primers can be recommended (see **Supplementary Table S1** for the above-mentioned primer sequences). In the future, the shotgun sequencing of roots, which provide no PCR amplification, while a fungus can be observed, may allow to unravel clades that escape all primers available, if any.

FUNGAL DIVERSITY AND SPECIFICITY IN ORCHIDS

Orchid-Fungal Diversity

Orchids are often associated with phylogenetically and ecologically diverse fungi. Basidiomycota and Ascomycota, with very few Chytridiomycota, Glomeromycota, Zygomycota, or Mucoromycota, are widely distributed in the aerial roots of epiphytic orchids and in the underground roots or rhizomes of terrestrial and lithophytic orchids (Martos et al., 2012; Waud et al., 2014; Cevallos et al., 2018a; Egidi et al., 2018; Novotná et al., 2018; Qin et al., 2019). Fungi in tissues can be divided into true OMF and orchid non-mycorrhizal fungi (ONF) based on the structures formed during in orchids. Coiled pelotons in root cortical cells are characteristic for OMF (Rasmussen, 1995; Suárez et al., 2009; Dearnaley et al., 2016); ONF are endophytic fungi that colonize roots or other tissues at a certain period during the life span of orchids but possess no peloton-like structures and cause no obvious pathogenic symptoms in host orchids (Rasmussen, 1995; Bayman and Otero, 2006; Selosse et al., 2018; Sisti et al., 2019). Most ONF in root tissues of a given orchid show no noticeable phylogenetic relationship with known OMF (Stark et al., 2009), even if some OMF may be ancient ONF that evolved mycorrhizal abilities (Jacquemyn et al., 2017b; Selosse et al., 2018).

Orchid Mycorrhizal Fungal Diversity

The most common OMF, belonging to Basidiomycota, are traditionally grouped under the name rhizoctonias, including Tulasnellaceae and Ceratobasidiaceae (belonging to Cantharellales) as well as Serendipitaceae (previously known as Sebacinales clade B). Most of these three fungal taxa have ecological niches ranging (and mixing in many cases) saprotrophy, i.e., exploiting decaying matter, and endophytism in non-orchid plants (Dearnaley et al., 2012; Selosse and Martos, 2014; Jacquemyn et al., 2017a; McCormick et al., 2018; Selosse et al., 2018). This clearly applies for Serendipitaceae at least: the famous endophytic model *Serendipita* (= *Piriformospora*) *indica* is orchid mycorrhizal in Brazilian orchids (Oliveira et al., 2014). In addition, Thelephoraceae fungi are also commonly found in some orchids, such as *Cephalanthera longibracteata* and *Liparis loeselii* (Bidartondo and Read, 2008; Jacquemyn et al., 2015a; Waud et al., 2017; Herrera et al., 2019a). Further, Selosse et al. (2004) observed Ascomycota from the genus *Tuber*, based on molecular identification, transmission electron microscope, and immunolabeling in *Epipactis microphylla*. Ascomycota were sporadically reported, e.g., as rare OMF in MX orchids, such as *Limodorum abortivum* and *Epipactis helleborine* (Girlanda et al., 2006; May et al., 2020; Xing et al., 2020), and in South-African orchids (Waterman et al., 2011).

Some AT orchids are associated with Atractiellales (Pucciniomycotina; Kottke et al., 2010; Cevallos et al., 2018b; Qin et al., 2019; Xing et al., 2019) and saprotrophic fungi from Mycenaceae or ECM fungi from Russulaceae, *Peziza*, and *Inocybe* (Waterman et al., 2011; Zhang et al., 2012; Esposito et al., 2016; Waud et al., 2017; Xing et al., 2020). Additionally, a few photosynthetic orchids associate with saprotrophic Auriculariales, Psathyrellaceae, *Tricharina*, *Clavulina*, *Armillaria*, *Marasmius*, and *Scleroderma* fungi belonging to the common ECM fungal taxa (Waterman et al., 2011; Yagame et al., 2013; Jacquemyn et al., 2016b; González-Chávez et al., 2018; Qin et al., 2019; May et al., 2020; Salazar et al., 2020). The presence of such fungi, likely in minor amounts, was greatly enhanced by the use of HTS and the reporting of all the diversity found, without *a priori* screening (Selosse et al., 2010). Although some MX orchids preserve their autotrophic ability, as shown by intact photosynthetic genes in plastid genomes (Lallemand et al., 2019), they possess few or no rhizoctonias in their roots, whereas ECM fungi

[1]https://unite.ut.ee/index.php

are dominant as far as we know (Paduano et al., 2011; Merckx, 2013; Gebauer et al., 2016; Schiebold et al., 2018). MH orchids associate with ECM fungi or non-rhizoctonia saprotrophic fungi, including wood or litter decomposers (Martos et al., 2009; Hynson et al., 2013; Lee et al., 2015; Kinoshita et al., 2016; Ogura-Tsujita et al., 2018). However, a few MH and MX orchids may superficially look like associated with rhizoctonias; for example, the MH *Rhizanthella gardneri* (Bougoure et al., 2009) or the MX *Platanthera minor* (Yagame et al., 2012) with *Ceratobasidium* spp., but which belong to a sub-clade forming ECM.

In all, OMF have been reported from at least 17 families of Basidiomycetes and five families/genera of Ascomycetes (Dearnaley, 2007; Waterman et al., 2011; Dearnaley et al., 2012). Notably, dark septate endophytes with melanized hyphae, mainly include the members of Helotiales with variable impact on roots (Newsham, 2011), have been observed in some AT and MX orchids, adding to the fungal diversity in orchid (Calvert, 2017; Schiebold et al., 2018).

During the orchid life cycle, OMF communities can change, although general trends are established from a limited number of species. Very often (but not always), OMF diversity tends to decline from the seed stage to the seedling stage and to often increase again in the adult stage in an hourglass-like pattern. In terrestrial orchids, OMF from protocorms or seedlings are conventionally subsets of those found in early germinating seedlings and adult plants (Bidartondo and Read, 2008; Jacquemyn et al., 2011a; Waud et al., 2017). This has been observed for culturable *Tulasnella* species (Meng et al., 2019a). In addition, recent investigations indicate continuous OMF community dynamics at the different stages of orchid life cycle (Cevallos et al., 2018b). To summarize, orchids are subject to internal and external natural conditions, potentially promoting symbiotic benefits (McCormick et al., 2016, 2018).

Orchid Non-mycorrhizal Fungal Diversity

The ecological adaptability of ONF facilitates their wide distribution, covering over 110 genera, of which 76 genera belong to Ascomycetes with much higher diversity and occurrence frequency than those of OMF (Sudheep and Sridhar, 2012; Ma et al., 2015). The cultivability of most of them makes their isolation relatively easy. Xylariales (e.g., *Xylaria* spp. and *Hypoxylon* spp.) and Helotiales (e.g., Helotiaceae and Hyaloscyphaceae) are the main ONF associated with tropical and temperate orchids, respectively; Chaetothyriales, Hypocreales, Helotiales, and Capnodiales are also frequent in tropical epiphytic orchids (Bayman et al., 1997; Dearnaley et al., 2012; Oliveira et al., 2014; Govinda Rajulu et al., 2016; Jacquemyn et al., 2016a; Beltrán-Nambo et al., 2018; Novotná et al., 2018). In addition, *Colletotrichum*, *Fusarium*, and *Trichoderma* fungi are generally found in the roots of various orchids from tropical and temperate zones (Martos et al., 2012; Tao et al., 2013; Sufaati et al., 2016; Salazar-Cerezo et al., 2018; Sisti et al., 2019; Sarsaiya et al., 2020).

These taxa are well-known endophytes in many plants (Rodriguez et al., 2009; Selosse et al., 2018) and entail no known disease symptoms. In contrast, a very small number of potential plant pathogens have been proven to support the growth and development of orchids to some extent. For example, *Fusarium* fungi are known to promote an early seed germination of some *Cypripedium* and *Platanthera* spp. (Vujanovic et al., 2000; Bayman and Otero, 2006), and *Fusarium* have been suggested to be OMF in a few orchids (Jiang et al., 2019; Sisti et al., 2019). Interestingly, *Colletotrichum* enhance the growth of adult individuals belonging to *Dendrobium* spp., despite its high pathogenicity on seedlings (Shah et al., 2019; Sarsaiya et al., 2020).

Currently, *in vitro* investigations on the functions of ONF during the life cycle of orchids are limited, even though at least 65 ONF genera have been successfully isolated and cultured (Novotná et al., 2018; Meng et al., 2019a,b,c; Sarsaiya et al., 2019; Shah et al., 2019; Bell et al., 2020). It is crucial to further investigate the ONF that frequently occurs in orchids roots, in order to determine their potential physiological and ecological advantages. Active compounds produced by some ONF may prove beneficial for orchids by improving their resistance to abiotic stresses, thereby promoting their adaptability to different environmental conditions (Ma et al., 2015) or by protecting against pathogens and herbivores. Some ONF may even decompose local substrates and provide some nutrients to the orchids (Tedersoo et al., 2009; Waterman et al., 2011; Herrera et al., 2019a). Considering the potential significance of ONF, it is crucial to analyze the balance between OMF and ONF (including potential pathogens) in orchid ecology (Nilsson et al., 2019).

Orchid Mycorrhizal Fungal Specificity

Orchids interact with a more limited set of mycorrhizal fungi as compared to other mycorrhizal plants, with a relatively higher degree of specificity for OM than ECM, AM, and even ErM fungi (Dearnaley et al., 2012; van der Heijden et al., 2015; Suárez and Kottke, 2016; Põlme et al., 2018). Mycorrhizal specificity, one of the core issues in OM research, ranges from low to high and can be quantified as the phylogenetic width of the range of associated mycorrhizal fungi (i.e., the ancientness of the last common ancestor). Moreover, despite some phylogenetic conservatism in mycorrhizal partners (Martos et al., 2012) and specificity in orchids (Jacquemyn et al., 2011b), specificity level is a labile evolutionary trait (Irwin et al., 2007; Shefferson et al., 2007, 2010). According to the degree of specialization and the ecological opportunities for interactions and following Shefferson et al. (2019), specificity can be subdivided into assemblage specialization (combined with specific orchid hosts; the interactions are less affected by the environment), apparent generalism (combined with a few orchid hosts; there is a certain correlation between interactions and the environment), and true generalism (combined with multiple orchid hosts; the interactions are greatly affected by the environment).

The OM specificity may be affected by environmental factors, climate changes, extreme host selections, evolutionary history, accompanying plant species, biocompatibility, biogeographic range, and density of OMF in the soil or, for epiphytes,

phorophytes (Jacquemyn et al., 2011b, 2017a; McCormick et al., 2012, 2018; Pandey et al., 2013; Waud et al., 2016a; Shefferson et al., 2019; Xing et al., 2019), thus showing strong and complex variations. For example, *Neottia* and *Caladenia* prefer symbiosis with sebacinales fungi (Těšitelová et al., 2015; Phillips et al., 2016; Reiter et al., 2020); the rare terrestrial orchid *Caladenia huegelii* specifically associates with *Serendipita (= Sebacina) vermifera* (Swarts et al., 2010); *Cypripedium*, *Ophrys*, and *Chiloglottis* prefer Tulasnellaceae (Shefferson et al., 2005, 2019; Roche et al., 2010; Schatz et al., 2010); *Dendrobium nobile* and *Liparis japonica* have high specificity for Tulasnellaceae (Ding et al., 2014; Xing et al., 2017); *Pterostylis nutans* and *Sarcochilus weinthalii* are only symbiotic with *Ceratobasidium* fungi (Irwin et al., 2007; Graham and Dearnaley, 2012); *Platanthera leucophaea* tends to be associated with *Ceratobasidium* fungi over a 10-year period (Thixton et al., 2020); *Corallorhiza trifida* shows high specificity for Thelephoraceae in different countries and varied habitats (McKendrick et al., 2000; Zimmer et al., 2008).

Jacquemyn et al. (2010) asserted the possibility of promoting widespread associations between orchids and available OMF in an environment devoid of water and nutrients. However, despite the diminished availability of groundwater and nutrients, the associated Tulasnellaceae showed an increasing trend of specificity from terrestrial to epiphytic and lithophytic orchids (Xing et al., 2019). Moreover, OMF of lithophytic orchids are mostly Tulasnellaceae. The specificity may also differ between biogeographic regions. Mainland Australia appears to have a relatively higher incidence of mycorrhizal specificity than, e.g., South Africa, Eurasia, and North America (Roche et al., 2010; Phillips et al., 2011, 2016; Davis et al., 2015; Jacquemyn et al., 2017a; Reiter et al., 2020). For instance, the genus *Corycium* of subtribe *Coryciinae* is almost exclusively symbiotic with *Peziza*, and all orchids in *Disperis* only associate with Ceratobasidiaceae (Waterman et al., 2011; Jacquemyn et al., 2017a). Furthermore, MX orchid mycorrhizae often have lower specificity than AT and MH and culmulatively associate with various ECM fungi or rhizoctonias (Abadie et al., 2006). However, there are a few specific MX orchids, such as *P. minor* and *L. abortivum* that specifically associate with ECM *Ceratobasidium* fungi and *Russula delica*, respectively (Girlanda et al., 2006; Yagame et al., 2012). In a *Pinus thunbergii* plantation, the MX *E. helleborine* displayed abundant *Wilcoxina* fungi, but whether this is an OMF remains unclear (Suetsugu et al., 2017), while this species is normally poorly specific (May et al., 2020).

A few studies address the specificity of OM at different stages of orchid life cycle. Bidartondo and Read (2008) detected fungus-specific bottleneck at the seedling stage of *Cephalanthera* spp. Těšitelová et al. (2012, 2015) studied the OMF assemblages of *Neottia* and *Epipactis* species in protocorms and adult, indicating that differences in OMF diversity were scarce (as for the single *Epipactis* species studied in Bidartondo and Read, 2008). Recently, OMF identification in a large number of samples of the Japanese epiphytic *Taeniophyllum glandulosum* revealed specific link to Ceratobasidiaceae throughout the entire life cycle and whatever the phorophyte (Rammitsu et al., 2019). *Ceratobasidium* spp. highly specifically associate with two rare and endangered *Bipinnula* orchids at the adult stage and effectively promote seed germination (Claro et al., 2020). However, the specificity of OMF sometimes varies at different developmental stages. For example, *Gastrodia elata* relies on fungi, such as *Mycena osmundicola*, during seed germination and protocorm development, while adults associate with *Armillaria mellea*, despite it inhibits the germination seeds to a certain extent (Ran and Xu, 1988; Xu and Guo, 1989). The invasive *Oeceoclades maculata* associates with multiple saprotrophic fungi and rhizoctonias at the adult stage; however, during *in vitro* germination, seeds retain high specificity for *Psathyrella* spp. (Bayman et al., 2016).

High OMF specificity is often observed in orchids with habitats specialized or those distributed in the southern hemisphere (Davis et al., 2015). On the one hand, this may be attributed to an extreme selection of host orchids during the recent historical adaptation; while on the other hand, it may be a result of a low local OMF diversity (Illyés et al., 2009; Swarts et al., 2010; Waud et al., 2017). Mycorrhizal specificity does not limit the distribution range or rarity of at least some orchids (Shefferson et al., 2007; Phillips et al., 2011, 2016; Bailarote et al., 2012; Pandey et al., 2013; Waud et al., 2017), since specificity for a widespread fungus is not limitative. Indeed, OMF associated with orchids exhibiting high mycorrhizal specificity tend to have a wide distribution (Jacquemyn et al., 2011b; Swift et al., 2019; Thixton et al., 2020). Even OMF promoting the germination of narrowly distributed orchids may display a wider distribution (Oktalira et al., 2019).

The adaptive significance of OM specificity relies on three points. First, specificity increases the germination rate of seeds and thus improves fitness by survival. Second, it may enhance the efficiency of nutrient exchange between orchids and OMF, thereby promoting the efficiency of bidirectional carbon flow in OM symbiosis, or even, facilitating the absorption of carbon by MH and MX orchids (Bonnardeaux et al., 2007; Suetsugu et al., 2017); such exchange improves fitness by seed set (Roy et al., 2013). Conversely, more effective nutrient exchange is hypothesized one of the driving factors for OM specificity (Selosse et al., 2002; McCormick et al., 2006; Nurfadilah et al., 2013). Third, specificity facilitates the formation of patchy distribution of orchids under natural conditions, and this affects gene flow, population dynamics, and pollination systems (Tremblay et al., 2005).

DIFFERENCE AND ADAPTABILITY OF OMF IN DIFFERENT HABITATS

Habitats, including macro- and micro-habitats, indirectly affect the coexistence and widespread distribution of orchid species by affecting the composition, structure, and richness of OMF communities (**Figure 1**). The community composition of OMF associated with the same orchid species distributed in different habitats, different orchids co-occurring in the same habitat, or different individuals of most orchid

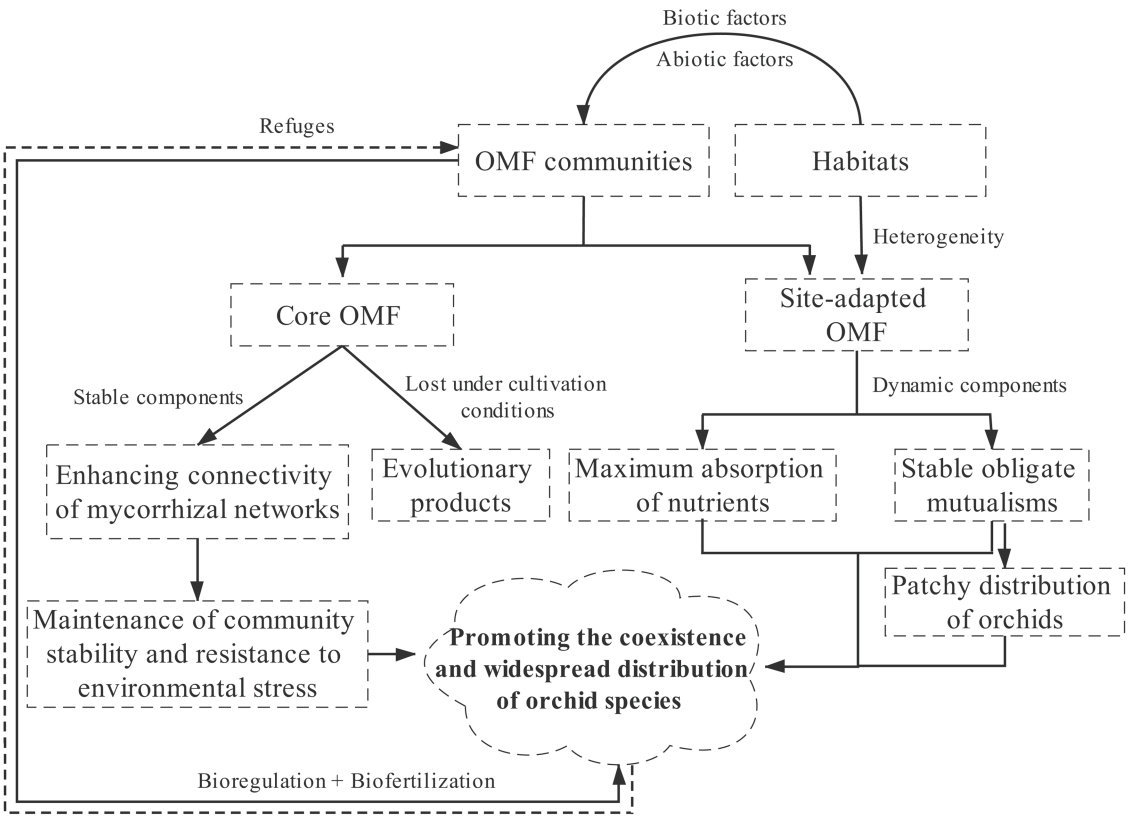

FIGURE 1 | A framework depicting how habitats indirectly affect the coexistence and widespread distribution of orchid species by affecting OMF communities. OMF communities shaped by different habitats affect the growth and development of orchids (bioregulation) by promoting nutrient absorption (biofertilization). The dotted arrow indicates that the current understanding of the impact of orchids on OMF life cycle is very limited.

species in the same habitat can differ to some extent (Martos et al., 2012; Jacquemyn et al., 2014, 2015a, 2016a, 2021; Waud et al., 2017; Pecoraro et al., 2018; Duffy et al., 2019).

From a macrohabitat perspective, most orchids distributed in the Mediterranean shrublands, temperate deciduous forests, boreal forests, and tropical forests are associated with a large number of mycorrhizal partners, with the most prominent OMF families being Tulasnellaceae and Ceratobasidiaceae, ECM basidiomycetes, Sebacinaceae and Thelephoraceae, and rhizoctonias (*Tulasnella* as the most common taxon; Jacquemyn et al., 2017a). Concurrently, habitat heterogeneity or local environmental conditions also substantially affect the OMF communities of different populations or at different sites of the same species of orchids. For example, for nine *Neottia ovata* populations of different sizes, the orchid individuals in the most central part exhibit significantly different OMF communities (Jacquemyn et al., 2015b). Oja et al. (2015) corroborated that *N. ovata* in grasslands and forests have different OMF compositions (see also Těšitelová et al., 2015). Of note, greater habitat differentiation may lead to more significant mycorrhizal association variation among *Dactylorhiza* species inhabiting different sites than among *Orchis* species (Jacquemyn et al., 2011b, 2016b). Considering the continent-wide scale, strong turnovers are witnessed in the OMF communities associated with *Gymnadenia conopsea* and *E. helleborine* from Europe to China (Xing et al., 2020), although the genetics of the plant itself can somewhat change over such a distance. Furthermore, some AT orchids distributed in the Mediterranean grasslands, Australia, and tropical montane rainforests also display significant changes the OMF in different habitats or populations (Ramsay et al., 1987; Pandey et al., 2013; Jacquemyn et al., 2014, 2016a; Cevallos et al., 2017; Waud et al., 2017; Herrera et al., 2018; Duffy et al., 2019). These reports indicate that the availability of OMF does not restrict the distribution of orchids, but that their occurrence is bounded by specific ecological habitats.

From a microhabitat perspective, sympatric orchids are usually associated with different OMF (Martos et al., 2012; Jacquemyn et al., 2015a, 2016a, 2021; Cevallos et al., 2017; Qin et al., 2019). If two or more species are equally dependent on the same available resources, theoretical models predict that they cannot coexist (niche exclusion); indeed, at small-scale, associating with different mycorrhizal fungi facilitates rational division of the niche space resources among cohabiting species by reducing competition and avoiding indirect exploitation due to asymmetrical benefits from shared fungi (Tilman, 1982; Selosse et al., 2004; McCormick and Jacquemyn, 2014; Gerz et al., 2018). Simultaneous association with multiple OMF taxa may

maximize the absorption of nutrients as well as stabilize obligate mutualisms with respect to robustness. Notably, the OMF composition and community structures of *Paphiopedilum dianthum* and *P. hirsutissimum* that often coexist (i.e., almost same microhabitats) on limestone vary significantly (Li et al., unpublished); hence, the co-occurring orchid species with different OMF compositions may also result from a strong OMF selection by the host orchids (Jacquemyn et al., 2015a; Xing et al., 2019).

However, some reports suggest that sympatric orchids, conspecific orchids in different populations or sites, or even epiphytic and terrestrial orchids in different habitats share some generalized OMF. These OMF may have stronger ecological adaptability and are commonly found in orchid mycorrhizal networks, which may maintain community stability under environmental stresses or variations (Kottke et al., 2013; Pandey et al., 2013; Suárez et al., 2016; Pecoraro et al., 2017; Xing et al., 2017; Herrera et al., 2018, 2019b). To better define these specific fungal guilds, Cevallos et al. (2017) proposed the "keystone species" theory based on their study of OMF communities associated with epiphytic orchids: the OMF community of a given orchid species in different sites contains a core (permanent components or keystone species), and site-adapted mycorrhizal fungi (dynamic components). This has also been observed in the OMF communities of seedlings transplanted at different elevation gradients (Cevallos et al., 2018b; Herrera et al., 2019b). Interestingly, studies have shown that some orchids either have completely different OMF or share few OMF under natural habitats and cultivation conditions (Downing et al., 2017; Qin et al., 2019). Therefore, the selection among habitats, host orchids, and OMF is more complicated than previously perceived. Comparative studies at larger scales will shed light on rules followed by these symbiont assemblies.

Similar to all soil fungi, AM, ECM, pathogens, and saprotrophic fungi, the composition and richness of OMF communities also differ according to various ecological factors, such as soil physical and chemical properties (e.g., P abundance), tree richness, altitude, mean annual precipitation, and mean annual temperature (Geml et al., 2014; Barnes et al., 2016; Geml, 2017; Hu et al., 2019; Mujica et al., 2020). Elucidating the interplay between biotic and abiotic factors in shaping OMF communities is vital for conservation practices such as introduction and artificially assisted colonization (the transfer of individuals from the current natural range to potential new habitats to adapt to climate change).

In terms of biotic factors, changes in habitat vegetation influence the OMF communities associated with orchid roots (Pandey et al., 2013; Herrera et al., 2018), by changing soil or even plants in which rhizoctonias are endophytes. Especially, some terrestrial MX or MH, if not AT, orchids often tend to share fungi with surrounding trees (Bidartondo and Read, 2008; McCormick et al., 2009; Jacquemyn et al., 2016a). Moreover, orchid species living on different phorophytes or different epiphytic niches on the same phorophyte tend to associate with different OMF (Wang et al., 2017; Rasmussen and Rasmussen, 2018), although broader validation of this expected.

With respect to abiotic factors, several studies suggest that many soil characteristics (e.g., soil water content, pH, organic matter content, nutrition level, trace elements, and textural components) explain the distribution of OMF in habitat patches (Jacquemyn et al., 2015b; Esposito et al., 2016; Waud et al., 2017; Kaur et al., 2019; Vogt-Schilb et al., 2020). Depending on the model, the OMF communities seem to be significantly related to the hydrolyzable and extractable nitrogen in the soil (Han et al., 2016; Duffy et al., 2019) and to the P availability (Mujica et al., 2020), but a general view is pending, beyond existing case studies. Factors, such as rainfall and humus type, predict and affect the presence and richness of OMF-associated epiphytic orchids (Izuddin et al., 2019). Interestingly, although epiphytic orchids and terrestrial orchids exhibit different responses to light at the early growth, OMF have parallel ecological importance in overcoming the photoinhibition during germination and early growth (Alghamdi, 2017).

Considering the effect of latitude on the species and functional group richness of OMF in orchids, diversity decreases with increasing latitude; however, whether the orchid diversity follows similar trends remains unclear (Jacquemyn et al., 2017a; Duffy et al., 2019). The diversity of orchids has been proven to initially increase and then decrease with increasing altitude, with a peak in the mid-altitude area (approximately 1,000–1,600 m; Küper et al., 2004; Cardelús et al., 2006; Acharya et al., 2011). At present, no reports suggest changes in OMF communities under an altitudinal gradient. Studies on high-altitude tropical southern Ecuador montane rainforests reported that some epiphytic orchids have higher richness or abundance of OMF at relatively high altitudes (Cevallos et al., 2018a; Herrera et al., 2019b). However, Cevallos et al. (2018a) insisted that high-altitude areas (3000–3,500 m) showed no significant difference in fungal communities among different orchid species or sites, while in low-altitude areas (2,050–2,800 m), host orchids and sites were the two major drivers of OMF communities' composition. Cevallos et al. (2018b) suggest that OMF display rich diversity in mid-altitude areas (approximately 2,050 m), but this is limited to a narrow altitude range (1,850–2,100 m). Therefore, whether the OMF composition and community structure are opportunistic associations (Dearnaley et al., 2012) or exhibit certain trends under large altitudinal gradients is still under scrutiny.

At the same latitude, orchids in areas with higher temperatures and fewer seasonal changes exhibit higher OMF diversity. Environmental conditions, such as high temperature or high humidity, may increase the activity of some saprotrophic fungi, enabling them to obtain more carbon so as to support the growth of MH orchids (Martos et al., 2009; Shefferson et al., 2019). Recently, it was proposed that the colonization of some dominant OMF in orchid roots is significantly affected by rainfall (McCormick et al., 2006; Kartzinel et al., 2013; Jasinge et al., 2018). Among these, Tulasnellaceae increase with increasing rainfall, indicating their hydrophilic nature. Similarly, Ceratobasidiaceae, a dominant assemblage in dry habitats, displays a higher resistance to drought. Thus, climate may also be a major driver for the turnover of orchids and OMF at different sites (Kottke et al., 2013).

In conclusion, climatic changes may strengthen the filtering effect of environmental factors on OMF associated with orchids by perturbing biological interactions. Furthermore, in addition to ecological factors, the phylogeny, ploidy levels, and genome

composition of host orchids as well as the sampling effort itself influences the OMF communities (Shefferson et al., 2007, 2019; Jacquemyn et al., 2011b, 2016b; Těšitelová et al., 2013). Therefore, OMF diversity is expected to intertwine multiple factors.

COMPARISON OF OMF IN ORCHID ROOTS AND SOIL

Despite extensive research on the diversity of OMF colonizing orchid roots, their spatial distribution and abundance in soil has received more limited attention. Several reports suggest that OMF either exist in saprophytic form in the soil or form ECM or endophytic colonization on adjacent plants (Girlanda et al., 2011; Dearnaley et al., 2012; van der Heijden et al., 2015; Oberwinkler et al., 2017); however, the distribution of OMF in soil remains unclear (Voyron et al., 2017). Analyzing and understanding this is vital for restoring the populations of endangered orchids and for artificially-assisted colonization.

Early seed germination experiments and a few pieces of molecular evidence revealed that most OMF distributed in orchid roots are also widely present in the soil. Moreover, with increase in radial distance between the soil sampling point and the adult orchid, the corresponding OMF richness and abundance often decreased (**Table 1**). Interestingly, the overall similarity between OMF communities in orchid roots

TABLE 1 | Comparison of OMF in orchid roots and soil.

Orchid species	Number of Orchid species[a]	Methods	PCR primers	Number of PCR primer pairs[b]	OMF in orchid roots		References
					was comparable to that in soil[c]	was sporadic or undetected in soil	
Caladenia arenicola	1	Seed germination	Uninvolved	Uninvolved	Yes	No	Batty et al., 2001
Neottia nidus-avis	1	Seed germination	Uninvolved	Uninvolved	Yes	No	McKendrick et al., 2002
Goodyera pubescens *Anacamptis morio*	1	Seed germination	Uninvolved	Uninvolved	Yes	No	Diez, 2007
Gymnadenia conopsea	3	Seed germination	Uninvolved	Uninvolved	Yes	No	Jacquemyn et al., 2012
Orchis mascula *Anacamptis morio*							
Gymnadenia conopsea	3	454 pyrosequencing	ITS86F/ITS4 ITS3/ITS4OF	2	Yes	No	Waud et al., 2016a
Orchis mascula							
Orchis mascula *Orchis purpurea*	2	Spatial point pattern analysis + qPCR	OTU1f_1g/Tul_r1 OTU2f_2g/Tul_r3 GIS-B159 F/R	2	Yes	No	Waud et al., 2016b
Goodyera pubescens *Liparis liliifolia* *Tipularia discolor*	3	Seed germination + ABI Sequencing + qPCR	SW-2779-59-1 F/R ITS-Lip1/ITS4-tul Tip14F/Tip14R Tip2_F1/Tip14R	6	Yes	No	McCormick et al., 2016
Corallorhiza odontorhiza	1	ABI sequencing + Seed germination	ITS5/ITS4-tul SSU1318-Tom/LSU-Tom2 ITS1-P/ITS4 SSU1318-Tom/ITS-Tom4 SSU1318-Tom/ITS4	5	Yes	Yes	McCormick et al., 2009
Cypripedium calceolus *Neottia ovata* *Orchis militaris*	3	454 pyrosequencing	ITS1-F/LSU-Tom2 ITS1ngs/ITS4ngs ITS1Fngs/ITS4ngs ITS1/ITS4-Tul2	3	No	Yes	Oja et al., 2015
Neottia ovata	1	454 pyrosequencing	ITS1OF-C/ITS4OF ITS1OF-T/ITS4OF	2	No	Yes	Jacquemyn et al., 2015b
Paphiopedilum spicerianum	1	Illumina MiSeq sequencing	ITS3/ITS4OF	1	No	Yes	Han et al., 2016
Anacamptis morio *Ophrys sphegodes*	2	Illumina MiSeq sequencing	ITS1F/ITS4 ITS3mod/ITS4	2	No	Yes	Voyron et al., 2017
Platanthera praeclara	1	Illumina MiSeq sequencing	ITS1-OF/ITS4-OF ITS1/ITS4-TUL	2	No	Yes	Kaur et al., 2019

[a]*number of orchid species in the first column corresponding to each reference in the last column.*
[b]*number of PCR primer pairs in the fourth column corresponding to each reference in the last column.*
[c]*distribution and abundance of the potential OMF in soil decreased with an increase in the sampling distance from adult orchids.*

among different populations was higher than that in the soil (Jacquemyn et al., 2015b), whereas many microhabitats near the adult orchids did not support seed germination due to the absence of suitable OMF (Taylor et al., 2010; McCormick et al., 2016; Waud et al., 2016a). These results clearly indicate that OMF are unevenly distributed in the soil and that OMF community display small-scale patchiness in the soil, as other mycorrhizal fungi (Richard et al., 2005; Pickles et al., 2010). This likely contributes to the clumped distribution and patchiness of orchids, as common observed for terrestrial orchids.

Growing evidence proves a higher abundance of OMF in orchid roots than in surrounding soil, thus deserving further investigation. Several studies have stated that the germination rate of seeds remains constant regardless of the presence or absence of adult orchids, whereas the physical and chemical properties of soil have significant effects on seed germination (McCormick and Jacquemyn, 2014; Waud et al., 2017). This suggests that the richness and abundance of OMF are not correlated with the distance from the host orchids, as previously reported. In concert with this, increasing molecular evidence supports the widespread dominance of OMF in orchid roots that either have sporadic occurrence or are undetected in the soil (Egidi et al., 2018), while a few OMF that specifically associate with roots exist with very low relative abundance in orchid-occupied locations (**Table 1**). Moreover, Voyron et al. (2017) found that OMF displayed significant horizontal spatial autocorrelation in soil, whereas their relative abundance had no significant correlation with the distance from adult orchids. In addition, dominant OMF in the tubers of the chlorophyll-free orchid *Gastrodia flavilabella* were rarely found in the surrounding soil (Liu et al., 2015b).

These reports further support the proposal put forth by Selosse (2014) that the orchid roots could possibly be a protective refuge for some fungi that protects them from external factors (**Figure 1**), at least in some seasons. Thus, some OMF abundant in orchid roots are absent in the surrounding soil, at least for a part of the year. On one hand, independent or opposed spatiotemporal changes in OMF distribution between orchid roots and soil could explain their absence in the soil at the time of root sampling, although the presence of OMF communities in soil looks relatively stable with almost no turnovers during the entire vegetation growth period (Oja et al., 2015; Voyron et al., 2017). On the other hand, it may be because the OMF belong to short-distance exploration type, i.e., the kind of mycorrhizal fungi that efficiently collect nutrients in a limited volume of soil around the rhizosphere (Peay et al., 2011; Voyron et al., 2017). This was confirmed by a study combining the rhizosphere soil and orchid-occupied bulk soil of the Australian *Diuris fragrantissima*, where a *Tulasnella* fungus was only detected in the rhizosphere soil (Egidi et al., 2018). Additionally, the fungi that take refuge in orchid roots may have distinct ecological characteristics. For instance, *Tulasnella* spp. cannot absorb nitrate in the soil, whereas habitats rich in nitrogen and phosphorus tend to have more abundant *Ceratobasidium* fungi (Nurfadilah et al., 2013; Mujica et al., 2016, 2020; Fochi et al., 2017; Vogt-Schilb et al., 2020).

In summary, discrepancies in the distribution of OMF between adult roots and surrounding soil were revealed by seed germination experiments and barcoding. Several factors may have affected the results in each study, such as the physical and chemical properties of the soil, the difference in amplification ability of the same primer in orchid roots and soil, the quality and volume of samples analyzed for molecular identification, the time and site of experiment, and the specificity of each species. Therefore, seed germination and barcoding should be optimized and combined in the future to explore the correlation between OMF communities in roots and in soil. The current discrepancies regarding this issue also pose a great challenge; specifically, do OMF affect the distribution of orchids or orchids affect the distribution of OMF?

SPATIOTEMPORAL VARIATION PATTERNS OF OMF

The spatial and temporal linkages between host orchids and their associated OMF communities remain largely unexplored. In the past decade, the combination of *in situ* experiments, isolation and culture *in vitro*, and molecular identification of extensive root samples under natural conditions has led to an understanding of spatiotemporal variations of OMF communities, giving some clues on their control.

Spatial Variation Patterns

As described in the previous section, there are significant spatial variations in OMF in soil. Similar to other mycorrhizal types and general soil fungal communities at small scales, the distribution of OMF in the soil shows non-random spatial distribution characteristics, and a significant horizontal spatial autocorrelation within 10 m of adult orchids is observed (Waud et al., 2016b; Voyron et al., 2017). In contrast, the detection results of fungi in the soil at a distance of 0–32 m from two host orchids (*Orchis militaris* and *Platanthera chlorantha*) in 21 semi-natural calcareous grasslands suggested that the available evidence for spatial clustering of OMF on a local scale was limited (weak spatial structure), while ONF displayed a more distinct spatial distribution pattern (Oja et al., 2017). The characterization of *Platanthera praeclara*-associated OMF naturally distributed in the tallgrass prairie in North America was studied for many years, and almost all populations and several phenological stages of different years demonstrated high spatiotemporal specificity to a certain Ceratobasidiaceae fungus (Kaur et al., 2019).

Although previous studies provided the evidence that there is no or only minute effect of geographical proximity on the similarity between OMF communities (Jacquemyn et al., 2015b, 2016a; Swift et al., 2019), the recent transcontinental comparisons of OMF associated with two widely distributed Eurasian terrestrial orchids revealed that the OMF community composition of the two orchids has significant turnover in Europe and China, and the similarity between OMF communities decreases significantly with increasing geographical distance (Xing et al., 2020). Significant progress has been made with respect to the spatial distribution of OMF in recent years; however, there are limited reports on the fine spatial distribution of OMF in the different parts of root segments of different ages, different

tissues of orchids (i.e., plant niches), and different parts of cortical cells (middle, close to the epidermis, close to the steles, etc.), which should be given more attention in the future.

Temporal Variation Patterns

Compared to spatial distribution, the contemporary understanding of OMF temporal dynamics is more limited. Orchids are often associated with different OMF at different stages of their life cycle and during their growth and development, and these shifts may mediate their health and ecosystem service functions. Early studies on the temporal variation of orchid-fungal symbionts revealed that seasonal OMF turnover might occur in orchids with annual underground structures (Taylor and Bruns, 1999), which was later confirmed in few tuber-shaped orchids (Huynh et al., 2009; Kohout et al., 2013; Oja et al., 2015). Nevertheless, there are increasing examples of variations in OMF associated with perennial root system orchids over time (Rasmussen and Whigham, 2002; Oja et al., 2015; Jasinge et al., 2018).

A recent study demonstrated that extremely heavy rainfall may drive *Ceratobasidium* sp., which is usually the dominant mycorrhizal fungus colonizer in *Pterostylis revoluta*, to elimination by *T. calospora*; *Ceratobasidium* sp. re-dominates after the magnitude of rainfall returns to a normal level (Jasinge et al., 2018). The fungal community composition and the most abundant fungal genera of the critically endangered *P. spicerianum* tend to vary in rainy and dry seasons, and the abundance of Tulasnellaceae declines significantly during the dry season (Han et al., 2016). Similarly, Kartzinel et al. (2013) found that the reduction in the diversity of Tulasnellaceae associated with a rare tropical epiphytic orchid was related to reduced seasonal precipitation. Furthermore, although the infection rate of *Tulasnella* fungi in the roots and tubers of *Pseudorchis albida* is higher during summer (Kohout et al., 2013), the molecular identification of pelotons isolated manually from *Anacamptis morio* during the complete growing season indicates that *Tulasnella* fungi are more common during winter and autumn, while *Ceratobasidium* and Pezizacean clade tend to dominate during summer and spring, respectively (Ercole et al., 2015). These results provide strong proof that interactions between orchids and OMF are closely related to the season, if not seasonal climatic changes. At a finer time scale, Oja et al. (2015) confirmed the turnover of OMF colonizing the roots of *N. ovata* to be 1 month and that their richness increases at the beginning of the flowering period. Interestingly, the diversity and richness of the detected total fungi tended to show a similar trend, and the flowering period was often accompanied by a significant enrichment of ^{15}N (Herrera-Rus et al., 2020; Zeng et al., 2021). One of the explanations for the high diversity of fungal partners at flowering period could be host orchids recruit a large number of fungal assemblages that can supplement the nutrients consumed by blooming by releasing specific signals, and that they may even be crucial to the successful completion of the pollination process. In addition, the lower proportion of OMF in the soil of meadow habitats also demonstrated a slight but significant turnover over time (Oja et al., 2015).

Most of the above-mentioned reports mainly focused on adult orchids, and other developmental stages were only studied by Cevallos et al. (2018b); they combined the seedling trapping experiment and molecular identification to confirm that the composition of the OMF community associated with the seedlings of two epiphytic orchids showed significant temporal variation. In addition, seasonal (or phenological) variation in the diversity of most mycorrhizal types (including OM) is generally only sampled for 1 year at present. Thus, whether these turnovers follow interannual cycles and whether the dynamic fungal assemblages can be attributed to the soil fungal community changes require in-depth explorations at interannual scales across several sampling sites.

Driving Force for Spatiotemporal Turnovers

Recent studies have proposed that the turnover rate of fungal communities observed in space is relatively smaller than the annual turnover rate; the short-term fluctuations in community abundance are mainly affected by spatial variability, while long-term fluctuations are influenced by the time variability factor (Averill et al., 2019; Ji et al., 2019). The mechanism that drives the spatiotemporal turnovers of OMF is unlikely to be the same; host orchids may select different fungal associates with spatiotemporal variation (Jacquemyn et al., 2015a; Oja et al., 2015; Xing et al., 2019), while different degrees of spatiotemporal dynamic turnovers of the fungal species pool in soil may exist (Ercole et al., 2015). Certain environmental and climate covariates can be used to deduce a few spatiotemporal effects.

Further, the extent to which these spatiotemporal variations depend on the nutrition strategies of host orchids, random dissemination process of OMF, habitat types, seasonality, functional differences within roots, and succession remains to be elucidated. From the perspective of nutrition strategies, the spatiotemporal turnovers of OMF can be attributed to (1) the different nutritional requirement of host orchids at different spatiotemporal points; (2) the differences in the ability of OMF to grow in soil and provide nutrients; and (3) the different adaptability of OMF to environmental conditions (especially harsh environments). Notably, fungal dormancy may play a key role in the spatiotemporal turnover of fungal communities, since dormancy can bypass environmentally imposed choices and allow the consistent presence of genetic variations in the environment, thereby increasing the genetic diversity of fungal communities (Cordovez et al., 2019).

FUTURE DIRECTIONS

Fungi are a major pillar of biodiversity in ecosystems, occupying a wide range of niches. Owing to the relatively easy growth of some fungi, fungal diversity and strain collection have always been hot spots in the field of fungal research (Hyde et al., 2019). OM symbiosis is an excellent model for investigating the biological interactions between plants and fungi that would help to solve the fundamental questions on interactions between aboveground and underground pollinators and fungi, respectively (Selosse, 2014; Favre-Godal et al., 2020). Orchids have complex symbiotic relationships with fungi at various stages of their life cycle. Understanding of OMF diversity is just the beginning of a series of interesting scientific explorations.

The emergence of culture methods that differ from traditional technologies, such as genomic information methods based on membrane protein expression and microfluidic technology (Tang, 2020), brings hopes for solving the fungal-specific bottleneck faced by some orchid species that need specific fungal switches to move onto the adult developmental stage after germination. Moreover, considering the continuous development of sequencing technologies, it is expected that the functional diversity of orchid-fungal communities and the complexity of spatiotemporal dynamics will be deciphered in the near future.

The roles played by the deterministic processes based on niche theory and the stochastic processes based on neutral theory in the construction of communities have been well-established in the field of microbial ecology (Dini-Andreote et al., 2015; Xun et al., 2019; Gao et al., 2020a). However, the relative importance of these two processes in shaping OMF communities and the factors controlling them are still undetermined. Nevertheless, based on this review, deterministic processes seem to contribute to the construction of OMF communities because environmental conditions significantly affect OMF diversity and abundance. The construction of any given ecological community involves four main ecoevolutionary processes (Vellend, 2016); in addition to the deterministic selection and the stochastic drift that regulates the relative abundance of species, the diversification that generates genetic variation and the dispersal (the movement of individuals across local communities) are also included. The latter two roles in fungal community assembly and function are frequently overlooked. However, they exhibit crucial functions; for example, priority effects and horizontal gene transfer play key roles in niche preemption/modification and adaptability, respectively (Pinto-Carbó et al., 2016; Toju et al., 2018).

Thus, future research on OMF diversity should primarily focus on how the four main ecological processes interact in the assembly and function of OMF communities. In addition to improve certain shortcomings mentioned in the various sections of this review, considering the overall framework required for further research on the fungal diversity of orchids, we have added several keys or difficult issues that are noteworthy for further explorations in the future:

1. Compared to terrestrial orchids, little is known about the OMF in tropical epiphytic orchids and their distribution on phorophytes. The phorophytes of orchids reportedly cover at least 46 plant families (Rasmussen and Rasmussen, 2018). However, the following two questions remain unanswered: why do orchids have a preference for certain phorophytes? Why can some orchids cohabitate on the same phorophyte but others cannot? Therefore, further investigations and experiments, such as *in situ* seed baiting, the transplantation of seedlings, and the identification of OMF contained in barks or substrates of phorophytes at different sites, are needed to elucidate the interactions among orchids, OMF, and phorophytes.
2. The selection method for OMF-specific primers, in addition to the method combining multiple pairs of complementary primers mentioned in this review, can be further improved by employing epicPCR technology, which can focus on two genes in a genome at the same time (Spencer et al., 2016). Moreover, ONF are frequently detected in different tissues of orchids, but their diversity, community structure, and functions exerted in the different stages of orchid life cycle remain ignored. Considering that OMF and ONF coexist in orchid tissues, there is an urgent need to incorporate the latter in the research goals, as an important turning point in orchid mycology. Moreover, promoting research on the interactions between plant pathogens (e.g., *Alternaria*, *Clonostachys*, *Aspergillus*, *Penicillium*, *Phomopsis*, etc.) and OMF in orchid tissues is strongly recommended.
3. Several recent studies have revealed that the difference in relative abundance generated by amplicon sequencing technology is unable to reflect the difference in true absolute abundance of microorganisms in samples, as it ignores the influences of changes in the overall microbial abundance on hosts (Props et al., 2017; Vandeputte et al., 2017; Guo et al., 2020). Although qPCR can be employed to estimate the absolute abundance of specific strains, some strains require specific qPCR primers that are difficult to evaluate and optimize at an early stage. Hence, qPCR is not suitable for studying complex environmental samples. Therefore, to truly reflect on interactions between orchids and fungi, a sophisticated and innovative absolute quantitative technology that integrates ITS amplicon sequencing, qPCR of total fungi, and qPCR absolute quantification of specific fungal strains is required.
4. The determination of whether some singletons and doubletons obtained *via* HTS are truly rare species remains controversial. However, an in-depth analysis of the fungal metabarcoding data obtained from 16 HTS analyses of representative ribosomal RNA gene regions indicates that less than half of these sequences are artifacts (Brown et al., 2015). Moreover, an increasing number of studies have confirmed that rare microbiological taxa are more active than dominant taxa, which play an over-proportional role in ecosystems multifunctionality (Jousset et al., 2017; Chen et al., 2019; Liang et al., 2020). Hence, extensive care should be taken when disregarding singletons and doubletons. It is recommended that subsequent studies concentrate on changes in orchid-associated fungal diversity after incorporating these low-abundance sequences (at least in a separate analysis) and intensify efforts to explore their functions in the construction and maintenance of orchid communities.
5. In recent years, the determination and analysis of core microbiota in environmental samples have attracted a great deal of attention. Current studies on orchid core fungal taxa mainly use the traditional Venn diagrams, which ignore some ecological characteristics (e.g., the coexistence of members in an ecosystem and some of their functions). To improve the efficiency and accuracy of such predictions, it is recommended to use platforms or software, such as MetaCoMET, COREMIC, BURRITO, and PhyloCore, that consider more critical ecological information (e.g., composition, persistence, connectivity, phylogeny, and functional redundancy) to predict and analyze key species

in a given microbial community. It is also necessary to standardize their application for orchid-fungal communities based on the effect of various methods, thus facilitating better comparability of core fungal taxa of different or the same orchid species from different case studies.

6. Clarifying the architecture of the OMF networks formed by co-existing orchids can help to comprehend how these hyper-diverse interacting guilds are maintained and co-evolve in their habitats. This can further reveal the species, lineages, or functional taxa that are significant to ecosystem services.
7. Research on orchid-fungal diversity often involves cryopreservation, especially in case of large-scale sampling to study the mycorrhizal networks. This may result in a decline in the vitality of orchid tissues, affecting the OMF. Therefore, it is highly critical to explore the influence of liquid nitrogen or cryopreservation at −80°C on the composition and structure of OMF communities – a prerequisite for truly reflecting the symbiotic patterns of orchids with OMF in natural habitats.
8. Although it is known that OMFs occur in various habitats, not only the rhizoctonia taxa (e.g., Tulasnellaceae and Ceratobasidiaceae) but also some frequent non-rhizoctonia taxa (e.g., *Mycena*, Thelephoraceae, and Russulaceae) demonstrate a relatively wide distribution in orchids. Further investigations are needed to identify (i) their exact ecology, between pure saprotrophy and pure endophytism in non-orchids and (ii) the geographical hot spots of these OMF. This is crucial for the conservation and regeneration of rare and endangered orchids.
9. Once the diversity of fungi associated with orchids distributed in different habitats is clearly understood, the next step is to evaluate and analyze their functions in these communities and in orchids. There are three methods for exploring the ecological functions of orchid-associated fungi. First, culture methods, such as long-term culture, *in situ* culture, or the dilution of culture medium can be used. In addition to the culture methods, which differ from the traditional technologies mentioned at the beginning of this section, the currently uncultured strains should be isolated to a maximum degree. Then, different isolated fungal sets can be used to evaluate their germination- and growth-promoting effects so as to determine their specific functions at the different stages of orchid life cycle. Second, selective fungicides can be used cautiously to eliminate the key fungi linked with orchid growth and stress resistance, and their respective functions may be preliminarily inferred by observing the growth and reproduction of the host orchids above- and under-ground (Bellino et al., 2014; Jacquemyn and Merckx, 2019). Third, the combination of high-resolution microscopy, metagenomes, whole genomes, or transcriptomes *in* and *ex situ* is expected to reveal the dynamic changes in fungal functions of orchids.
10. The exploration of a mycorrhizal fungal colonization model is highly important in determining the carrying capacity of fungal communities and the function of individual fungi in the construction of these communities. In future research, it is recommended to carry out proactive exploration of OMF colonies in the rhizospheres of orchids through a combination of green fluorescent protein-labeled bioreporter systems, isotope labeling, and HTS. Moreover, how OMF propagate, adapt to the microenvironment of orchid roots, and achieve mutual recognition with the host orchids needs to be elucidated.

To summarize, considering the importance of microbiota in plant growth and health, synthetic communities (SynComs) have increasingly become a hot issue in the study of ecology and evolution of plant microbiomes (Cordovez et al., 2019; Liu et al., 2019). The reconstruction of orchid-fungal communities requires a comprehensive understanding of all the aspects mentioned above, and the design of different fungal assemblages with complementary or synergistic traits is recommended. Simultaneously, the relationship among key fungi, pathogens, and other key microbial assemblages (e.g., bacteria and archaea) that are directly or indirectly associated with the orchid-fungal community should be further studied, and the association pattern of orchid-fungal interaction network should be focused on to predict the fungal SynComs that affect orchid development.

With the joint efforts of the growing number of orchid experts from all over the world, the exploration and understanding of elusive and complex mechanisms underlying orchid-fungal interactions are expected to gradually gain momentum and reveal the much sought-after answers.

AUTHOR CONTRIBUTIONS

JG and M-AS designed the outline of the manuscript. TL, JG, M-AS, WY, and SW collected the data and wrote the manuscript. M-AS and JG polished the article. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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Rhizosphere Bacterial Networks, but Not Diversity, Are Impacted by Pea-Wheat Intercropping

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Plant-plant associations, notably cereal-legume intercropping, have been proposed in agroecology to better value resources and thus reduce the use of chemical inputs in agriculture. Wheat-pea intercropping allows to decreasing the use of nitrogen fertilization through ecological processes such as niche complementarity and facilitation. Rhizosphere microbial communities may account for these processes, since they play a major role in biogeochemical cycles and impact plant nutrition. Still, knowledge on the effect of intecropping on the rhizosphere microbiota remains scarce. Especially, it is an open question whether rhizosphere microbial communities in cereal-legume intercropping are the sum or not of the microbiota of each plant species cultivated in sole cropping. In the present study, we assessed the impact of wheat and pea in IC on the diversity and structure of their respective rhizosphere microbiota. For this purpose, several cultivars of wheat and pea were cultivated in sole and intercropping. Roots of wheat and pea were collected separately in intercropping for microbiota analyses to allow deciphering the effect of IC on the bacterial community of each plant species/cultivar tested. Our data confirmed the well-known specificity of the rhizosphere effect and further stress the differentiation of bacterial communities between pea genotypes (Hr and hr). As regards the intercropping effect, diversity and structure of the rhizosphere microbiota were comparable to sole cropping. However, a specific co-occurrence pattern in each crop rhizosphere due to intercropping was revealed through network analysis. Bacterial co-occurrence network of wheat rhizosphere in IC was dominated by OTUs belonging to Alphaproteobacteria, Bacteroidetes and Gammaproteobacteria. We also evidenced a common network found in both rhizosphere under IC, indicating the interaction between the plant species; this common network was dominated by Acidobacteria, Alphaproteobacteria, and Bacteroidetes, with three OTUs belonging to Acidobacteria, Betaproteobacteria and Chloroflexi that were identified as keystone taxa. These findings indicate more complex rhizosphere bacterial networks in intercropping. Possible implications of these conclusions are discussed in relation with the functioning of rhizosphere microbiota in intercropping accounting for its beneficial effects.

Keywords: bacterial community, biodiversity, intercropping, networks, pea, rhizosphere, wheat

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INTRODUCTION

Rhizosphere is a dynamic zone of interactions between microorganisms and their host plants (Hiltner, 1903; Hartmann et al., 2008). These interactions can be assimilated to a feedback loop: plants release a significant part of their photosynthates in the form of rhizodeposits, which results in the recruitment of a microbial community best adapted to the rhizosphere environment; rhizosphere microorganisms interact with each other and with the host plant, and impact plant growth, nutrition and health (Philippot et al., 2013). Rhizosphere ecology has received a great deal of attention with major progress made in understanding plant-microorganism interactions (Philippot et al., 2013; Guttman et al., 2014). They have allowed to demonstrate the specificity of the so-called rhizosphere effect at the species (Lemanceau et al., 1995; Grayston et al., 1998; Berg and Smalla, 2009; Lakshmanan et al., 2014; Tkacz et al., 2020) and even at the genotype level, for maize (Peiffer et al., 2013), soybean (Zhong et al., 2019), and medic (Pivato et al., 2007). The importance of the rhizosphere microbiota in terms of abundance, diversity and beneficial effects for the host plant has led to an holistic vision of the plant and its microbiota, rather than considering plants and microbiota as standalone entities (Hacquard and Schadt, 2015; Vandenkoornhuyse et al., 2015; Theis et al., 2016). Plant growth, development, health and fitness are mediated by plant but also microbial traits, with variations in plant phenotypes directly linked to their rhizosphere microbiota (e.g., biomass: Swenson et al., 2000; flowering time: Panke-Buisse et al., 2015). Thus, the holobiont concept has been recently proposed as encompassing the plant *per se* and its associated microbiota (Vandenkoornhuyse et al., 2015). Lemanceau et al. (2017) have further proposed the concept of functional core microbiota, in which plants recruit given microbial functional genes whatever the soils in which they are cultivated. Identification of plant and microbial traits involved in positive feedback loops has become a major target for plant-breeding in order to take better advantage of beneficial effects of rhizosphere microbiota (Wei and Jousset, 2017) for decreasing the use of chemical inputs in a more sustainable agriculture (Lemanceau et al., 2015).

Agroecology aims at valuing biotic interactions in agroecosystems in order to reduce the use of chemical inputs. A specific attention is given to crop diversification to promote agriculture sustainability (Altieri, 1999; Wezel et al., 2014; Bedoussac et al., 2015; Lemanceau et al., 2015). A classic strategy for increasing plant diversity in cropping systems is the intercropping (IC) that consists in cultivation of different plant species or cultivars on the same field and at the same time (Willey, 1979). Intercropping is a longstanding and widespread practice in low-input cropping systems throughout the world (Altieri, 1999; Knörzer et al., 2009; Maitra et al., 2021). Intercrop area represents 20-25% of arable land in China, and 17% in India, and up to 83% in Northern Nigeria, and 94% in Malawi (Knörzer et al., 2009). Indeed, in Europe, intercropping systems, such as wheat-pea, encounter different obstacles that contribute to their slow adoption and dissemination (Mamine and Farès, 2020). Varietal selection is one of the main technical limit identified by authors (Mamine and Farès, 2020).

Intercropping may allow to increasing yields (Bedoussac and Justes, 2010a; Mamine and Farès, 2020), while reducing or even avoiding the use of nitrogen fertilizers when using legumes thanks to their ability to fix atmospheric nitrogen (Pelzer et al., 2012). Indeed, legumes promote the uptake of nutrients (e.g., nitrogen, phosphorus and iron, . . .; Hinsinger et al., 2011; Zuo and Zhang, 2009; Xue et al., 2016) and grain protein content of the associated cereals (Bedoussac et al., 2015). Additionally, IC allows to: (i) reducing the pressure of weeds, by occupying available ecological niches, and that of pests, through the physical barrier effect (Corre-Hellou et al., 2011), and to (ii) providing mechanical support to the peas by the cereals (Bedoussac et al., 2015).

Nitrogen-fixing bacteria contribute to the above-referred beneficial effects of the associated legumes. More generally, it has been proposed that rhizosphere microbiota may account for the added value of IC. Thus, attempts have been to assess the impact of IC on rhizosphere microbiota. Total microbial communities (Taschen et al., 2017; Li et al., 2018; Gao et al., 2019; Liu et al., 2021; Tang et al., 2021) and specific functional guilds (e.g., ammonia oxidizing bacteria, Song et al., 2007; diazotrophic Proteobacteria, Solanki et al., 2020) from the total root systems of plant genotypes associated in IC have been analyzed. These reports evidenced that rhizosphere microbiota from IC and sole-cropping (SC) differ significantly, these differences being more strongly expressed for bacteria than for fungi (Gong et al., 2019). Changes in bacterial communities were mostly associated with differences in the abundance of specific phyla. These phyla were either increased (e.g., Proteobacteria, Chloroflexi, Gemmatimonatedes, Acidobacteria, Nitrospirae, and Firmicutes in proso millet and mung bean; Rhizobiales, Burkholderiales, Pseudomonadales and Bacillus populations in wheat and alfalfa; Actinobacteria in wheat and pea) or decreased (Actinobacteria in proso millet and mung bean; Sphingomonadales and Xanthomonadales populations in wheat and alfalfa, α-Proteobacteria and Acidobacteria in wheat and pea) (Taschen et al., 2017; Gong et al., 2019; Li et al., 2020). However, considering separately the roots of each plant genotypes cultivated in IC, no consistent conclusion can be drawn. No difference could be detected between microbiota from IC and SC of fababean and wheat (Tang et al., 2016), or between rhizobia populations in IC and SC of maize and soybean (Herrmann et al., 2014). In contrast, abundance of ammonia oxidizing bacteria was increased in maize and fababean in IC compared to the respective SC (Song et al., 2007). Conclusion variations between reports could possibly be ascribed to differences in compatibility between plant genotypes cultivated together. Optimization of plant-plant interactions in intercropping by an appropriate choice of plant genotypes and cultivars is a major issue. It has been hypothesized that this choice is also crucial to value beneficial plant-plant-microbe interactions. This is supported by the different responses of the root bacterial community of two sugarcane varieties when intercropped with soybean (Liu et al., 2021). Thus, identifying the appropriate plant partners in IC represent a major issue, this require to test different

combinations of plant genotypes/cultivars. Biotic interactions do not only occur between plant-plant and plants-microbes, but also among microorganisms. An increasing attention is given to interaction networks between rhizosphere organisms (van der Heijden and Hartmann, 2016) and the impact of IC on these networks has recently been stressed (Liu et al., 2021; Tang et al., 2021). Thus, co-occurrence networks have been proposed as an additional parameter to characterize microbial communities (Barberán et al., 2012; Berry and Widder, 2014; Morriën et al., 2017; de Vries et al., 2018; Li and Wu, 2018; Jacquiod et al., 2020). Concerning IC co-occurrence network, Tang et al. (2021) showed, through a shotgun metagenome analysis, that sugarcane and peanut IC increased the abundance of bacterial genes involved in organic matter turnover comparing to SC, without correlating these differences to changes in microbiota diversity. Liu et al. (2021) showed differences between the co-occurrence networks between two sugarcane varieties in IC. None of these studies analyzed the differences in co-occurring network between bacterial taxa in IC and SC.These complex interactions may account for the increased yield of the IC but also of the following crop, indicating a positive legacy effect of multispecies cropping systems (Wang et al., 2020).

In the present study, we evaluated the impact of pea-wheat intercropping on rhizosphere microbiota. More specifically, we assessed how this impact may differ according to the pea and wheat cultivars. Biodiversity, structure and network of co-occurrence of bacterial community from roots of plants cultivated separately and in combination were characterized by high throughput sequencing of 16S rRNA genes. Results are discussed in the impact of IC of wheat and pea on the diversity, structure and networks of bacterial communities.

TABLE 1 | Description of first and second experiments main characteristics: year of culture, previous culture, and soil physico-chemical parameters.

		Experiment 1	Experiment 2
Year of culture		2016-2017	2017-2018
Previous culture	2016-2017	—	Spring oat
	2015-2016	Spring oat	Soft winter wheat
	2014-2015	Winter barley	Sunflower
	2013-2014	Soft winter wheat	Soft winter wheat
Soil physico-chemical parameters	Fine soil (<2 mm)	997 g/kg	996 g/kg
	Gravels (0.2-1.5 cm)	1.82 g/kg	2.32 g/kg
	Pebbles (>0.5 cm)	1.59 g/kg	1.9 g/kg
	Clay (<2 μm)	463 g/kg	403 g/kg
	Fine silt (2/20 μm)	289 g/kg	334 g/kg
	Coarse silt (20/50 μm)	187 g/kg	219 g/kg
	Fine sand (50/200 μm)	31 g/kg	25 g/kg
	Coarse sand (200/2000 μm)	30 g/kg	19 g/kg
	pH	7.81	6.87
	Total carbon (C)	19 g/kg	12.6 g/kg
	Total nitrogen (N)	1.69 g/kg	1.08 g/kg
	Calcium carbonate (CaCO)	10 g/kg	< 1 g/kg
	Phosphorus (P2O5)	0.23 g/kg	0.09 g/kg
	Calcium (Ca)	28 cmol + /kg	18.3 cmol + /kg
	Magnesium (Mg)	1.13 cmol + /kg	1.53 cmol + /kg
	Sodium (Na)	0.04 cmol + /kg	0.05 cmol + /kg
	Potassium (K)	0.56 cmol + /kg	0.45 cmol + /kg
	Iron (Fe)	0.01 cmol + /kg	0.01 cmol + /kg
	Manganese (Mn)	0.01 cmol + /kg	0.04 cmol + /kg
	Aluminum (Al)	0.03 cmol + /kg	0.04 cmol + /kg

MATERIALS AND METHODS

Site Description, Experimental Design, and Sampling

The impact of a given plant species on the rhizosphere bacterial community of the other plant species, cultivated in intercropping (IC), was tested in two independent experiments. Both experiments were performed at the Experimental Unit INRAE-Epoisses, France (47°14′11.2″ N 5°05′56.1″ E), the first from October 2016 to July 2017, the second from October 2017 to July 2018 (**Table 1** and **Supplementary Figure 1**). Both followed a spring oat crop (**Table 1**). Soil physico-chemical parameters are indicated in **Table 1**.

In the first experiment, emphasis was given to the impact of wheat on pea bacterial community, by testing the effect of seven winter wheat (*Triticum aestivum*) cultivars (CF11007, CF14336, Ehogold, Flamenko, Forcali, RE13003, Renan) on the bacterial community of three winter pea (*Pisum sativum*) cultivars (cv. Fresnel - hr genotype, Geronimo and Spencer - Hr genotypes).

In the second experiment, emphasis was given to the impact of pea on wheat bacterial community, by testing the effect of 11 winter pea cultivars (Aviron, China S-29, Fresnel, Furious, Geronimo, Isard, Isard H3 1.2, Isard ttl, Joker, IVD 304/10, Spencer) on the bacterial community of two winter wheat cultivars (Ehogold and Flamenko). Each pea and wheat cultivars were cultivated in SC, and each pea cultivars was cultivated in IC with one of the two wheat cultivars. Six pea cultivars (Aviron, Fresnel, Furious, Isard, Isard H3 1.2, Isard ttl) belong to the conventional winter pea genotype hr and five (China S-29, Geronimo, Joker, Hr IVD 304/10, Spencer) to the photo-responsive winter pea genotype Hr (**Table 2**).

In both experiments, each wheat and pea cultivars were cultivated in sole cropping (SC) and in IC.

The IC set up was full mixed of the two plant species on the row IC, as previously described to be the best suitable for cereals and herbaceous legumes in intercropping (Malézieux et al., 2009). Sowing rates varied according to experimental treatments as follows: (i) wheat in SC: 300 grains/m^2, in IC: 150 grains/m^2; (ii) hr peas in SC: 80 grains/m^2, in IC: 60 grains/m^2; (iii) Hr peas in SC and IC: 40 grains/m^2. Plants did not receive any chemical inputs, or watering. The sowing rate was optimized in order to reach 50% wheat and 50% pea at harvest in IC. In all cases, sowing rate was at 50% for wheat, that of pea differed upon cultivars. It was at 75% and 100% for hr and Hr genotypes, respectively, to take in account the difference of competitiveness of the pea genotypes (Bedoussac and Justes, 2010b).

These sowing rates allowed a plant emergence, expressed as the average ratio between IC/SC, equal to 48% for the wheat and

TABLE 2 | Wheat and pea cultivars used in the present study.

		Pea genotype	Experiment 1	Experiment 2
Wheat cultivars	CF11007 = Geny		X	
	CF14336		X	
	Ehogold		X	X
	Flamenko		X	X
	Forcali		X	
	RE13003		X	
	Renan		X	
Pea cultivars	Aviron	hr		X
	China S-29	Hr		X
	Fresnel	hr	X	X
	Furious	hr		X
	Geronimo	Hr	X	X
	Isard	hr		X
	Isard H3 1.2	hr		X
	Isard ttl	hr		X
	Joker	Hr		X
	HR IVD 304/10	Hr		X
	Spencer	Hr	X	X

Wheat and pea cultivars cultivated both in the first than in the second experiment are highlighted in bold.

87% for the hr genotype, and 47,5% for the wheat and 104,5% for the Hr genotypes in the first experiment, and 62% for the wheat, 89% for the pea hr genotype, and 78% for the Hr genotypes in the second experiment.

Treatments were replicated in three blocks, each encompassing 31 plots (1.5 m × 8 m) in the first experiment and 35 in the second.

Ten root systems were randomly sampled per plot to a depth of 20 cm. In intercropping cultures, only wheat and pea root systems in close contact were sampled and their roots were further carefully separated on site. Samplings were performed at an early flowering stage for peas, which was reached 15 days earlier in hr than in Hr pea genotypes. Wheat roots were sampled at both these dates corresponding to heading stage. Bare soil was collected in three uncultivated plots integrated into blocks in each experiment and at each sampling date.

Root systems and bare soils were kept cold and transferred immediately to the laboratory. Rhizosphere soils were taken from the root systems as described by Offre et al. (2007). Samples of rhizosphere and bare soils were lyophilized at −80°C and stored at −20°C.

Molecular Characterization of Bacterial Communities

One hundred fifty-six samples of rhizosphere soil were analyzed from the first experiment (84 from the wheat rhizosphere and 72 from the pea rhizosphere) and 177 from the second (78 from the wheat rhizosphere, 99 from the pea rhizosphere), for a total of 333 rhizosphere samples. Moreover, a total of 12 bare soil samples were also analyzed as controls, for a total of 345 samples.

DNA was extracted from soil samples (1 g dry weight) according to ISO standard 11063 (Petric et al., 2011). The library for MiSeq sequencing was generated through two PCR steps according to Berry et al. (2011). The first step consisted in amplifying all the taxa present in the samples. The bacterial 16S rRNA gene V3-V4 hypervariable region was amplified using primers Pro341F (5′-CCTACGGGAGGCAGCAG-3′) and Pro805R (5′-CCTACGGGNBGCASCAG-3′) (Takahashi et al., 2014). The PCR1 mix was prepared by adding 10X Advantage 2 PCR Buffer (Ozyme, Saint-Cyr-l'École, France), 10 mM each dNTP Mix (Thermo Fisher Scientific, Waltham, MA, United States), 10 μM of Pro805R and Pro341F each (Eurogentec, Liège, Belgium), 1.5U of 50X Advantage 2 Polymerase Mix (Ozyme, Saint-Cyr-l'École, France), 10 ng of DNA to be amplified and water up to 25 μl of final volume for each tube. Thermal cycling conditions were 2 min at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 1 min at 72°C, with a final extension at 72°C for 1 min. Duplicate first step PCR (PCR1) products were pooled and then used as template for the second step PCR (PCR2). PCR2 amplification added multiplexing index-sequences to the overhang adapters using a unique multiplex primer pair combination for each sample according to Illumina guidelines. The conditions for PCR2 were the same as for PCR1, except for the number of cycles (8 cycles instead of 30).

Both PCR steps were performed on the Applied Biosystem 9700 thermal cycler (Applied Biosystem, Foster City, CA, United States). The PCRs were checked by electrophoresis (1.5% agarose, TAE1X, 100V). Two technical replicates of each PCR (1 and 2) were made, the products were then pooled and purified by Agencourt AMPure XP magnetic beads (Beckman Coulter, Brea, CA, United States), according to the supplier's recommendations. The amplified DNA was finally quantified at StepOnePlus Real Time PCR Systems (Thermo Fisher Scientific, Waltham, MA, United States). The equimolar mixture of the samples was prepared before being sent for sequencing (GenoScreen, Lille, France). Sequencing was performed using 300-bp paired-end sequencing chemistry on the Illumina MiSeq platform (Illumina, San Diego, CA, United States). Raw paired-end reads were then demultiplexed and assembled per sample, with the Illumina MiSeq Reporter software (version 3.1).

Bioinformatic Analyses

Sequence data were analyzed using an in-house developed Python notebook piping together different bioinformatics tools (available upon request). Briefly, quality checks of the 16S rRNA sequences were conducted using the QIIME pipeline (Caporaso et al., 2010b) and short sequences were removed (<400 bp). Reference-based and *de novo* chimera detection as well as clustering in Operational taxonomic Units (OTUs) were performed using VSEARCH (Rognes et al., 2016) and RDP representative set of 16S rRNA sequences as the reference database. The identity thresholds were set at 97%. Representative sequences for each OTU were aligned using PyNAST (Caporaso et al., 2010a) and a 16S rRNA phylogenetic tree was constructed using FastTree (Price et al., 2010). Taxonomy was assigned using UCLUST (Edgar, 2010) and the SILVA database (SILVA SSU 138 update release; Quast et al., 2012).

The raw sequences for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB42023[1].

After cleaning, a total of 5 824 759 sequences (a mean of 16 316 sequences for each sample) were kept for OTU picking. 10 549 OTUs were delineated, and 5 713 OTUs were considered for further analysis after rarefying using the "rarefy_even_depth" function in phyloseq package.

Statistical Analyses

Statistical analyses were conducted using R statistical software version 4.0.2 (R Development Core Team, 2014). The α-diversity of the bacterial communities was assessed by quantifying the number of OTUs per sample (richness), and by calculating the Shannon (both richness and evenness) and Simpson (evenness) indexes. β-diversity was investigated through Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances. Both α and β diversity were calculated using phyloseq package (McMurdie and Holmes, 2013). Effects of plant species, cultivars, and associated plant species and cultivars on bacterial communities were tested using one- and two-way Permutational MANOVA analyses (PERMANOVA; this statistical test allows a direct additive partitioning of the variation for complex models), with 999 permutations and, when necessary, applying the 'strata' correction in order to restrict permutations between the 2 years of culture. Significant tests were further carried on individual pair-wise comparisons between experimental treatments, as described by Anderson (2001). PERMANOVA analyses were run using the Vegan package (Oksanen et al., 2019). OTUs explaining differences between treatments were identified by differential OTU abundance analysis. This was achieved by fitting a generalized linear model with a negative binomial distribution to normalized values for each of the OTUs and testing for differential abundance using a likelihood ratio test under DESeq package (Anders and Huber, 2010), after performing the extension DESeq with phyloseq (McMurdie and Holmes, 2014).

Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were applied to identify possible significant effects on α-diversity of plant species and cultivars in monocropping and in intercropping. Kruskal-Wallis tests were followed by pair-wise comparisons with Dunn test. Wilcoxon-Mann-Whitney and Kruskal-Wallis tests were run under the dplyr package (Wickham et al., 2021) and Dunn test under the dunn.test package (Dinno, 2015).

Network Analyses

The interactions between coexisting OTUs in rhizosphere and in bare soils were further analyzed through co-occurrence network. As suggested by Berry and Widder (2014), data of the two experiments were pooled for constructing the corresponding matrices in order to increase the size of the sample dataset and thus obtain a more stable co-occurrence network and accurate correlation estimation. Before outputting the five matrices (bare soil, SC and IC wheat, SC and IC pea), only OTUs represented in at least 50% of the samples of the entire dataset were kept for the co-occurrence network computation. Thus, OTUs only occurring in one of the two experiments were eliminated during this initial trimming, in order to keep only the common to the two experiments. The five correlation matrices amongst OTUs were calculated using Poisson Log Normal models (PLN, Chiquet et al., 2020). The models were validated by using the Bayesian Information Criterion (BIC, only r2-values provided here) and the significance of partial correlations was evaluated by a resampling of each matrix (n = 30) to test the robustness of the networks, using the Stability Approach to Regularization Selection (StARS) method (Liu et al., 2010). StARS method was developed for high dimensional problems and is based on random subsamples (30 iterations in our study, as stated before) and the construction of an highly stable graph from subsamples, in order to evaluate the robustness of the network along the path of solutions (Liu et al., 2010). Moreover, StARS method also allowed reducing possible biais in OTUs relative abundances between the two experiments, through the use of partial correlations. Hereafter we elaborated a network approach based on edge arithmetic (Jacquiod et al., 2020) to identify OTU correlations that were specific of intercropping (**Supplementary Figure 2**). Briefly, in the matrix of the pea and the wheat intercropping, we systematically removed all correlation interferences that were attributed to (i) the bare soil, (ii) the pea monocropping, and (iii) the wheat monocropping (**Supplementary Figure 2**). This resulted in two trimmed networks of OTUs showing specific links in the pea and in the wheat intercropping rhizosphere, respectively. Then, we intersected these two networks in order to retain the unique fractions of wheat, the unique fraction of pea, and the common conserved links found in both rhizospheres only in intercropping context. Complexity of networks was investigated by means of the degree index, the node betweenness and the edge betweenness (Newman, 2003).

RESULTS

Effect of Plant Species and Cultivars Grown in SC on the Rhizosphere Bacterial Communities

The effects of the plant species and cultivars on the rhizosphere bacterial communities were analyzed on either the separate or the pooled dataset of the two experiments.

Microbial α-diversity was significantly higher in wheat than in pea rhizosphere in the first experiment including seven wheat cultivars (Wilcoxon-Mann-Whitney, p = 7.46e-04; p = 3.3e-04; p = 1.27e-03 for Observed, Shannon and Simpson diversity indices, respectively), but not in the second only including two wheat cultivars (**Supplementary Figure 3A**). In the pooled dataset, microbial α-diversity was significantly higher in wheat than in pea rhizosphere (Wilcoxon-Mann-Whitney, p = 3.65e-06; p = 1.08e-05; p = 6.52e-05 for Observed, Shannon and Simpson diversity indices, respectively) (**Supplementary Figure 3B**).

β-diversity was significantly different between wheat and pea rhizosphere in both experiments (separate datasets,

[1]https://www.ebi.ac.uk/ena/browser/view/PRJEB42023

PERMANOVA analysis; F-model = 2.61, p = 0.001, based on 999 permutations; pooled datasets, PERMANOVA analysis; F-model = 3.11, p = 0.001, based on 999 permutations) (**Supplementary Figure 3C**). Differences between the species in the pooled data sets were explained by five OTUs of Proteobacteria, three Alphaproteobacteria (two Rhizobiales and one Sphingomonadales orders) and two Betaproteobacteria (Burkholderiales order) that were preferentially associated with pea (**Supplementary Data Sheet 1**).

Within wheat species, no significant differences between cultivars were detected in microbial α- and β-diversity in the first experiment (**Supplementary Figures 4A,B**). Within pea species, α-diversity was significantly higher in Hr than in hr cultivars in the second experiment (**Supplementary Figures 5A,B**; Wilcoxon-Mann-Whitney, p = 9.8e-04; p = 8.59e-04; p = 5.67e-03 for Observed, Shannon and Simpson diversity indices, respectively). β-diversity also differed significantly between Hr and hr cultivars (**Supplementary Figure 5C**; PERMANOVA analysis; F-model = 1.38, p = 0.001, based on 999 permutations). These differences were significantly most expressed when comparing Aviron and Furious (hr) with Geronimo (Hr) cultivars (**Supplementary Figure 5C** PERMANOVA analysis; F-model = 1.07, $p < 0.02$, based on 999 permutations).

Compared Effects of SC and IC on the Diversity of the Rhizosphere Bacterial Communities

α-diversity of wheat bacterial communities did not differ between SC and IC with pea in any of the two experiments (**Figure 1A**), nor in the pooled dataset (**Figure 1B**). Similarly, β-diversity of wheat bacterial communities did not differ in SC and IC (**Figure 1C**).

α- and β-diversity of pea bacterial communities did not either differ in SC and in IC in any of the experiments (**Figures 1A,C**), although significant differences were detected in the pooled dataset, in which the richness of the bacterial communities associated to IC pea plants was higher than the one associated to SC pea plants (**Figure 1B**, Kruskal-Wallis, p = 2.35e-09, p = 5.26e-08, p = 3.05e-06, for Observed, Shannon and Simpson, respectively).

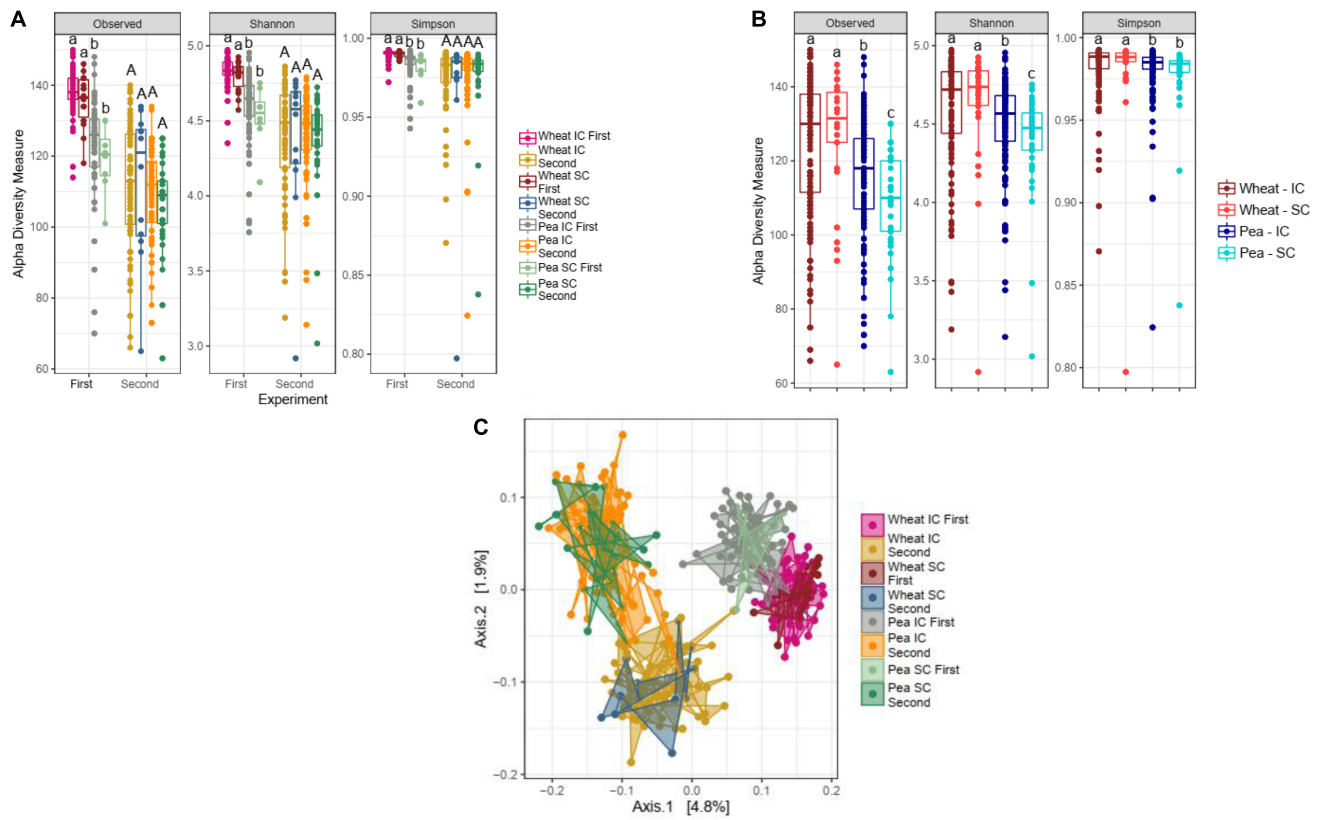

FIGURE 1 | Impact of the sole- (SC) and intercropping (IC) on rhizosphere microbiota. **(A)** Box-plots illustrate α-diversity indices (Observed, Shannon and Simpson) in bacteriobiota of wheat and pea cultivated in SC- and IC, in the first and the second experiment. Median values and interquartile ranges are indicated in the plots. Different letters indicate significant differences according to Wilcoxon-Mann-Whitney test. **(B)** Box-plots illustrate α-diversity indices (Observed, Shannon and Simpson) in bacteriobiome of wheat and pea cultivated in SC and IC, in a dataset pooling results from the two experiments. Median values and interquartile ranges are indicated in the plots. Different letters indicate significant differences according to Wilcoxon-Mann-Whitney test. **(C)** PCoA (with unweighted UniFrac), representing the β-diversity results of rhizosphere microbiota profiles of wheat and pea cultivated in sole- and intercropping, in the first and the second experiment.

In order to better explore the impact of the IC on the β-diversity of wheat and pea bacterial communities, a PERMANOVA with two covariate (plant species –wheat or pea- and culture –SC or IC-) has further been performed. In all cases (separate and pooled experiments), this test confirmed that only plant species had a significant impact on β-diversity (PERMANOVA analysis for plant species factor; $p = 0.001$, based on 999 permutations).

No differences in α- and β-diversity was either detected between intercropping and monocropping when testing a data subset only including cultivars which were shared in the two experiments (i.e., wheat: Ehogold and Fresnel, and pea: Fresnel, Geronimo and Spencer).

Effect of Mono- and Intercropping on the Co-occurrence Network of the Rhizosphere Bacterial Communities

Co-occurrence networks were produced from the pooled dataset, as a high number (>25) of samples is required to obtain a stable network with accurate correlation estimation, as recommended by Berry and Widder (2014).

The BIC R^2 (0.98 for monocropped and intercropped wheat, 0.99 for monocropped and intercropped pea, and 0.97 for bare soil) clearly showed that the PLN models fitted the dataset, resulting in accurate correlation matrices.

After removing the edges observed in the bare soil and in the SC rhizospheres (**Supplementary Figure 2**), we obtained cleaned intercropping networks featuring edges only present in the rhizosphere of each IC plant rhizosphere.

The resulting cleaned intercropped wheat microbial network consisted of 573 nodes (OTUs) and 1673 edges (1462 positive and 211 negative edges; mean degree or node connectivity 5.8). The mean node and edge betweenness centrality were 851.4 and 389.6 respectively. The resulting cleaned intercropped pea microbial network consisted of 451 nodes (OTUs) and 1189 edges (1112 positive and 77 negative edges; mean degree or node connectivity 5.3). The mean node and edge betweenness centrality were 716.4 and 357.1 respectively. Pea microbial network showed a higher positive to negative edge ratio in comparison to wheat network (14.4 vs. 6.9 respectively, **Supplementary Figure 6A**). Mean degree, node and edge betweenness were significantly higher in wheat than pea network (Wilcoxon-Mann-Whitney, $p < 0.05$; **Supplementary Figures 6B-D**). 50% of the OTUs in wheat and pea networks were affiliated to Acidobacteria, Bacteroidetes and Alphaproteobacteria phyla (**Supplementary Figure 6E**).

We then applied a network intersection (**Supplementary Figure 2**) between the pea and wheat networks under IC to specifically identify: (i) the unique fraction of the pea network that was only seen in the pea under IC; (ii) the unique fraction of the wheat network that was only seen in the wheat under IC; and (iii) the common network that was shared amongst both rhizosphere under IC.

The network of intercropped wheat was characterized by a dominance of Alphaproteobacteria, Bacteroidetes and Gammaproteobacteria OTUs (**Figure 2A**); but that intercropped pea did not show any significant taxonomic dominance (**Figure 2B**).

Regarding the common network fraction shared amongst both pea and wheat rhizosphere under IC, a clear organization in

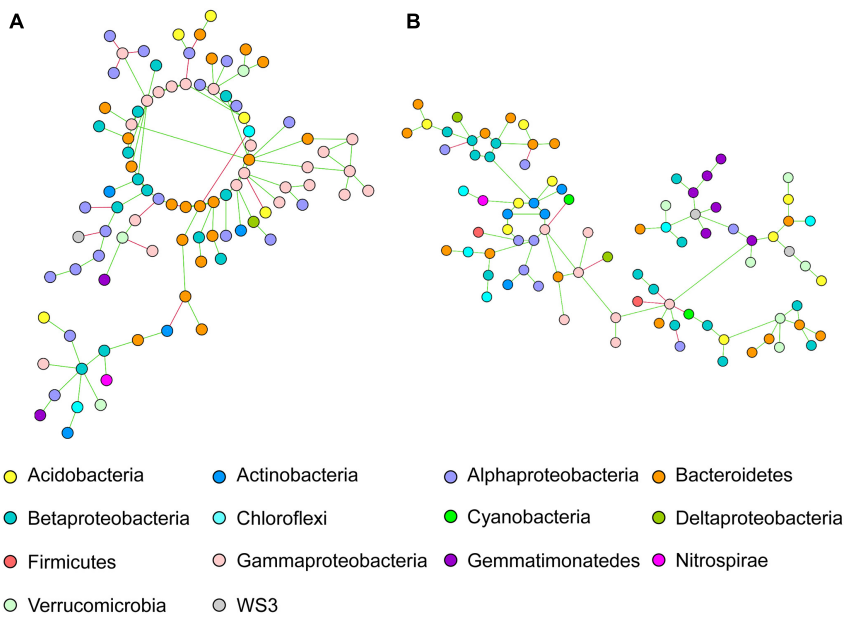

FIGURE 2 | Co-occurring bacterial network of OTUs belonging to intercropped **(A)** wheat and **(B)** pea rhizosphere network. Each network node (individual circle) represents an OTU. Network edges are represented as straight lines connecting the nodes and indicate significant co-occurrences based on partial correlation obtained from a Poisson Log Normal model ($r > |0.06|$, $p < 0.05$, $n = 30$ iterations); green for positives and red for negative co-occurrence.

modules was observed, with three keystone OTUs that had a very strong degree compared to the others. Networks belonging to intercropped wheat and pea shared three main modules including OTUs assigned to Acidobacteria, Alphaproteobacteria and Bacteroidetes phyla (**Figure 3**). Three keystone OTUs were further identified (**Figure 3**): OTU-496 belonging to Acidobacteria, OTU-152 belonging to Betaproteobacteria (order Burkholderiales, family Alcaligenaceae) and OTU-233 belonging to Chloroflexi (order Thermomicrobia).

DISCUSSION

An increasing attention is given to intercropping in agroecology to better value resources and to decrease the use of synthetic inputs (i.e., fertilizers, pesticides). In wheat-pea intercropping, reports indicated the promotion of nitrogen nutrition of wheat (Ghaley et al., 2005; Bedoussac and Justes, 2010a; Guiducci et al., 2018) and suggested a promotion of iron nutrition for pea (Zuo and Zhang, 2009), and of phosphorus nutrition for both plant species (Li et al., 2003, 2007; Hinsinger et al., 2011). The possible contribution of rhizosphere microbiota of plants to these beneficial effects mostly remains to be untapped. This requires to disentangle the complex interactions between the plants grown in association and their rhizosphere microbiota. A major issue is to determine whether the rhizosphere microbiota of the plant species cultivated together differ or not from that of the plant species cultivated separately, and how the IC impact would vary upon the cultivars chosen to be cultivated together. For this purpose, we have compared the rhizosphere bacterial communities of wheat and pea when cultivated in intercropping and in sole cropping, and have tested different cultivar combinations. Bacterial communities were characterized on the basis of their biodiversity, structure and of co-occurrence network of 16S rRNA genes.

In sole cropping, we confirmed the specificity of the rhizosphere effect, which was reported for long (Lemanceau et al., 1995; Grayston et al., 1998; Berg and Smalla, 2009; Lakshmanan et al., 2014; Tkacz et al., 2020). Indeed, bacterial communities from wheat and pea differed significantly (**Supplementary Figure 3**). Both richness and evenness of bacterial OTUs were higher in wheat than in pea rhizosphere, this greater number of bacterial OTUs is in agreement with previous reports (Turner et al., 2013; Taschen et al., 2017; Cordero et al., 2020). Differences in richness and evenness were at least partly explained by a higher representation of Proteobacteria in pea rhizosphere, especially Comamonadaceae, Sphingomonadaceae, but also, as expected, Bradyrhizobiaceae and Rhizobiaceae (**Supplementary Data Sheet 1**), known to be leguminous-associated bacteria (Wielbo, 2012; Chaudhari et al., 2020). Differences were less clear-cut at the cultivar level. In wheat, no difference could be detected between the rhizosphere microbiota of the tested cultivars (**Supplementary Figure 4**). This is in agreement with Corneo et al. (2016), Simonin et al. (2020). However, Kavamura et al. (2020) identified differences of richness between tall and semi-dwarf cultivars, which contrast with the lack of differences recorded in the present study between the four tall cultivars (RE 13 088, CF 14 336, RE 13 003 and Ehogold) and the three semi-dwarf (Flamenko, Forcali, and Renan) tested. In pea, we detected a significant higher richness of the bacterial

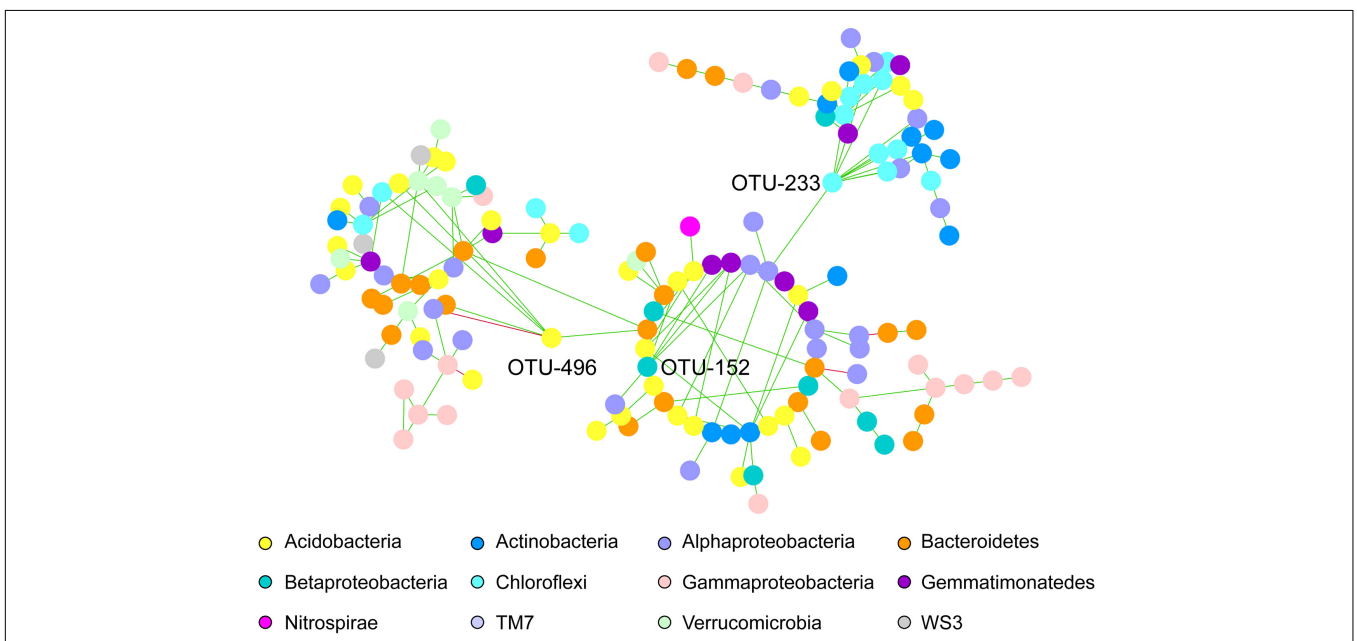

FIGURE 3 | Co-occurring bacterial network of common OTUs belonging to intercropped wheat and pea rhizosphere. Each network node (individual circle) represents an OTU. Network edges are represented as straight lines connecting the nodes and indicate significant co-occurrences based on partial correlation obtained from a Poisson Log Normal model ($r > |0.03|$, $p < 0.05$, $n = 30$ iterations); green for positives and red for negative co-occurrence. OTU-496, OTU-152 and OTU-233 have been identified as keystone OTUs.

communities in Hr than in hr genotypes (**Supplementary Figure 5**). This observation is consistent with the physiological differences between the genotypes known for their differential sensitivity to photoperiod, that leads to a later floral initiation (Murfet, 1973; Alcalde et al., 1999), flowering and maturity in Hr genotypes. Still, to our knowledge, this is the first report pinpointing significant discriminating effect of Hr/hr genotypes on their rhizosphere bacterial communities.

In intercropping, biodiversity and structure of rhizosphere bacterial community of plant species/cultivars did not differ significantly from sole cropping in any of the two experiments (**Figure 1**). This is consistent with previous reports made on wheat-fababean IC (Tang et al., 2016) and on sugarcane varieties IC (Liu et al., 2021). Differences between intercropping and sole cropping were only detected in pea, when pooling data from the two experiments. Then, bacterial community richness appeared to be higher in intercropping than in sole cropping. Our observation is consistent with previous research on intercropping involving other plant species (Li and Wu, 2018).

However, in overall, our results on biodiversity and structure are not in favor of a differential effect of IC compared to SC on wheat and pea bacterial communities. This would suggest that both plant species have similar impact on their bacteria independently of their neighboring plant. This is in agreement with Tkacz et al. (2020), who showed that bacterial community is influenced more by the root fraction than by the soil or plant species. However, the lack of differences between IC and SC could also be ascribed to the characterization methods of the bacterial community. Indeed, they provided information on the taxonomic composition and diversity, but not on the interactions between microbial groups or on their functions. Pivato et al. (2017) previously reported that despite their low impact on the total bacterial community, combination of plant species had a significant effect on the functional bacterial community mediating nitrification. Thus, additional analyses were performed to compare the co-occurrence networks in IC and SC. More specifically; we searched for the possible existence of specific co-occurrence links amongst rhizosphere OTUs that would only be recorded in IC. After filtering for all potential interference sources in our data (**Supplementary Figure 2**), we identified three networks whose edges were only observed in IC, only found in wheat cultivated in IC, another specific to pea cultivated in IC and finally a common network for wheat and pea cultivated in IC.

The specific wheat IC co-occurrence network was characterized by a dominance of OTUs belonging to Alphaproteobacteria, Bacteroidetes and Gammaproteobacteria (**Figure 2**). Alphaproteobacteria (e.g., Rhizobiales order) are known to be well represented in wheat rhizosphere (Bartoli et al., 2020), both in wild and domesticated cultivars, and thus to show a high heritability (Spor et al., 2020). Higher abundance of Bacteroidetes and Proteobacteria (e.g., Alphaproteobacteria, Gammaproteobacteria) was reported in tall than in semi-dwarf wheat cultivars (Kavamura et al., 2020). Connector bacterial OTUs belonging to Gammaproteobacteria, Alphaproteobacteria and Bacteroidetes were previously shown to be more represented in wheat rhizosphere than in bulk soil (Fan et al., 2018). The rationale for the further increase of these nodes recorded here in intercropping remained to be investigated. In the specific pea IC co-occurrence network, no dominant OTUs nodes were observed. Larger and stronger networks in bacterial communities were also described in pea when combining two cultivars (Horner et al., 2019). Lastly, wheat and pea IC specific networks had three common main modules with dominant OTUs belonging to Acidobacteria, Alphaproteobacteria and Bacteroidetes phyla. Three additional keystone OTUs were identified to be shared in networks of wheat and pea cultivated in intercropping: OTU-496, OTU-152, OTU-233, belonging to Acidobacteria (class RB41, order Ellin6075), Betaproteobacteria (order Burkholderiales, family Alcaligenaceae), Chloroflexi (order Thermomicrobia). Acidobacteria was described to be a key taxa in microbiota network associated with wheat (Kavamura et al., 2020), and among this taxa, order Ellin6075 to be part of the core microbiota of *Brassica napus* rhizosphere (Taye et al., 2020). A lot of attention has recently been dedicated to Acidobacteria in rhizosphere ecology (da Rocha et al., 2013; Kielak et al., 2016a,b; Kalam et al., 2020) with populations of Acidobacteria enriched in the rhizosphere (da Rocha et al., 2013; Kielak et al., 2016b). Genomic and metagenomic analyses allowed Kielak et al. (2016a) to predict a range of activities (e.g., the ability to attach roots thanks to exopolysaccharide production, promotion of plant iron uptake, indole-3-acetic acid production) in Acidobacteria with populations beneficial to the host-plant (Afzal et al., 2019). Acidobacteria have frequently been described as co-occurring with Proteobacteria, however it is not yet clear if this co-occurence stems from overlapping niches and/or from metabolic interactions. Alcaligenaceae have been identified as being associated with soil suppressiveness to soilborne diseases (Chapelle et al., 2016; Gómez Expósito et al., 2017). Abundance of Chloroflexi, known for their ability to oxidize nitrites, varies in wheat rhizosphere upon N addition (Ma et al., 2020). Since legumes may transfer ammonium from nodules to the surrounding soil and plants (Zhang et al., 2017), wheat intercropped with pea may benefit from an increased content in ammonium that would promote nitrifiers. Indeed, *amoA* genes appear to be more represented in maize-peanut intercropping than in maize monocropping. Among Chloroflexi, Thermomicrobia are dominant taxa in wheat rhizosphere (Latif et al., 2020) and key taxa in microbial network associated with tall wheat cultivars (Kavamura et al., 2020).

In conclusions, the present study shows that bacterial communities associated with wheat and pea differ between IC and SC, despite the lack of significant differences of their biodiversity and structure. Among the key taxa of specific of IC networks, some could be candidate promoting plant growth, nutrition and health. Our data also point out more complex networks within bacterial communities in the IC rhizosphere of wheat and pea, whereas their biodiversity and structure were not impacted. Co-occurring networks of plant microbiome were described to be more structured and complex in rhizosphere than in bare soil (van der Heijden and Hartmann, 2016). How

this increased complexity may account for the beneficial effects of the intercropping on the plant growth and nutrition remains to explored. Still, recent studies clearly showed an increased function expression in belowground communities when the networks of co-occurence between populations were more complex, despite the lack of biodiversity variations (Morriën et al., 2017). The possible enhancement of functionalities in more complex microbial networks could be assumed to be related to modified activities of the populations when closely interacting. This hypothesis is currently being tested with the help of transcriptomic approaches in synthetic bacterial communities.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/ **Supplementary Material**.

AUTHOR CONTRIBUTIONS

BP, JB, and PL conceived the study. TG, NM, CL, and JB designed the field experiments. TG and JM performed the field experiments. BP and FD performed the sampling and were involved in the experiments in molecular biology. BP and AS performed bioinformatics and statistical analysis. BP and SJ conceived the analysis of the co-occurrence networks. SJ performed the analysis of the co-occurrence network and revised the manuscript. BP and PL wrote and revised 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.2021.674556/full#supplementary-material

Supplementary Figure 1 | Plan of the Experimental Unit INRAE-Epoisses, France (47°14'11.2″ N 5°05'56.1″ E) in panels **(A)** 2016-2017 and **(B)** 2017-2018. Blue rectangles with red outline indicate the experimental site in panels **(A)** 2016-2017 and **(B)** 2017-2018. The dark green circles with black outline indicate the sampling site for soil physical-chemical analyses.

Supplementary Figure 2 | Tailored approach to identify OTU correlations that are specific of the intercropping.

Supplementary Figure 3 | Impact of wheat and pea on rhizosphere bacterial community. **(A)** Box-plots illustrate α-diversity indices (Observed, Shannon, and Simpson) in bacterial communities of wheat (red) and pea (light blue) rhizosphere in the first and the second experiments. Median values and interquartile ranges are indicated in the plots. Different letters indicate significant differences according to Wilcoxon-Mann-Whitney test. **(B)** Box-plots illustrate α-diversity indices (Observed, Shannon and Simpson) in bacteriobomes of wheat (red) and pea (light blue) rhizosphere corresponding to the pooled dataset of information from the first and second experiments. Median values and interquartile ranges are indicated in the plots. Different letters indicate significant differences according to Wilcoxon-Mann-Whitney test. **(C)** PCoA (with unweighted UniFrac) showing the β-diversity results of rhizosphere bacterial community profiles of wheat and pea rhizosphere ine the first (First) and the second (Second) experiment.

Supplementary Figure 4 | Impact of the wheat cultivars in sole cropping on rhizosphere bacterial community. **(A)** Box-plots illustrating α-diversity indices (Observed, Shannon, and Simpson) in bacterial community of wheat cultivars in the first experiment. Median values and interquartile ranges are indicated in the plots. **(B)** PCoA (with unweighted UniFrac) showing the β-diversity results of rhizosphere microbiota profiles of wheat cultivars corresponding to the first experiment.

Supplementary Figure 5 | Impact of the pea cultivars in sole cropping on rhizosphere bacterial community. **(A)** Box-plots illustrateα-diversity indices (Observed, Shannon and Simpson) in bacterial community of pea cultivars in the second experiment. Median values and interquartile ranges are indicated in the plots. **(B)** Box-plots illustrating α-diversity indices (Observed, Shannon and Simpson) in bacterial community of pea hr and Hr genotypes corresponding to the pooled dataset. Median values and interquartile ranges are indicated in the plots. Different letters indicate significant differences according to Wilcoxon-Mann-Whitney test. **(C)** PCoA (with unweighted UniFrac) showing the β-diversity results of rhizosphere microbiota profiles of pea cultivars corresponding to the first experiment.

Supplementary Figure 6 | Comparison of the architecture characteristics of co-occurring network between intercropped wheat and pea networks: **(A)** positive to negative edge ratio, **(B)** mean degree, **(C)** node betweenness, and **(D)** edge betweenness. Significant differences were indicated by ***, according to Wilcoxon-Mann-Whitney test ($p < 0.05$). **(E)** OTUs affiliations in intercropped wheat and pea networks.

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ORIGINAL RESEARCH
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Native AMF Communities in an Italian Vineyard at Two Different Phenological Stages of *Vitis vinifera*

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Arbuscular mycorrhizal fungi (AMF) are beneficial soil microorganisms that can establish symbiotic associations with *Vitis vinifera* roots, resulting in positive effects on grapevine performance, both in terms of water use efficiency, nutrient uptake, and replant success. Grapevine is an important perennial crop cultivated worldwide, especially in Mediterranean countries. In Italy, Piedmont is one of the regions with the longest winemaking tradition. In the present study, we characterized the AMF communities of the soil associated or not with the roots of *V. vinifera* cv. Pinot Noir cultivated in a vineyard subjected to conventional management using 454 Roche sequencing technology. Samplings were performed at two plant phenological stages (flowering and early fruit development). The AMF community was dominated by members of the family Glomeraceae, with a prevalence of the genus *Glomus* and the species *Rhizophagus intraradices* and *Rhizophagus irregularis*. On the contrary, the genus *Archaeospora* was the only one belonging to the family Archaeosporaceae. Since different AMF communities occur in the two considered soils, independently from the plant phenological stage, a probable role of *V. vinifera* in determining the AMF populations associated to its roots has been highlighted.

Keywords: *Vitis vinifera*, arbuscular mycorrhizal fungi, biodiversity, conventional management, soil, grapevine roots

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INTRODUCTION

Arbuscular mycorrhizal fungi (AMF) are beneficial symbiotic soil microorganisms that improve the plant nutritional state by increasing the interface area between roots and soil (Hodge et al., 2010). Moreover, the arbuscular mycorrhizal symbiosis provides other advantages for plants, such as better tolerance versus biotic or abiotic stresses (Hodge et al., 2010; Bona et al., 2011; Lingua et al., 2012; Degola et al., 2015) and improved fruit yield and quality (Baslam et al., 2013; Berta et al., 2014; Bona et al., 2015, 2017, 2018; Todeschini et al., 2018).

Grapevine (*Vitis vinifera* L.) is an important perennial crop cultivated in all continents where the climatic conditions are permissive. Italy is one of the five major grape producers in the world, with about 8,600,000 tons which represent 11% of the production in the world (IOV, 2019). It has been widely demonstrated that the inclusion of fresh grape and its derivates in the diet (Vislocky and Fernandez, 2010) and also the reasonable consumption of wine (Georgiev et al., 2014;

Artero et al., 2015) can give beneficial effects for human health, decreasing the risk factors associated with cancer and age-related cognitive decline as well as cardiovascular and neurodegenerative diseases (Torres et al., 2018). Pinot Noir is a grapevine cultivar from which both white and red fine wines, with typical organoleptic characteristics, are produced all over the world. In particular, the surface area dedicated to this cultivation corresponds to 112,000 ha. Germany, Italy, and Switzerland in Europe and United States, New Zealand, and Australia in non-European countries are the main producers (IOV, 2017). Grapevines, during their life cycle, are subjected to various cultivation practices which can interfere with the native microbiota and also the fungal soil population. It is well documented that practices like tillage, as well as the use of fertilizers and/or pesticides, can reduce soil microbial biodiversity (Berruti et al., 2014; Trouvelot et al., 2015; Zaller et al., 2018; Nogales et al., 2019). The intensity and frequency of these practices vary according to the type of vineyard management, which can be classified into conventional, organic, and/or integrated (Likar et al., 2017; Zaller et al., 2018). The growth and development of grapevines are dependent on AMF (Linderman and Davis, 2001; Schreiner, 2005), and the occurrence of species specificity between *V. vinifera* and AMF has been observed (Holland et al., 2014). In addition, if compared to non-native ones, AMF native of a certain area are often reported to be more effective in plant growth promotion (Schreiner et al., 2006). In order to realize and manage a sustainable agricultural ecosystem, the study of AMF communities associated with grapevines, in the context of conventional management, becomes of great importance (Likar et al., 2013). Several works described the biodiversity of AMF in vineyards subjected to conventional management. The AMF community of two differently managed vineyards (tilled and covered) in Sardinia was characterized by Lumini et al. (2010). Conventional management of the vineyard leads to the development of different fungal and bacterial microbial communities according to specific local biogeographic factors (Likar et al., 2017). The differences between AMF communities in a vineyard and in nearby unmanaged areas were analyzed in order to highlight the impact of viticulture on AMF community diversity and composition (Holland et al., 2016). Finally, AMF biodiversity was studied in the roots of *V. vinifera* cv. Pinot Noir and Chardonnay in Burgundy (France) and Oregon (United States) (Bouffaud et al., 2016; Schreiner, 2020). In the past, the study of AMF communities was exclusively based on the morphological identification of isolated spores. More recently, this methodology has been complemented and/or replaced by molecular techniques, applied both to roots and soils as, for example, cloning followed by Sanger sequencing (Vasar et al., 2017). Then, since the early 2000s, the use of next-generation sequencing, including Roche 454 platform, allowed the analysis of a huge number of sequences (hundreds of thousands) per sample, enormously increasing the depth of investigation. Molecular approaches are based on nuclear ribosomal markers such as the small subunit (SSU) rRNA gene, the internal transcribed spacer region (ITS), and the large subunit (LSU) rRNA gene (Öpik and Davison, 2016). Based on the idea that Piedmont has winemaking tradition that we can define as historic, it becomes of extreme ecological and applicative importance to get information on the AMF communities associated with the vines. In this geographical zone, we therefore identified a vineyard cultivated with grapevine cv. Pinot Noir and subjected to conventional management. A detailed description of the native AMF communities of the soils associated or not with the grapevine roots at two plant phenological stages (flowering and early fruit development) was obtained.

MATERIALS AND METHODS

Soil Sampling

The experimental vineyard is located in Mantovana (Predosa municipality, Alessandria, Southern Piedmont, Italy – altitude: 215 m a.s.l., latitude: 44.730294° N, and longitude: 8.6226556°E), and it is subjected to conventional management. Glyphosate treatment was performed in the vineyard in June. Trifloxistrobin and Fosetyl-Al + copper were employed as fungicides against *Oidium* spp. and *Peronospora* spp., respectively, and were distributed in June and July, coupled with one insecticide (thiamethoxan) and two sulfur treatments in July.

The soil, hereafter indicated as Bs, was sampled close to the vineyard, in a not cultivated area covered in part with grasses (**Figure 1**). The soil associated to the roots (Rs) of *V. vinifera* cv. Pinot Noir, grafted onto SO4 rootstock, was collected from grapevine roots entrapped in the soil cores taken near the plant. Samplings were carried out in May 2014 (Bs1S and Rs1S, flowering) and July 2014 (Bs2S and Rs2S, early fruit development). For each soil (Bs or Rs) and time point (1S or 2S), five samples were collected from the topsoil (5–30 cm). According to the Italian guide for soil analysis (GU 179/2002), for each plant, three soil cores were taken, pooled, and mixed to prepare one sample. The soil samples were stored at −20°C until DNA extraction.

The soil of the vineyard was clay-loam and acidic, as reported in Gamalero et al. (2020); moreover, the climatic conditions of the area, such as temperature, humidity, and rainfall, are detailed in Bona et al. (2019).

AMF Root Colonization

Mycorrhizal colonization was evaluated microscopically following the method of Trouvelot et al. (1986) as mycorrhizal frequency, degree of AMF root colonization, and arbuscule and vesicle abundance. Briefly, from grapevine roots, 30 randomly chosen 1-cm-long pieces were cut, cleared at 80°C for 90 min in 10% KOH, stained with 1% methyl blue in lactic acid, and mounted onto slides. The results were analyzed by ANOVA; differences were considered statistically significant for p-values less than 0.05.

DNA Extraction and Amplification

Power Soil R DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, CA, United States) was used, following the manufacturer's instructions, to extract DNA from five samples, both of Bs and of the soil associated with the roots of *V. vinifera*

FIGURE 1 | Vineyard aerial view showing the sampling points. The vineyard is located in Mantovana (Predosa municipality, Alessandria, Italy). The two soil sampling sites included one in an area just outside the borders of the vineyard in the absence of grapevines (Bs, white dots) and one inside the vineyard corresponding to the grapevine plants (Rs, cyan dots). Google Earth online version was used to produce this image (https://earth.google.com/web/).

(Rs), collected at flowering (1S) or at fruiting (2S) times. A hemi-nested PCR, employing as template the previously extracted DNA, was performed using LR1 and FLR2 (Cesaro et al., 2008) primers for the first amplification and LR1 and FLR4 (Cesaro et al., 2008) primers tagged with Multiplex Identifier sequences for 454 Pyrosequencing (Roche) for the second one. In particular, the FLR2 and FLR4 primers are specific for fungi and for Glomeromycota, respectively (Farmer et al., 2007). The reactions were performed at the conditions described in Massa et al. (2020).

Pyrosequencing employing 454 technology was performed on the products of the second PCR (size, 700 bp). DNA-carrying beads were loaded on a PicoTiterTM plate and surrounded by enzyme beads (sulfurylase and luciferase). The light signals were represented in flow grams and analyzed; a nucleotide sequence was determined for each read with the GS Amplicon Variant Analyzer software.

Bioinformatic Analysis

Data were analyzed using a custom bioinformatic pipeline as fully described in Massa et al. (2020). Raw sequence reads were demultiplexed. The reads with the following characteristics were discarded: (1) read length less than 200 nucleotides, (2) average Phred quality score less than 25 (Ewing et al., 1998), and (3) presence of at least one ambiguous base inside the read. Then, an alignment of each sequence was performed against our AMF LSU rDNA database, consisting of 3.803 univocal sequences downloaded from online sources: EBI and SILVA databases, and from the web site[1] (Krüger et al., 2011). Our database was prepared as described in Massa et al. (2020). The alignment of each sequence was performed using BLASTN (Altschul et al., 1997). Two criteria were applied in order to identify the taxa at species level (named "known"): coverage ≥80% and similarity of sequences ≥97% according to Lindahl et al. (2013) and Hart et al. (2015). Following these criteria, chimeras were also removed. All the sequences that did not satisfy both afore-mentioned criteria were then aligned against themselves. After comparing one sequence to each other, all those with coverage ≥80% and similarity of sequences ≥97% were grouped together, and each group was named *de novo* (Massa et al., 2020).

Bioinformatic analysis was performed on a database containing the results normalized at 8,000 sequences. The rarefaction curves were plotted with the RAM package of R (R Core Team., 2018; **Supplementary Figure 1**).

Taxa Abundance and Biodiversity Analysis

In order to describe the distribution of "known" and *de novo* taxa in the different samples, the number of taxa with at least 10

[1]http://www.amf-phylogeny.com/amphylo_species.html

sequences in one replicate was calculated, considering altogether the replicates for each sample (if the same taxon was present more than one time in the different replicates of the sample, it was counted as one). The freely available Venny version 2.1 software[2] was used to construct the Venn diagrams of AMF taxa in Bs and Rs soils.

For abundance analysis, only taxa present with at least 10 sequences in one replicate were used ("known" reported in **Supplementary Table 1** and *de novo* in **Supplementary Table 2**). Then, the *de novo* taxa were BLASTed against the NCBI database to give them a name (**Supplementary Table 2**) as fully described in Massa et al. (2020).

Using these data, the four AMF communities were compared also by analysis with MicrobiomeAnalyst, a freely available online software[3], according to Berlanas et al. (2019) and to Sergaki et al. (2018), which allow community description by alpha diversity, heat trees, beta diversity, and linear discriminant analysis effect size (LDA-LEfSe).

In particular, alpha diversity analysis was performed using the phyloseq package (McMurdie and Holmes, 2013). The results were represented as box plots for each sample. The statistical significance was also estimated using either parametric or non-parametric tests.

Heat tree method was used to compare abundance at the species taxonomic level for space and time factors. Heat tree uses a hierarchical structure of taxonomic classifications to quantitatively (median abundance) and statistically (non-parametric Wilcoxon rank-sum test) describe taxon differences among communities. The resulting differential heat tree shows the relative abundance of each taxon in two different samples. Heat tree analysis was performed using R metacoder package (Foster et al., 2017).

Beta diversity was analyzed using the phyloseq package (McMurdie and Holmes, 2013). Principal coordinate analysis (PCoA) was applied using Bray–Curtis distance-based method. Permutational ANOVA (PERMANOVA) was employed for the evaluation of the statistical significance of the clustering pattern in ordination plots.

Moreover, LDA-LEfSe analysis using the non-parametric factorial Kruskal–Wallis sum-rank test was applied. Features were considered significant for adjusted *p*-value cutoff at 0.05 and LDA score at 1.0.

Finally, for each sample, considering the sequence abundance in the single replicates, the median value of the number of sequences was calculated, and only the taxa with a median higher than 0 (yellow lines in **Supplementary Tables 1, 2**) were considered to be assigned to the different taxonomic groups.

Data Availability

The genomic datasets are available in NCBI using BioProject ID PRJNA613620 containing the following BioSamples: SAMN14411203, SAMN14411449, SAMN14411451, and SAMN14411452 (project name: *Vitis vinifera* association with local AMF communities in an Italian vineyard at two different phenological stages).

[2]www://bioinfogp.cnb.csic.es/tools/venny

[3]https://www.microbiomeanalyst.ca

RESULTS

AMF Root Colonization

Arbuscular mycorrhizal fungi root colonization was checked in grapevine plants in both sampling times. The frequencies of colonization were 92.0 ± 2.5% at the first sampling and 94.2 ± 3.3% at the second sampling (*p*-value = 0.3658). The degrees of mycorrhizal colonization were 35.4 ± 5.5 and 44.5 ± 8.0% (*p*-value = 0.3379), the arbuscule abundances were 13.9 ± 3.6 and 16.8 ± 5.3% ($p = 0.6547$), and the vesicle abundances were 11.3 ± 3.0 and 17.6 ± 7.7% ($p = 0.9491$) in the first and the second sampling, respectively. No significant differences between the two sampling times were detected in all the considered parameters.

Taxa Abundance and Analysis of Biodiversity

Table 1 shows the real number of sequences for each replicate of Bs and Rs soils. On average, the number of obtained sequences was about 9,000. As the rarefaction curves reached a plateau (**Supplementary Figure 1**), the number of obtained sequences was adequate to properly describe the biodiversity of the AMF community in the samples. A total of 467 taxa (305 univocal taxa) were obtained from the two soils at the two sampling times, including 177 (87 univocal taxa) "known AMF" and 290 (218 univocal taxa) *de novo* taxa (**Supplementary Tables 1, 2**). **Figure 2A** shows the distribution of taxa in the different samples. In Bs soil, 108 and 118 taxa were obtained in the first and the second sampling time, respectively. In particular, 42 "known AMF" taxa occurred in Bs1S sample and 49 in Bs2S (factor time), while the number of *de novo* AMF taxa at the two sampling times was 66 and 69, respectively. In Rs soil, a total of 104 and 137 taxa was found in the first and the second sampling time, respectively. In particular, in this soil, 48 "known AMF" taxa were observed

TABLE 1 | Number of sequences obtained from the different replicates of the soils associated (Rs) or not (Bs) with the roots of *Vitis vinifera* cv. Pinot Noir at the two sampling times (1S = flowering; 2S = fruit development).

Soil sample	Replicate	Number of sequences		Average number of sequences
		1S	2S	
Bs	1	2,492	9,670	
	2	10,949	9,730	
	3	6,252	11,639	9,133
	4	9,685	12,169	
	5	11,600	7,145	
Rs	1	6,286	8,153	
	2	13,603	6,405	
	3	9,910	6,623	9,224
	4	10,229	12,570	
	5	9,641	8,816	

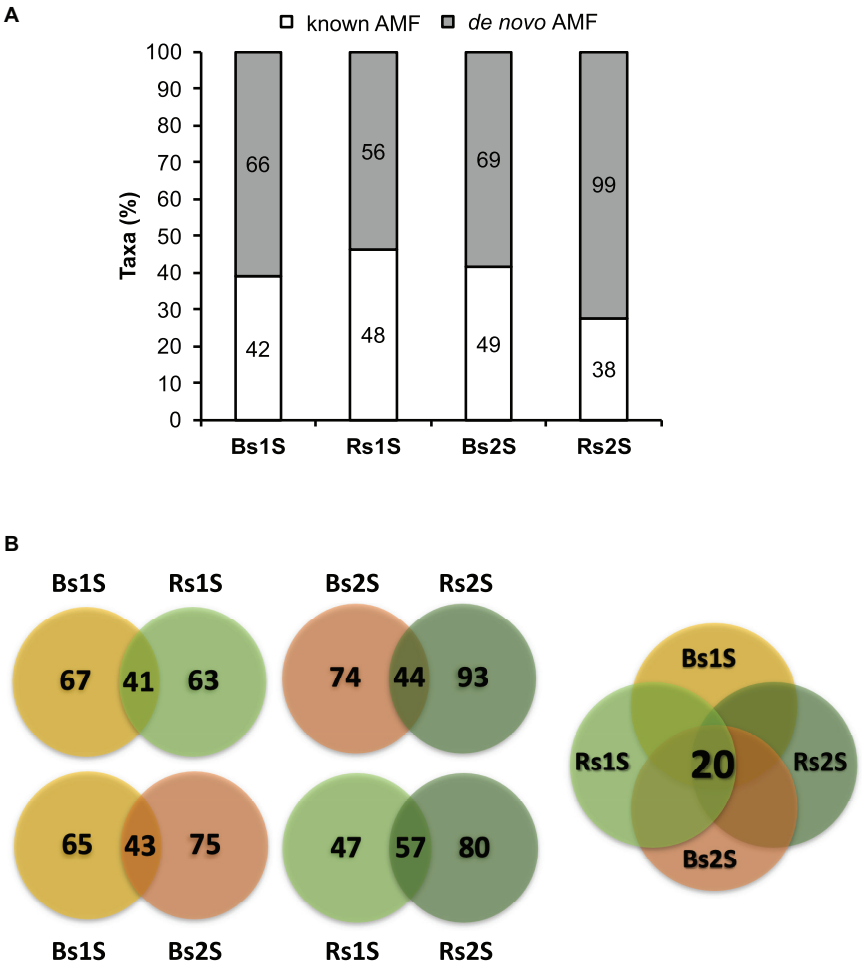


FIGURE 2 | (A) Abundance of the taxa obtained from the soils associated (Rs) or not (Bs) with the roots of *Vitis vinifera* cv. Pinot Noir during the two sampling times (1S = flowering and 2S = fruit development). Bars represent the percentage of known (white) or *de novo* (gray) arbuscular mycorrhizal fungi taxa, while labels inside the bars indicate the actual number of taxa. **(B)** Venn diagrams showing the number of taxa that were exclusive or common to (a) Bs and Rs soils in the first (1S) or in the second (2S) sampling time (upper part on the left of the figure), (b) the first (1S) and the second (2S) sampling times in Bs or in Rs (lower part on the left of the figure), and (c) the four soil samples (Bs1S, Rs1S, Bs2S, and Rs2S—on the right of the figure). The Venn diagrams were calculated by the freely available Venny version 2.1 software (http://bioinfogp.cnb.csic.es/tools/venny).

at the first sampling time while 38 taxa in the second one; on the contrary, the number of *de novo* AMF taxa was 56 and 99, respectively (factor time).

Bs1S and Rs1S or Bs2S and Rs2S (factor space) shared 41 and 44 taxa, respectively (**Figure 2B** and **Supplementary Table 3**). The AMF communities in Bs soils at the two sampling times showed 43 taxa in common. On the other hand, the number of taxa shared between the two sampling times in Rs soil was 57. Finally, all samples had 20 taxa in common (**Figure 2B** and **Supplementary Table 3**).

To compare AMF alpha diversity, the number of observed species, Simpson and Shannon indices were calculated (**Figure 3**). For all these indices, differences were not significant, even if an increased number of observed species occurred in Rs2S compared to the other samples.

The heat trees reported in **Figures 4**, **5** represented time and space effect on the AMF community. In particular, **Figure 4A** is relative to time effect in Bs and displays the increased (blue line) abundance of *Rhizophagus irregularis* in Bs2S compared to Bs1S (**Supplementary Table 4**). **Figure 4B** reports the time effect in the soil associated to *V. vinifera* roots and shows the increase (blue lines) of *de novo*_570 (uncultured *Glomus*), *de novo*_660 (*R. irregularis*), and *de novo*_10711 (uncultured *Glomus*) and the decrease (red line) of *Septoglomus viscosum* in the Rs2S compared to Rs1S samples (**Supplementary Table 4**). In **Figure 5A**, is represented the heat tree related to the space effect in the first sampling time: while *Glomus* sp., *Rhizophagus irregularis*, *de novo*_1903 (*R. irregularis*), and *de novo*_4639 (*Glomus* sp.) increased (blue lines) in Rs1S compared to Bs1S soil, *de novo*_358 (uncultured *Archaeospora*), *de novo*_581 (uncultured *Archaeospora*), *de novo*_627 (uncultured *Archaeospora*), and *de novo*_681 (uncultured *Archaeospora*) decreased (red lines) (**Supplementary Table 4**). Finally, **Figure 5B** shows the heat tree related to the space effect in the second sampling time.

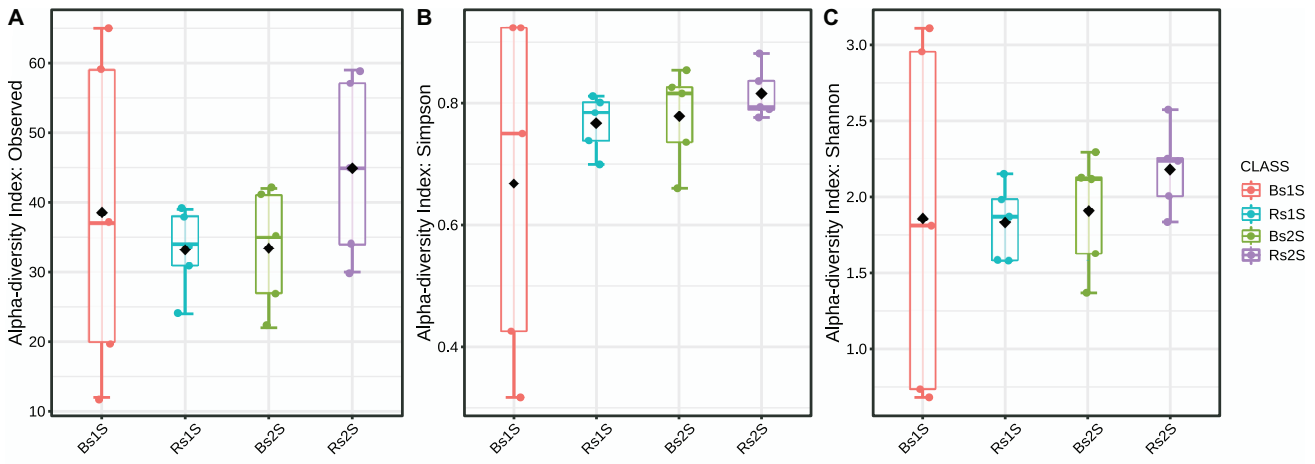


FIGURE 3 | Alpha diversity indices. **(A)** Number of observed arbuscular mycorrhizal fungi species (*p*-value, 0.5393), **(B)** Simpson index (*p*-value, 0.4700), and **(C)** Shannon's index (*p*-value, 0.8177) of biodiversity detected in the soils associated (Rs) or not (Bs) with the roots of *Vitis vinifera* cv. Pinot Noir at the two sampling times (1S = flowering and 2S = fruit development). Alpha diversity analysis was performed using the phyloseq package of MicrobiomeAnalyst, a freely available online software (https://www.microbiomeanalyst.ca).

Although *de novo*_660 (*R. irregularis*) and *de novo*_10711 (uncultured *Glomus*) increased (blue lines) in Rs2S compared to Bs2S soil, *de novo*_358 (uncultured *Archaeospora*), *de novo*_479 (uncultured *Glomerales*), and *de novo*_581 (uncultured *Archaeospora*) decreased (red lines) (**Supplementary Table 4**).

Data analyzed by PCoA underlined a different community composition between the two soils (PERMANOVA *p*-value, <0.005), with Rs samples distributed more homogeneously than Bs ones at both sampling times (**Figure 6A**), but no difference occurred between the two sampling times (PERMANOVA *p*-value, 0.994) (**Figure 6B**).

The LEfSe results, presented in **Figure 7** and **Supplementary Table 5**, showed the 13 taxa that better explained the differences in the AMF community analyzed. In particular, *Glomus* sp. showed a LDA score of 6.1 (*p*-value, 0.033341), the highest values in Rs1S followed by Bs2S. Other important taxa present in the soil associated to *V. vinifera* roots (Rs) were *de novo*_10711 that was uncultured *Glomus* (LDA score, 3.8), *de novo*_570 that was uncultured *Glomus* (LDA score, 3.79), *de novo*_10732 that was *Rhizophagus intraradices* (LDA score, 3.77), *de novo*_660 that was *R. irregularis* (LDA score, 3.58), and *de novo*_11975 that was uncultured *Rhizophagus* (LDA score, 3.51). On the contrary, the seven *de novo* taxa – *de novo*_358 that was uncultured *Archaeospora* (LDA score, 5.62), *R. irregularis* (LDA score, 5.47), *de novo*_561 that was uncultured *Archaeospora* (LDA score, 5.16), *de novo*_581 that was uncultured *Archaeospora* (LDA score, 4.62), *de novo*_627 that was uncultured *Archaeospora* (LDA score, 4.52), *de novo*_681 that was uncultured *Archaeospora* (LDA score, 3.63), and *de novo*_919 that was uncultured *Archaeospora* (LDA score, 3.58) – mostly explained the differences in Bs soil.

The *de novo* AMF taxa were named after being BLASTed against NCBI database (**Supplementary Table 2**). Then, for each sample, all the "known AMF" (yellow lines in **Supplementary Table 1**) and the *de novo*-BLASTed (yellow lines in **Supplementary Table 2**) taxa belonging to the same taxonomic group were added (**Figure 8A**). Two taxa corresponded to higher AMF classification levels (subphylum and order; **Figure 8A** – cyan area on the left). Many taxa were included in the family Glomeraceae (**Figure 8A** – central green area); the genus *Glomus* was the most abundant group (22 taxa in Rs2S). All the other remaining taxa of the family Glomeraceae belonged to the genus *Rhizophagus*, with the exception of *S. viscosum*. All the taxa belonging to the family Archaeosporaceae were included in only one genus (*Archaeospora*; **Figure 8A** – orange area on the right).

Most of the taxonomic groups were detected in both soils, with the exception of unidentified species of the genus *Rhizophagus* that were observed only in Rs2S and *S. viscosum* that was detected only in Bs soils at both sampling times (**Figure 8A**). Moreover, the species *Rhizophagus diaphanum* was present in both soils, but only at the second sampling time (**Figure 8A**).

The *de novo* AMF taxon identification by NCBI permitted to highlight the presence of the genus *Archaeospora* that did not appear among the "known AMF" taxa (**Figure 8B**). In fact, the "known AMF" taxa belonged only to the family Glomeraceae (**Figure 8B**).

All the identified *de novo* AMF taxa corresponded to uncultured AMF, with the exception of four taxa: one belonging to *Glomeromycota*, five to the genus *Glomus*, three to the species *R. irregularis*, and one to the species *R. intraradices* (**Supplementary Table 2**).

DISCUSSION

Arbuscular mycorrhizal fungi are widespread symbionts able to colonize the roots of a lot of terrestrial plant species, including *V. vinifera* (Trouvelot et al., 2015). The characterization of AMF communities associated to grapevines is of interest by both economic and historical viewpoints. In fact, Piedmont is one the most important Italian regions for vineyard cultivation and wine

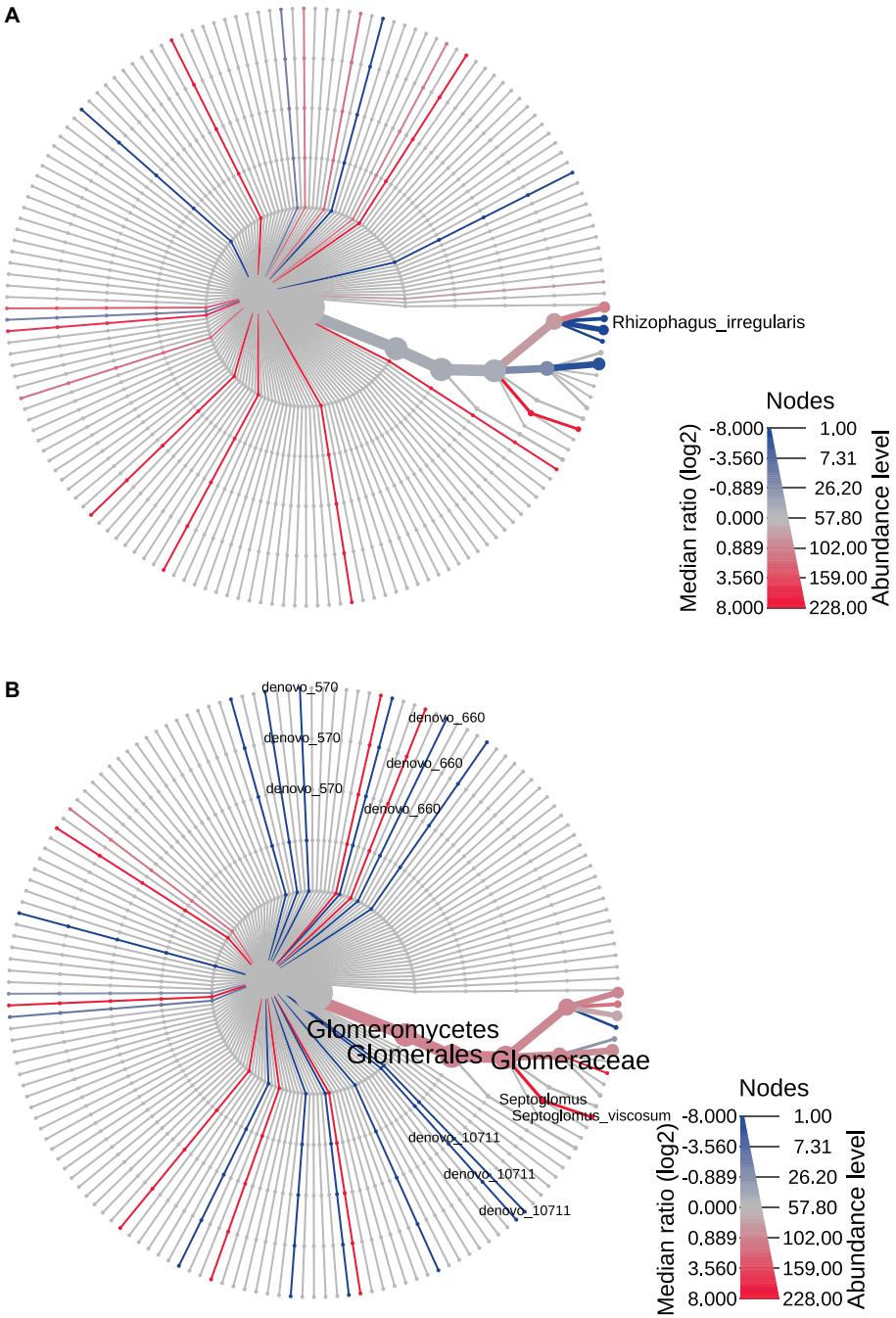


FIGURE 4 | Heat tree based on the factor "time", reporting the effect of sampling time on the hierarchical structure of taxonomic classifications (median abundance, non-parametric Wilcoxon rank–sum test). **(A)** Bs1S vs. Bs2S. **(B)** Rs1S vs. Rs2S. Heat tree analysis was performed using the R metacoder package of MicrobiomeAnalyst, a free available on-line software (https://www.microbiomeanalyst.ca).

production (ISTAT, 2018), and since 2014, the UNESCO World Heritage list has included the hills of the Piedmont area covering the Langhe, Roero, and Monferrato[4]. Therefore, we focused our attention on the AMF community associated to the Pinot Noir grapevine cultivar, which is largely cultivated in Piedmont.

At the beginning, the mycorrhizal colonization of grapevine roots was evaluated to assess the actual interaction between the plant and the AM fungi present in the soil. The plant roots were colonized by AMF at levels that were similar to those reported in the literature (Schreiner, 2005, 2020; Likar et al., 2013; Bouffaud et al., 2016; Massa et al., 2020), and no significant differences between the two sampling times were observed. This

[4]http://whc.unesco.org/en/list/1390

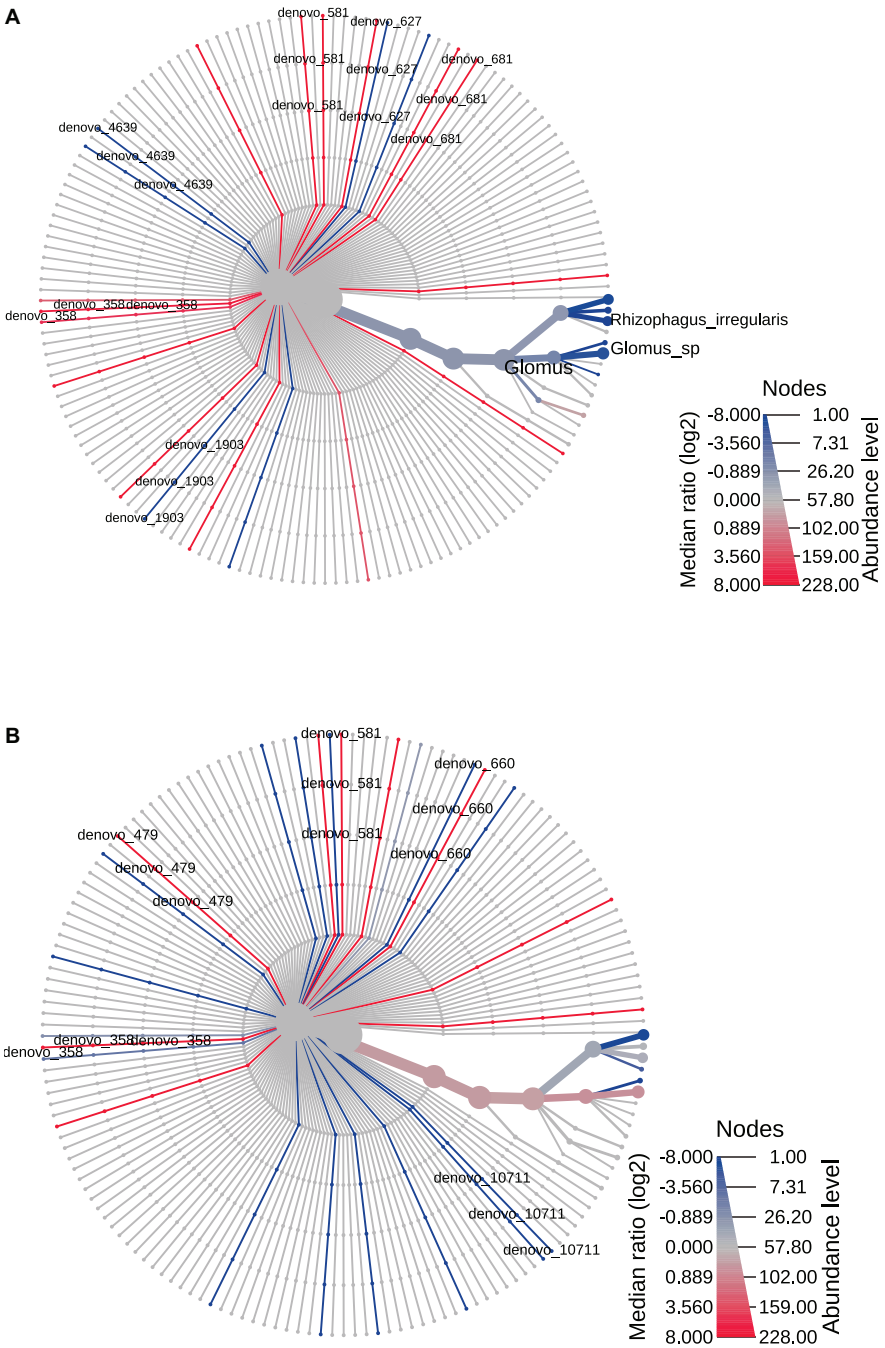


FIGURE 5 | Heat tree based on the factor "space" reporting the effect of the presence of *Vitis vinifera* roots on the hierarchical structure of taxonomic classifications (median abundance, non-parametric Wilcoxon rank–sum test). **(A)** Bs1S vs. Rs1S. **(B)** Bs2S vs. Rs2S. Heat tree analysis was performed using the R metacoder package of MicrobiomeAnalyst, a freely available online software (https://www.microbiomeanalyst.ca).

is in contrast with the findings of Schreiner (2005) who reported that the mycorrhizal colonization of *V. vinifera* cv. Pinot Noir roots increased before bud break in the spring, reaching values of about 50–60% of root length in early summer, that remained constant until leaf senescence in late fall.

Fragments resulting from pyrosequencing analysis were 700 bp in length, so they have a larger size if compared to those obtained in previously published works (Lumini et al., 2010; Holland et al., 2014). A total of 467 taxa have been found. This result is consistent with that reported by Holland et al. (2016), in which 816 taxa were obtained using the SSU rDNA marker for studying the AMF biodiversity in different vineyards in Canada. Similarly, Massa et al. (2020) found 528 taxa of AMF in an integrated pest-managed (IPM) vineyard in Piedmont

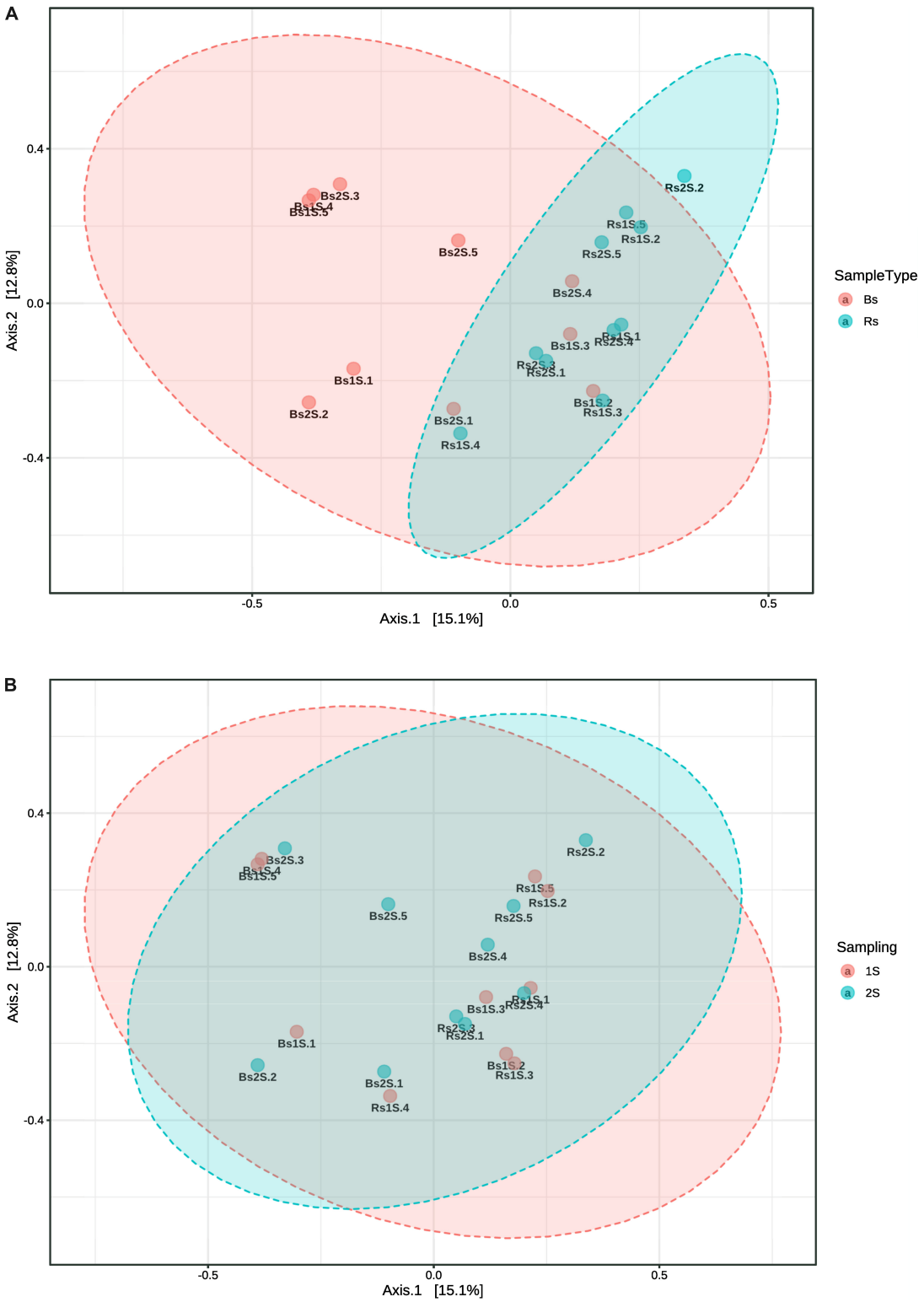


FIGURE 6 | Comparison by principal coordinate analysis of the ecological distance (based on Bray–Curtis distance method) of the different compartments. **(A)** Soil effect (Bs = soil not associated to grapevine roots, Rs = soil associated with the roots of *Vitis vinifera* cv. Pinot Noir); PERMANOVA *F*-value: 1.787, *R*-square: 0.090312, and *p*-value < 0.005. **(B)** Sampling time effect (1S = flowering and 2S = fruit development); PERMANOVA *F*-value: 0.48119, *R*-square: 0.026037, and *p*-value < 0.994. Beta diversity analysis was performed using the phyloseq package of MicrobiomeAnalyst, a freely available online software (https://www.microbiomeanalyst.ca).

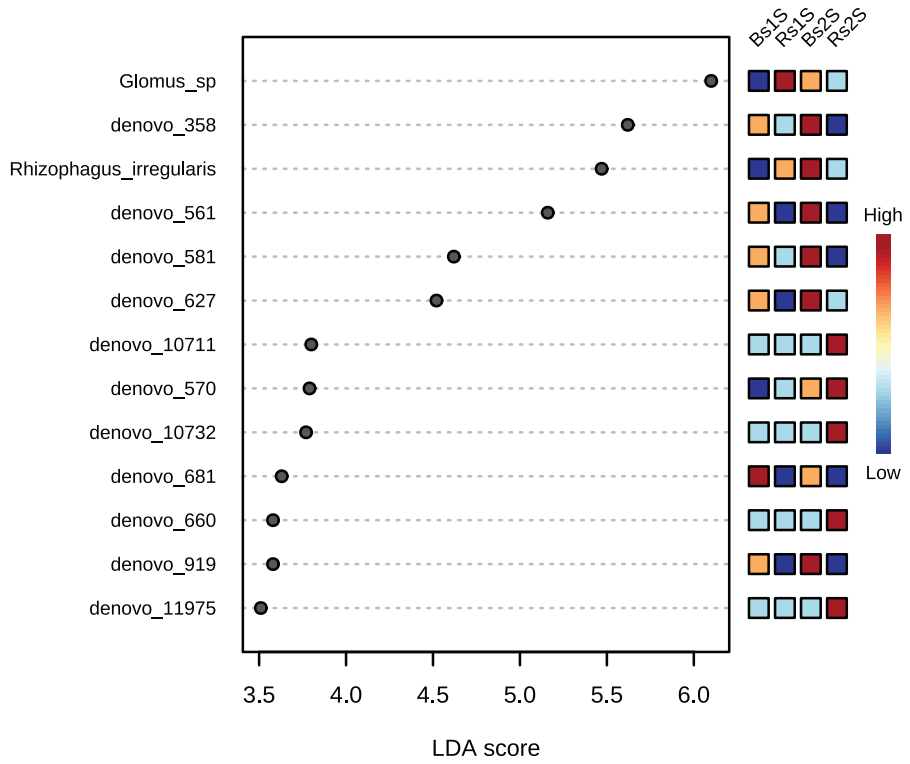


FIGURE 7 | Linear discriminant analysis–effect size (LDA–LEfSe) results using non-parametric factorial Kruskal–Wallis sum–rank test. Adjusted *p*-value cutoff = 0.05 and LDA score = 1.0; *Glomus* sp. (LDA score 6.1); *de novo*_358 – uncultured *Archaeospora* (LDA score 5.62), *Rhizophagus irregularis* (LDA score 5.47), *de novo*_561 – uncultured *Archaeospora* (LDA score 5.16), *de novo*_581 – uncultured *Archaeospora* (LDA score 4.62), *de novo*_627 – uncultured *Archaeospora* (LDA score 4.52), *de novo*_10711 – uncultured *Glomus* (LDA score 3.8), *de novo*_570 – uncultured *Glomus* (LDA score 3.79), *de novo*_10732 – *Rhizophagus intraradices* (LDA score 3.77), *de novo*_681 – uncultured *Archaeospora* (LDA score 3.63), *de novo*_660 – *Rhizophagus irregularis* (LDA score 3.58), *de novo*_919 – uncultured *Archaeospora* (LDA score 3.58), and *de novo*_11975 – uncultured *Rhizophagus* (LDA score 3.51). LEfSe analysis was performed with MicrobiomeAnalyst, a freely available online software (https://www.microbiomeanalyst.ca).

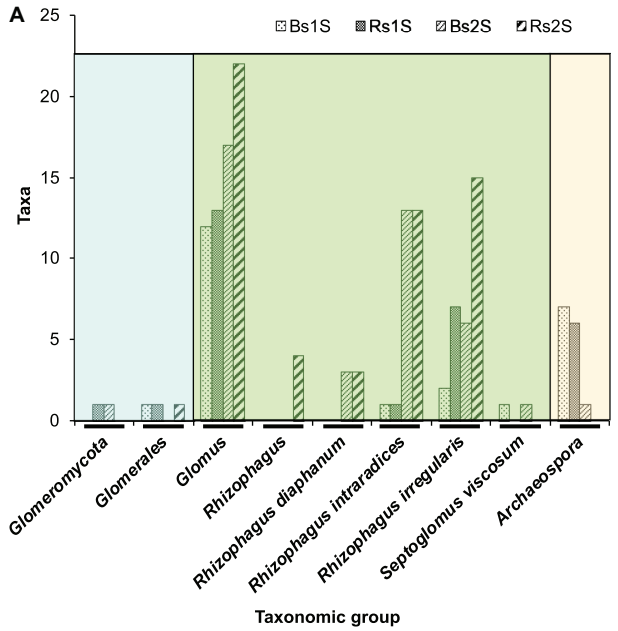
(Italy). Considering the distribution of taxa among the different samples, the highest number of taxa was observed in Rs2S. The two soils showed different taxa: in the second sampling, only 44 taxa were shared by both soils, while 74 were present only in Bs2S and 93 only in Rs2S. Moreover, only 20 taxa were common to the two soils at the two sampling times. Compared to the AMF community characterization performed in the IPM vineyard considered in Massa et al. (2020), in the present work, focused on a conventionally managed vineyard, a higher number of taxa shared between the two soils and a lower number of taxa exclusive of each soil were observed at the two sampling times. On the contrary, considering the sampling time, both Bs and Rs soils shared numbers of taxa similar to those reported in Massa et al. (2020). However, a lower number of exclusive taxa for each sampling time was observed. The differences between the AMF community described in these two studies could be due to the different vineyard management (IPM vs. conventional), but the impact of chemical–physical soil parameters cannot be ruled out.

The biodiversity indices (number of observed species – Shannon and Simpson's indices) did not show significant differences according to soil type (Bs vs. Rs) and sampling time (1S vs. 2S). Our results confirmed what was previously reported by Schreiner and Mihara (2009), demonstrating that AMF vineyard communities did not change with season succession, but differed according to the vineyard age and type of soil. Consistently, the impact of seasonality on the biodiversity of AMF has been described in crops other than grapevines (Dumbrell et al., 2011; Bouamri et al., 2014; Vargas-Gastélum et al., 2015).

Going into deeper detail and considering time and space as separate factors, some significant differences have been highlighted. In particular, an increase of *R. irregularis* in Bs and an increase of *R. irregularis* and uncultured *Glomus*, combined with a reduction of *S. viscosum* in Rs, were observed in the second sampling time compared to the first one. Moreover, in both sampling times, an increase of *Glomus* and *R. irregularis* and a decrease in uncultured *Archaeospora* were recorded in Rs compared to Bs.

Finally, the phylogenetic distance obtained by PCoA supported the difference between Bs and Rs soils, confirming what was previously discussed. These data are in accordance with what was reported in other scientific works, which demonstrated the effect of the plant in selecting the associated AM fungal population (Cesaro et al., 2008; Holland et al., 2014).

Consistently with Massa et al. (2020), no fungal sequences belonging to Claroideoglomeraceae, Acaulosporaceae,



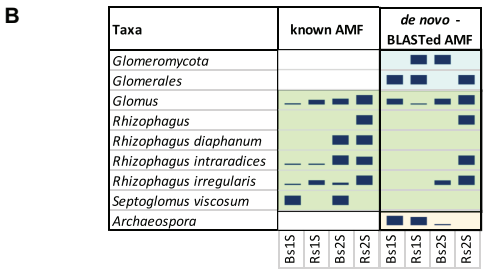


FIGURE 8 | (A) Number of taxa belonging to the different taxonomic groups – obtained by adding the "*de novo*-BLASTed AMF" taxa with the "known AMF" ones on the basis of the group to which they belonged – in the different samples (Bs = soil not associated to grapevine roots, Rs = soil associated with the roots of *Vitis vinifera* cv. Pinot Noir) at the two sampling times (1S = flowering and 2S = fruit development). **(B)** Relative abundance of taxa obtained considering separately "known AMF" and "*de novo*-BLASTed AMF", belonging to each taxonomic group, in Bs and Rs soils at the two sampling times (1S = flowering and 2S = fruit development).

Gigasporaceae, and Diversisporaceae were detected even if the primers used were specific for all AMF. Many fungal taxa corresponded to Glomeraceae, which is one of the most represented family in agricultural lands (Cesaro et al., 2008; Berruti et al., 2014) and also in vineyards (Balestrini et al., 2010; Lumini et al., 2010; Holland et al., 2014; Trouvelot et al., 2015; Bouffaud et al., 2016; Massa et al., 2020). This abundance could be explained by the high growth rate and the fast recovery of the hyphal network following the disturbance caused by agricultural practices (Berruti et al., 2014; Trouvelot et al., 2015) that are typical of the fungi belonging to this family. Although the genus *Glomus* was the most abundant, all the other remaining taxa of the family Glomeraceae, with the exception of *S. viscosum*, belonged to the genus *Rhizophagus*.

In agreement with Schreiner and Mihara (2009); Balestrini et al. (2010), Oehl and Koch (2018); Massa et al. (2020), and Schreiner (2020), we found AMF members of the family Archaeosporaceae represented only by one genus, *Archaeospora*.

The AMF community of a conventionally managed vineyard (with a prevalence of Glomeraceae and Archaeosporaceae) described in this work partly overlapped with those characterized in other vineyards subjected to a different management (Schreiner and Mihara, 2009; Balestrini et al., 2010; Holland et al., 2016; Massa et al., 2020). However, a lot of uncontrolled variables such as soil tillage, cover crops, manure application, and quality and amount of herbicides, fertilizers, and pesticides can influence the AMF diversity or community composition (Turrini et al., 2017).

In conclusion, in this work, a difference in AMF communities was observed between the two considered soils (Bs and Rs) independently from the plant phenological stage, suggesting a possible role of *V. vinifera* in modulating the AMF populations associated to its roots.

Overall, looking at the improvement in the global sustainability of viticulture practices, this study broadens the knowledge already gained by other works (Novello et al., 2017; Bona et al., 2019; Gamalero et al., 2020; Massa et al., 2020) regarding the microbiota associated with Pinot Noir grapevines cultivated in a geographic region historically dedicated to viticulture.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA613620.

AUTHOR CONTRIBUTIONS

PC cooperated in biological experiments, performed sample preparation for pyrosequencing, prepared the figures, analyzed data, and wrote the manuscript. NM organized the sampling, cooperated to data elaboration, prepared the figures, and wrote the manuscript. EB wrote the manuscript. GN participated to the sampling and performed DNA extraction. VT, EG, and GB cooperated in manuscript writing. LB performed pyrosequencing and bioinformatic analyses. FM cooperated in bioinformatic analyses. GL coordinated biological experiments, bioinformatic analyses, and manuscript writing. All authors revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2021.676610/full#supplementary-material

Supplementary Figure 1 | Rarefaction curves.

Supplementary Table 1 | List of IDs for "known" taxa identified by large subunit rDNA database.

Supplementary Table 2 | Identification of *de novo* taxa by BLAST analysis against the NCBI database.

Supplementary Table 3 | List of the IDs of "known" taxa (identified by large subunit rDNA database) and *de novo* taxa that were common between different soils (Bs or Rs) and in different sampling times (1S or 2S).

Supplementary Table 4 | Heat tree difference table reporting the results (*p*-values) of the non-parametric Wilcoxon rank–sum test used to depict the taxonomic differences between AMF communities.

Supplementary Table 5 | Linear discriminant analysis effect size results using non-parametric factorial Kruskal–Wallis sum–rank test.

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Ancient Relatives of Modern Maize From the Center of Maize Domestication and Diversification Host Endophytic Bacteria That Confer Tolerance to Nitrogen Starvation

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Plants can adapt to their surroundings by hosting beneficial bacteria that confer a selective advantage in stressful conditions. Endophytes are a class of beneficial bacteria that exist within the internal spaces of plants and many species can improve plant nitrogen use efficiency. Nitrogen is an essential plant macronutrient, and is often a limiting factor to plant growth, especially in cereal crops such as maize. Every year farmers apply over 100 million metric tonnes of synthetic nitrogen fertilizer to meet the growing demand for stable food crops. Breeding efforts in maize over the past several decades has focused heavily on yield in response to nitrogen inputs, and so may have selected against adaptations that allow plants to survive in nitrogen stressed conditions. Data suggests that our heavy dependence on synthetic nitrogen fertilizer is not sustainable in the long term, and so there is on-going research efforts to reduce and replace this currently essential part of modern agriculture. Bacteria that improve plant tolerance to nitrogen stressed environments would allow farmers to reduce the amount of fertilizer they apply. The selection of maize under high nitrogen conditions to create modern varieties may have caused the plant to lose these beneficial bacteria that allowed wild maize ancestors to thrive in low nitrogen soil. Here in this study, we examine the root and shoot microbiomes of the wild ancestor of all maize, Parviglumis, and an ancient Mexican landrace (Mixteco) from Oaxaca, the area of early maize diversification. Both of these maize genotypes have thrived for thousands of years with little to no nitrogen inputs and so we hypothesized that they host beneficial bacteria that allow them to thrive in nitrogen stressed conditions. We identified multiple root endophyte species from each ancient maize relative that increased the growth of annual ryegrass (model maize relative) under nitrogen starvation. Furthermore, research infers these strains were vertically transmitted to new generations of plants, potentially through seed, indicating selection pressure for Parviglumis and Mixteco to maintain them in their microbiome.

Keywords: endophyte, diazotroph, nitrogen, microbiome, maize, teosinte, landrace, domestication

INTRODUCTION

Maize (corn) is among the world's top three most important food crops, with over 1 billion tonnes produced in 2016 globally (FAO, 2016). Maize is cultivated around the world with large amounts of genetic diversity (Diggle and Friedman, 2011), but phylogenetic analysis traces all modern maize (*Zea mays* ssp. *mays*) to a single domestication event that occurred in southern Mexico 9,000 years ago from its wild ancestor *Z. mays* ssp. *parviglumis* (Parviglumis teosinte) with a minor contribution from *Z. mays* ssp. *mexicana* (Matsuoka et al., 2002). Parviglumis teosinte shows tolerance to low nitrogen stress (Gaudin et al., 2011; Han et al., 2015), and as a wild plant evolved in the absence of human nitrogen inputs, unlike its cultivated progenitors. Parviglumis can still be found thriving in nutrient poor soil in the Balsas River valley of Mexico. Elegant research has revealed the genetic differences that contribute to the dramatic morphological differences between modern maize and its Parviglumis ancestor (Matsuoka et al., 2002; Doebley et al., 2006), however, there has been little study on the microbiomes of these plants.

Endophytes are plant associated microbes that live inside host plants without causing disease (Johnston-Monje and Raizada, 2011b; Hardoim et al., 2015; Brader et al., 2017). Research suggested that a large fraction of the bacterial seed endophytes from Parviglumis were no longer present in modern maize (Johnston-Monje and Raizada, 2011a). Another study suggested that many root and shoot endophytes in Parviglumis could likely be vertically transmitted to subsequent generations presumably via the seed microbiome, and that certain bacterial species would be maintained regardless of the soil they were grown in, including sterile sand (Johnston-Monje et al., 2014). The latter study showed similar results with root and shoot endophytes of Mixteco (*Z. mays* ssp. *mays* landrace Mixteco), a giant, ancient pre-Columbian landrace of maize from the state of Oaxaca in Mexico, near the site of early maize diversification, that reportedly is grown by indigenous farmers on low-nutrient soils and may represent a missing link between modern maize and Parviglumis (Matsuoka et al., 2002). Recently, another giant farmer-selected landrace from Oaxaca growing on nutrient-poor soils was shown to possess nitrogen-fixing endophytes in its above ground brace roots (Van Deynze et al., 2018). This landrace, known as Sierra Mixe, secretes large amounts of sugar rich mucilage from brace roots as the plant matures, and this creates an environment suitable for nitrogen fixation and transfer to the plant host (Van Deynze et al., 2018). Researchers have speculated that the ability of these primitive maize genotypes to thrive in nutrient poor conditions may be attributed to beneficial endophytic bacteria (Triplett, 1996; Van Deynze et al., 2018). Moreover, it has previously been hypothesized that the root microbiome of Parviglumis hosts robust nitrogen fixing endophytes that perhaps contribute to its tolerance to nitrogen stress (Han et al., 2015). We previously demonstrated that the bacterial endophytes isolated from the below ground roots of Mixteco and Parviglumis could grow *in vitro* on nitrogen-free media (Johnston-Monje and Raizada, 2011a) and speculated that they might contribute nitrogen to their host plants, i.e., that they are diazotrophs.

Modern agriculture demands vast quantities of synthetic nitrogen fertilizer to grow cereal crops such as corn, wheat, and rice, which unlike their wild relatives have been bred with high levels of synthetic nitrogen fertilizer. These grasses are essential to feeding our growing population, but the burning of natural gas to create the 100 teragrams of nitrogen fertilizer produced every year has proven unsustainable in the context of our knowledge of global warming (Galloway et al., 2003, 2004; Cheng, 2008). Therefore, there is incentive to find alternative ways to provide bioavailable nitrogen to our cereal crops, and the use of diazotrophic endophytes has shown potential (Iniguez et al., 2004; Pankievicz et al., 2015; Rosenblueth et al., 2018).

Screening for diazotrophic endophytes that can provide bioavailable nitrogen to their host plants is somewhat complicated. Screening bacteria for plant growth promoting properties *in vitro* is fast (Narula et al., 1981; Ahmad et al., 2006, 2008), however the plant environment is vastly different, and so microbial *in vitro* performance may not correspond to their *in planta* performance. At the same time, high-throughput *in vitro* experiments can act as preliminary screens to reduce sample sizes based on mechanism of action.

The gold standard for determining microbial capacity for nitrogen fixation is the acetylene reduction assay. However, this method is difficult to scale for a larger library of candidates in a high throughput screen. A simpler and more scalable alternative is to screen for microbial growth in nitrogen free media, although this method has several limitations. Both these methods do not give information about potential bacterial nitrogen transfer to plant hosts. Measuring bacterial ammonium secretion is a common method to assess nitrogen transfer from bacteria to their environment (Pankievicz et al., 2015). However, the direct product of nitrogen fixation, ammonia, is toxic to maize at locally high concentrations (Schortemeyer et al., 1997; Britto et al., 2001), whereas the downstream product, glutamine (Gln), is the compatible, primary assimilate and major transport form for external nitrogen (Magalhães et al., 1990; Hirel et al., 2001) and so may be relevant in diazotroph-maize relationships. Here, we modify an existing Gln biosensor protocol to develop a high throughput assay to identify bacteria that secrete bioavailable nitrogen in the form of Gln. This biosensor was previously validated as a measure of nitrogen fixation derived from rhizobia bacteria inhabiting legume nodules (Thilakarathna et al., 2017, 2018).

In addition to nitrogen fixation, plant associated bacteria can improve plant growth and nutrient use efficiency by modulating and secreting phytohormones such as auxins, cytokinins, and ethylene (Cassán et al., 2009; Santoyo et al., 2016). Secretion of the auxin, indole acetic acid (IAA), by root associated bacteria has been shown to alter root morphology in grasses (Dobbelaere et al., 1999). Stimulating increased root growth and surface area to improve nutrient uptake is a mechanism by which plant associated bacteria can improve nitrogen use efficiency (NUE) (Di Benedetto et al., 2017, 2019). In addition to nitrogen, bacteria can assist in the uptake of important micronutrients such as Fe, Cu, and Cd. Collectively these bacteria are classified as

biofertilizers for their ability to provide and promote uptake of nutrients. Testing bacterial isolates *in planta* under nitrogen starvation would also identify these potentially useful isolates that confer tolerance to nitrogen stress by improving NUE and nutrient uptake. To develop a high-throughput *in planta* model system to identify nutrient promoting endophytes from maize, a recent study from our group assessed a diversity of forage and turf grass species (Shehata et al., 2017b). Annual ryegrass proved to be a good model and belongs to the family Poaceae along with maize, and hence they are genetic relatives. Annual ryegrass can be grown in test tubes and was shown to be highly growth/biomass responsive to nitrogen inputs, and identified a maize seed endophyte that could increase root biomass in the absence of external nitrogen (Shehata et al., 2017b).

In this study, we used the described combination of *in vitro* screens and the annual ryegrass *in planta* assay to screen the previously isolated root and shoot endophytes (Johnston-Monje et al., 2014) of the wild ancestral teosinte Parviglumis and the ancient Mexican landrace Mixteco for strains that confer tolerance to nitrogen starvation. Both of these maize genotypes thrive in nutrient poor soil with little to no N-fertilizer inputs, and this may in part be due to beneficial N-fixing and growth promoting endophytes. Altered root/shoot biomass ratio was also calculated, since plants have been shown to acclimate to low nitrogen stress by proliferating their roots to scavenge for nitrogen while limiting shoot growth and hence nitrogen demand (Gaudin et al., 2011).This study tests the hypothesis that Parviglumis and Mixteco possess nitrogen fixing endophytes that can act as nitrogen biofertilizers to promote plant growth under nitrogen stressed conditions.

MATERIALS AND METHODS

This study used two simple preliminary *in vitro* experiments to narrow the number of microbial targets we would test *in planta:* first, assessing their growth in N-free liquid media to identify candidates that can support their growth on N_2 gas alone; and second, testing for bacterial Gln secretion on N-free agar using a previously engineered Gln biosensor (Tessaro et al., 2012). This biosensor was previously validated as a measure of nitrogen fixation derived from rhizobia bacteria inhabiting legume nodules (Thilakarathna et al., 2017, 2018); here we use a modified protocol to identify maize endophytes that can secrete this bioavailable form of nitrogen while growing on nitrogen free agar media.

Source of Endophytes

The bacterial endophytes used in this study were previously reported, with the corresponding Genbank accession numbers located within (Johnston-Monje et al., 2014). Briefly, they were isolated from surface-sterilized root and shoot tissues from the wild ancestor of modern maize, *Z. mays* ssp. *parviglumis* (Parviglumis), and an ancient Mexican landrace (*Z. mays* ssp. *mays*, landrace Mixteco), growing on three types of soil in pots in a Canadian greenhouse: a Canadian agricultural soil that has grown modern hybrids for decades; a non-agricultural Mexican soil on which Parviglumis was observed growing; and sterile sand (**Figure 1**).

Endophyte Growth in Nitrogen Free Medium

Glycerol stocks of endophytes stored in 96 well plates at −80°C were used to inoculate 900 μL Burks N-free media using a 96-pin replicator. This medium consisted of (per L): 0.2 g $MgSO_4$, 0.8 g K_2HPO_4, 0.2 g KH_2PO_4, 0.130 g $CaSO_4$, 0.00145 g $FeCl_3$, 0.000253 g Na_2MoO_4, and 20 g sucrose. Phosphates were autoclaved separately and cooled before mixing to avoid formation of a precipitate which would interfere with OD_{600} readings in the spectrophotometer. Plates were sealed with a sterile breathable membrane (BF-400-S, Corning) and incubated in an anaerobic chamber (Shellab Bactron IV, Sheldon Manufacturing, Inc., Cornelius, OR, United States) for 6 days at 25°C. After growth in N-free medium, cells were resuspended, and the OD_{600} was measured using a 96-well spectrophotometer (Molecular Devices, SpectraMax 384 Plus). Each endophyte strain was tested with three replicates across three different 96-well plates, with two independent trials.

Burk's GlnLux Gln Secretion Assay

Preparation of GlnLux Biosensor Cells

From a glycerol stock, single streaked GlnLux colonies grown on LB agar + carbenicillin (100 mg/ml) and kanamycin (50 mg/ml) at 37°C were used to inoculate 25 ml of LB in 50 ml tubes. LB media was supplemented with 50 μL 2 M glucose, 25 μL 0.2 M Gln, and 25 μL kanamycin and 25 μL carbenicillin. These antibiotics at these concentrations were used to supplement all GlnLux media in this report. Liquid cultures were grown overnight at 37°C with shaking at 200 rpm in Burk's nitrogen free Burk's media to deplete endogenous nitrogen. The cultures were centrifuged at 2,500 × *g* at 20°C for 10 min, then washed 2× in nitrogen free Burk's media. Cells were resuspended to an OD_{595} of 1.0 in the same media.

Endophyte Colony Assay

The protocol was adapted from Tessaro et al. (2012). Glycerol stocks of endophytes stored in 96 well plates at −80°C were spotted on *Burk's* GlnLux plates using a 96-pin replicator. Burk's GlnLux plates were made as follows: per L, 800 ml of M9 medium (no NH_4, pre-warmed to 42°C) was mixed with 100 ml of molten agar (100 g/L), cooled to 42°C, to which 100 ml of GlnLux Burk's liquid culture (OD595 = 1.0) was added. Subsequently, 75 ml of this mixture was poured into Petri dishes (150 × 15 mm), cooled at room temperature, and stored at 4°C. Molten agar was mixed with nitrogen-free Burk's media to a final concentration of 10 g/L and cooled to 42°C. Washed GlnLux culture in nitrogen free Burk's media was added to the molten agar media to make 10% of the final volume. 75 ml of this mixture was poured in large Petri dishes, cooled at room temperature, and stored at 4°C. Inoculated plates were incubated at 30°C for 3 days to allow endophytes to fix nitrogen and grow. After 3 days, GlnLux plates were moved

to 37°C incubator to allow the GlnLux biosensor cells to grow for 24 h. These plates were then imaged using a ChemiProHT Luminescence Imaging System (Roper, United States) with Winview 32 software with a 10-min exposure. The CCD chip was cooled using liquid nitrogen for 1 h prior to imaging to reduce background dark noise. Treatments and replicates were normalized by equating minimum and maximum light intensities across plates in the Winview 32 software. Each endophyte plate had three replicates.

Annual Ryegrass Biofertilizer Experiment

Endophyte Seed Coat Preparation

Root and shoot endophytes that showed either Gln secretion or growth in N-free liquid media were selected for testing as potential nitrogen biofertilizers using annual ryegrass. The forage species annual ryegrass (*Lolium multiflorum*) was selected as the model species and used to screen a library of maize seed endophytes for candidates that could increase plant biomass in the absence of bioavailable nitrogen (Shehata et al., 2017b). Glycerol stocks stored at −80°C of selected root/shoot endophytes were inoculated on LB agar plates. The sample set of 51 candidate endophytes tested in the annual ryegrass biofertilizer experiment can be seen in **Table 3**. Single colonies were used to inoculate LB liquid medium and incubated at 37°C shaking at 200 rpm for 2 days. Cells were centrifuged, washed twice in 10 mM tris HCl (pH 7), then suspended to OD_{595} = 0.5 ± 0.1. From each suspension, 500 μl were diluted in 5 ml of 9.3% PVP aqueous solution. Sterilized seeds were mixed with endophyte solutions and placed on a rotary shaker for 1 h with shaking at 200 RPM to coat the seeds.

Plant Growth System

This consisted of glass culture tubes with 15 ml of 0.5 strength MS medium with no nitrogen (pH 5.8) (M531, Phytotech, United States). The media was supplemented (per L) with 250 μl nicotinic acid (1 mg/ml), 500 μl pyridoxine HCl (0.5 mg/ml), 5 ml thiamine HCl (100 mg/l), 500 μl glycine (2 mg/ml), and 2 g Phytagel (P8169, Sigma, United States) in double distilled water (Shehata et al., 2017b). To solidify Phytagel, 0.166 g/l $CaCl_2$ and 90 mg/l $MgSO_4$ were added. Seeds were soaked in 70% ethanol for 1 min then in bleach for 20 min. Seeds were then rinsed 6 times in sterile ddH_2O.

Plant Growth

Seven endophyte coated seeds were planted per tube (1 replicate), and each endophyte was tested with three replicates, randomized. There were 2–3 independent trials per treatment. Plants were moved to the dark to germinate for 7 days then grown at room temperature in a 16/8 h light dark cycle under 100–120 μmol m^{-2} s^{-1} cool white fluorescent light. The large number of candidate endophyte strain treatments meant the experiment was performed in batches, each with a Tris-PVP uninoculated control for comparison. After growing for 4 weeks, plants were removed from the tubes, and the gel substrate was removed manually and by soaking in a 60°C water bath. Plants were placed between two dry paper towels for 10 s, then placed on paper to air dry for 10 min. Shoots were separated from roots, and each was weighted as a total mass per tube, divided by the number of plants per tube to calculate the mean fresh weight per experimental unit (tube).

Statistical Methods

Growth in Burk's N-Free Liquid Media Experiment

The experiment was set up in a block design with 3 blocks. The fixed effect and independent variable was the endophyte strain, and the random effect was the block. The dependent variable was growth as measured by OD_{600}. The study had a type 1 error rate of $\alpha = 0.05$. The statistical analysis was performed on SAS version 9.4 software using the general linearized mixed model (Glimmix) procedure with a lognormal distribution. The assumptions of this model are: normal distribution of residuals, homogenous error variance across fixed and random effects, and error that is independent of treatment effects. To test the assumption of normality, a Q–Q plot of studentized residuals was created using the UNIVARIATE procedure; the distribution was assessed for normality. In addition to the Q–Q plot, a formal test of normality was conducted (Shapiro-Wilk test). To test the assumption of homogenous error variance, scatter plots of studentized conditional residuals across the fixed effect of strain and random effect of block were created. The distribution of these was assessed to ensure there were no patterns observed in the distribution of studentized residuals. Heterogeneous error variance was not seen and so the model was not modified. The fixed effect was tested using an *F* test, and the random effect was tested using a log likelihood test. To assess outliers in the data, the studentized residuals were analyzed to see if any treatment data was beyond the envelope of ± 3.4 as outlined by Lund's test, but no data points were removed. The mean OD_{600} growth values of each endophyte strain was compared pairwise with no adjustment. The letter values were generated using the Pdmix800 program.

Annual Ryegrass Biofertilizer Experiment

The experiment was a completely randomized design with 51 treatments, each with 3 replicates. The fixed effect (independent variable) was the endophyte strain, and the dependent variables were root and shoot biomass. The large sample size meant that the experiment was divided into batches, each with an uninoculated control, and batch was included as a random effect in the analysis. This exploratory study had a type 1 error rate of $\alpha = 0.1$, although *P*-values < 0.05 and 0.01 were noted. Statistical analysis was performed on SAS version 9.4 software using the general linearized mixed model (Glimmix) procedure with an identity link function. The model assumes a normal distribution of residuals, homogenous error variance across fixed effects and random effects, and error that is independent of treatment effects. Normality of residuals was tested with UNIVARIATE procedure to create a Q–Q plot of studentized residuals as well as a Shapiro-Wilk test; both tests showed residuals were normally distributed. Scatter plots of studentized conditional residuals were created to test the assumptions of homogenous error variance across treatments (fixed effect) and batches (random effect). The Covariance structure did not need to be modified as there were no obvious patterns. The fixed effect

was tested against the control with an *F*-test using a series of contrast statements in order to identify endophyte treatments that significantly increase plant biomass against the uninoculated controls. Outliers in the data were analyzed by looking at the studentized residuals, ensuring all were within the envelope of ±3.4 as outlined by Lund's test.

Whole Genome Sequencing

Candidate strains were grown on LB at 30°C overnight prior to DNA being isolated using the Norgen Bacterial Genomic DNA Isolation Kit (17900). A Nextera XT DNA sample prep kit (Illumina, San Diego) was used to create a paired-end library, and sequencing was done using Illumina Miseq. Genome assembly was completed using the CLC Genomics Workbench (10.0.1), while gene annotations were performed using the RAST genome annotation pipeline (Aziz et al., 2008). Genes in the nitrogen fixation operons were confirmed and reclassified using BLAST (Camacho et al., 2009).

RESULTS

Root and Shoot Endophyte Growth in N-Free Liquid Media

Growing 92 maize root and shoot endophytes from Parviglumis and Mixteco in N-free Burk's media anaerobically showed that many of these bacterial species showed modest growth or did not grow at all (**Figures 2A,B**). The ability to effectively colonize N-free liquid media to produce turbid cultures indicates a strain that is not limited by the nitrogen free conditions presented. There were six endophyte strains that were able to produce visibly turbid cultures and were the highest OD_{600} values seen in both trials of this experiment (**Table 1**). We refer to these endophytes as robust diazotrophs in this report as they can effectively colonize the N-free liquid media with only N_2 gas to support their growth. Interestingly, all 6 of these robust diazotroph endophyte strains were isolated from root tissue (**Table 1**). Another point of interest is that 5 of the 6 robust strains were isolated from Parviglumis, the direct wild ancestor of modern maize (**Table 1**). Endophyte strains from Parviglumis and Mixteco that showed growth in N-free Burk's media were added to the list of candidate endophytes to be tested *in planta* as nitrogen biofertilizers.

Ability for Endophytes to Secrete Gln *in vitro*

The Burk's GlnLux colony assay identified many root and shoot endophytes from Parviglumis and Mixteco that could grow on Burk's N-free agar and secrete Gln to support the growth of GlnLux cells as indicated by emission of luminescence (**Figure 3**). In total, 34/92 of the tested root/shoot endophytes secreted Gln on N-free agar in at least 2 out of 3 independent trials. These endophytes were added to the pool of candidates to test as nitrogen biofertilizers, given their ability to secrete this plant-available form of nitrogen. The summary of this assay is shown in **Table 2**.

Ability of Candidate Maize Endophytes to Promote Growth of Annual Ryegrass in the Absence of Any Nitrogen Fertilizer

The *in vitro* assays above identified root and shoot endophytes from maize that could either grow in N-free liquid media and/or secrete Gln on N-free agar (**Figures 2**, **3**), both of which are potential plant growth promoting mechanisms. To identify endophytes that have the potential to either increase NUE or provide bioavailable nitrogen to their plant hosts, we conducted an experiment testing a total of 51 candidate diazotrophs/Gln secretors for biomass effects in annual ryegrass following endophyte seed coating and growth in N-free media. It is important to note that some endophytes were excluded from this experiment if they showed poor growth in LB media which limited their potential as inoculants. Furthermore, certain wells in the 96 well endophyte source plate were each shown to have more than one colony morphology upon streaking, and so these mixed cultures were separated prior to ryegrass testing and given a letter suffix (e.g., A,B) (taxonomic confirmations for some strains are

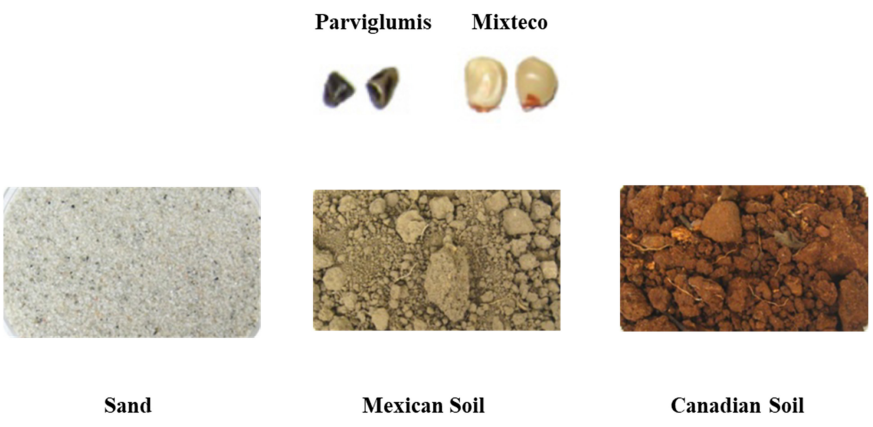

FIGURE 1 | Sources of root and shoot endophytes tested in this study. Endophytes were isolated from surface sterilized roots and shoots of Parviglumis and Mixteco grown in three different soils (including sterile sand) as part of a past study (Johnston-Monje et al., 2014).

pending, F2018). Endophyte strain *Enterobacter* 3D9 was shown to increase ryegrass biomass in a past experiment (Shehata et al., 2017b; Dumigan et al., 2018), and hence it was included as a positive control in Trial 1 for comparison (from 4 independent batches). Given the large number of strains to be tested, the experiment was broken up into batches where a subset of endophytes was tested in parallel with an uninoculated negative control (endophyte buffer), and the control was included in each batch; batches were separated by time (see section "Materials and Methods"). All candidate endophytes were tested by seed coating in two independent trials; a third trial was conducted in parallel to Trial 2 on a select group of endophytes, in part based on early promising results.

The fresh weights of annual ryegrass roots and shoots for all trials following seed inoculation are shown (**Figure 4**, and **Supplementary Tables 1–3**) in addition to changes in the root/shoot biomass ratio known to be involved in plant acclimation to nutrient stress (**Figure 5**). The change in percentage root or shoot biomass was calculated relative to the respective mean negative control from all batches of that trial (**Table 3**). The screen identified four strains that increased annual ryegrass biomass when seed coated and grown in N-free media in at least two independent trials with a *P*-value less than 0.05: *Klebsiella* 4F1, *Microbacterium* 4B5A, *Bacillus* 4E2 and *Stenotrophomonas* 4A12 (**Table 3**):

Klebsiella 4F1 increased root biomass compared to the uninoculated control by 67.8% ($p = 0.0012$), 35.2% ($p = 0.056$), and 51.79% ($p = 0.0008$) in Trials 1, 2, and 3, respectively (**Table 3** and **Figure 4**). This endophyte significantly increased the root to shoot ratio in two independent trials (**Figure 5**)

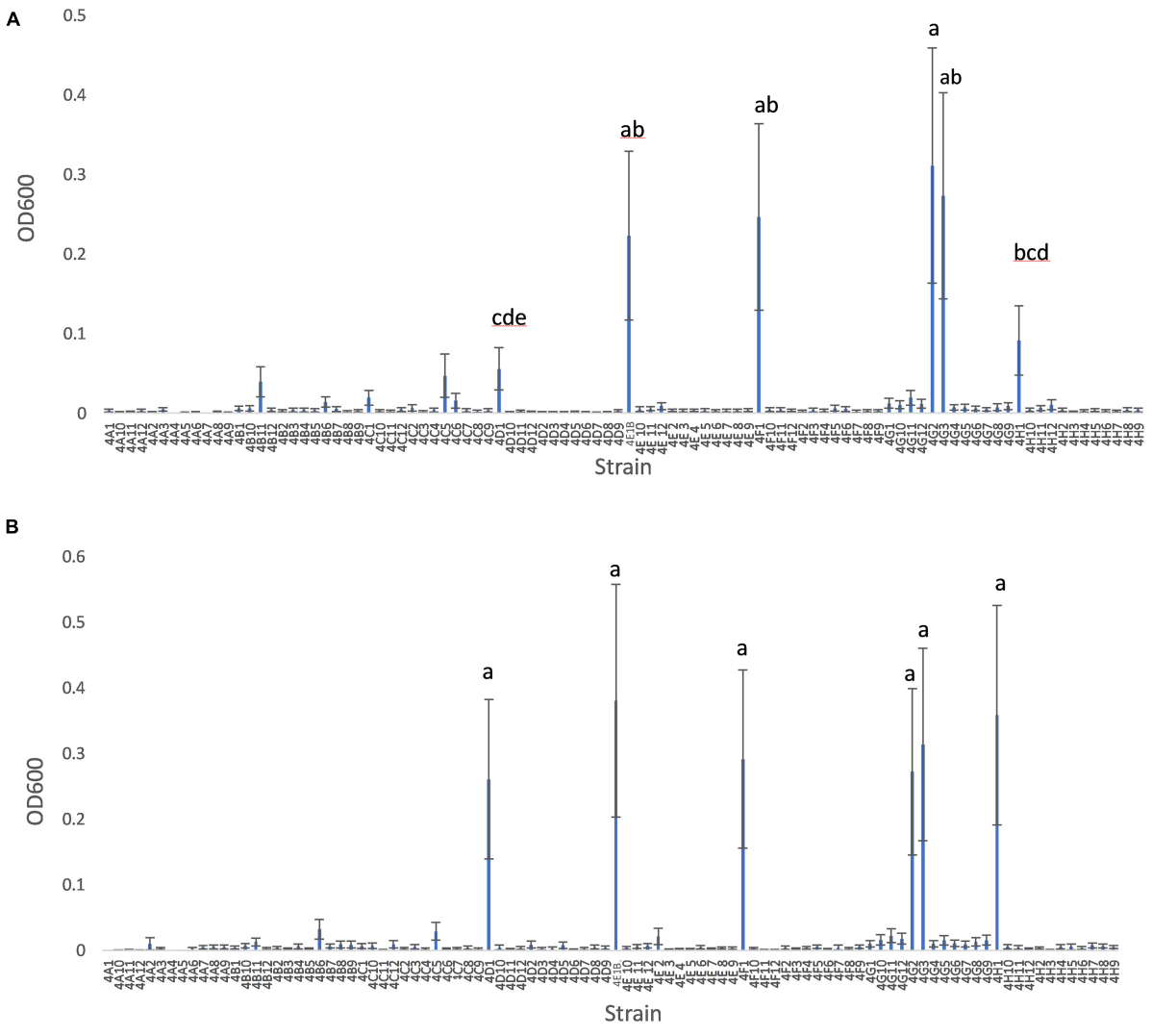

FIGURE 2 | *In vitro* growth of root and shoot maize endophytes in nitrogen free medium. **(A)** Trial 1. **(B)** Trial 2. Root and shoot endophytes stored in glycerol stocks in 96-well plates were inoculated into 900 μl Burk's N-free media in 96-deep well plates ($n = 3$). These plates were incubated anaerobically at room temperature for 6 days. After 6 days, cultures were resuspended and OD_{600} values were measured using a spectrophotometer. The error bars represent the standard error, and means with the same letter value are not statistically different.

TABLE 1 | Summary of endophytes* that grew robustly in N-free growth trials *in vitro*.

Endophyte	Host genotype	Tissue	Soil	Mean OD600
Trial 1				
4G2 – *Klebsiella*	Parviglumis	Root	Canada	0.311 a
4G3 – *Klebsiella*	Parviglumis	Root	Canada	0.273 ab
4F1 – *Klebsiella*	Parviglumis	Root	Canada	0.247 ab
4E1B – *Klebsiella*	Parviglumis	Root	Sterile sand	0.223 ab
4H1 – *Klebsiella*	Parviglumis	Root	Mexico	0.092 bcd
4D1 – *Bacillus*	Mixteco	Root	Mexico	0.056 cde
Trial 2^				
4G2 – *Klebsiella*	Parviglumis	Root	Canada	0.272 a
4G3 – *Klebsiella*	Parviglumis	Root	Canada	0.314 a
4F1 – *Klebsiella*	Parviglumis	Root	Canada	0.291 a
4E1B – *Klebsiella*	Parviglumis	Root	Sterile sand	0.381 a
4H1 – *Klebsiella*	Parviglumis	Root	Mexico	0.359 a
4D1 – *Bacillus*	Mixteco	Root	Mexico	0.261 a

**Both root and shoot endophytes were screened, and shown are those that showed the highest growth in both trials.*
^These six endophytes were not statistically different from one another in Trial 2.

and did not significantly increase shoot biomass in either trial. *Microbacterium* 4B5A significantly increased root biomass by 64.3 and 29.7% in Trials 2 and 3, respectively ($p = 0.0006, 0.041$), however showed a non-significant increase of 33.2% in Trial 1 ($p = 0.1129$) (**Table 3** and **Figure 4**). This candidate endophyte significantly increased the root/shoot biomass ratio in only one trial (**Figure 5**). *Bacillus* 4E2 did not increase root biomass in the first trial, however, in two subsequent independent trials this candidate endophyte significantly increased root biomass by 46.13% ($p = 0.0128$) and 28.66% ($p = 0.0483$), respectively. This candidate endophyte did not significantly increase the root/shoot ratio in any trial ($p > 0.05$) (**Figure 5**). *Stenotrophomonas* 4A12 showed significant increases in shoot biomass compared to uninoculated controls in both trials, with a 34.0% increase in Trial 1 ($p = 0.0108$) and a 36.9% increase in Trial 2 ($p = 0.0082$) (**Table 3** and **Figure 4**). Surprisingly, a third independent trial of 4A12A showed a non-significant decrease of 0.5% in shoot biomass compared to the negative control (**Table 3**). The root to shoot ratio of 4A12 coated ryegrass did not significantly change in any trial compared to the negative control (**Figure 5**).

Only endophytes with significant increases in plant biomass were considered interesting in these initial high-throughput *in planta* screens. It is important to note that there are many endophyte seed treatments that significantly increased root or shoot biomass compared to the control in a single trial, however, only endophyte seed treatments that gave a consistent result are highlighted in this Results text section. All biomass increases can be seen on the graphs (**Figure 4**) or in the summary (**Table 3**).

Whole Genome Sequencing of Top Microbial Candidates

Four strains were selected for whole genome sequencing based on their performance at improving biomass of annual ryegrass under nitrogen starvation. Strain 4F1 was identified as belonging to the species complex *Klebsiella pneumoniae-variicola*, and its genome was predicted to encode 19 *nif* open reading frames (ORFs) involved in nitrogen fixation (**Supplementary Figure 1**). Nitrogenase genes were not found in the other candidate strains. Strain 4E2 was identified as belonging to the species complex *Bacillus velezensis-amyloliquefaciens.* Strain 4A12 was identified as *Stenotrophomonas indicatrix.* Stain 4B5A was identified as *Microbacterium barkeri*.

DISCUSSION

Organisms including plants can adapt to stressful environments by adopting beneficial bacteria in their microbiomes (Johnston-Monje and Raizada, 2011b; Hardoim et al., 2015; Mousa et al., 2015, 2016; Brader et al., 2017; Shehata et al., 2017a; Van Deynze et al., 2018). Nitrogen (N) is a vital plant macronutrient needed for protein, DNA, and chlorophyll biosynthesis, therefore nitrogen stress is critically damaging to growing plants. Today, modern maize is grown with large amounts of synthetic N-fertilizer inputs, and breeding efforts in maize over the past several decades have focused on increasing yield responses to increasing N supply (Neill et al., 2004; Kant et al., 2011). Research has suggested that the domestication of maize caused a community shift in the rhizosphere microbiomes, in comparisons of modern hybrids to ancestral teosintes (Brisson et al., 2019). Interestingly, the largest shift in the plant-microbe community structure appears to be from the domestication of teosinte to landraces, while modern agronomic practices show the largest effect on potential microbe-microbe interactions (Schmidt et al., 2020).

A past study from our group showed that the seed microbiomes of 14 wild and domestic maize varieties have undergone significant changes over the course of evolution, and subsequent human selection and migration in the Americas (Johnston-Monje and Raizada, 2011a). The study further showed that approximately half of the endophytes in the direct, wild ancestor of modern maize, Parviglumis, are no longer present in modern maize. It is possible that humans have selected against

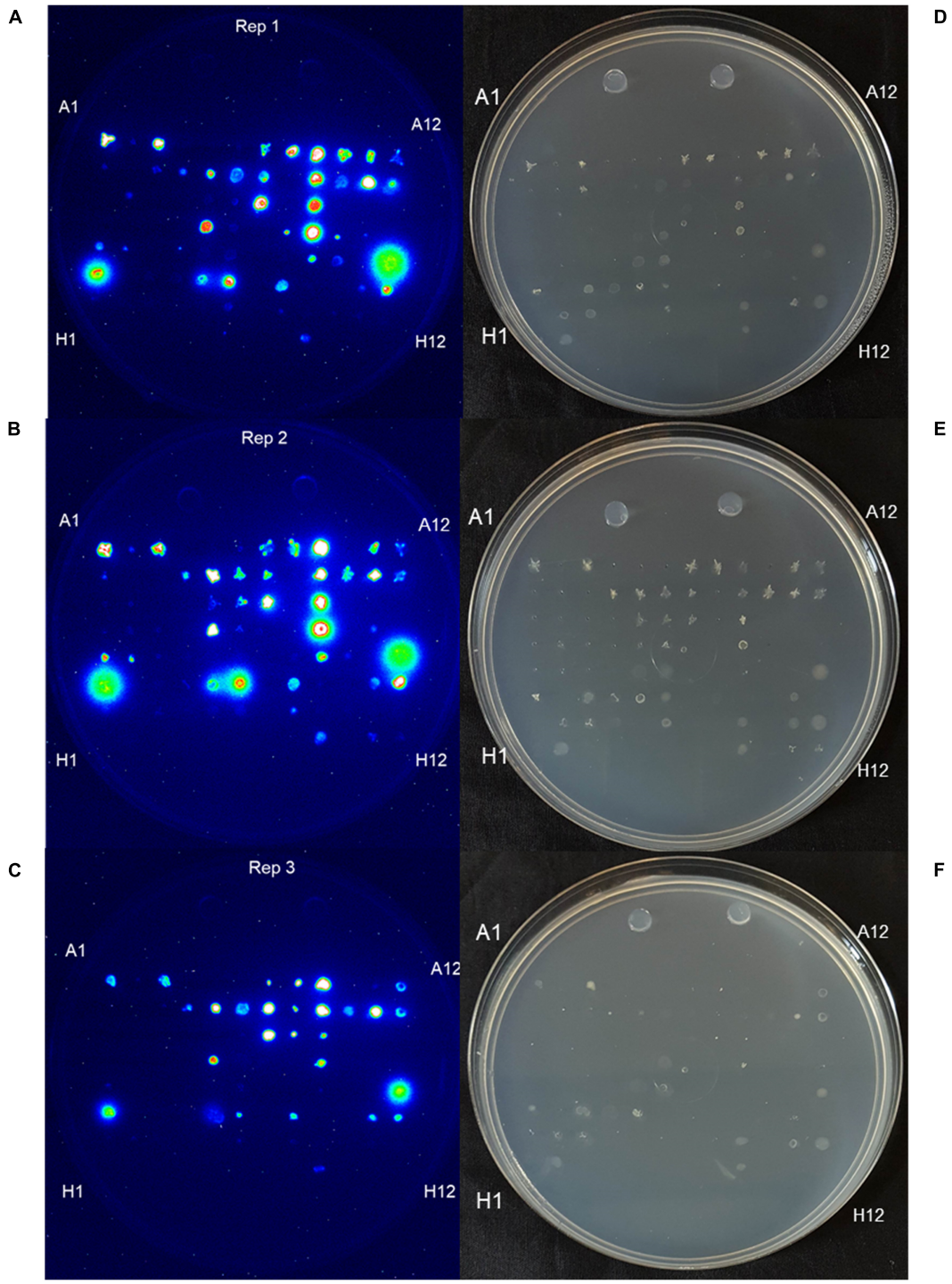

FIGURE 3 | Burk's GlnLux colony assay to screen the maize endophytes for Gln secretion *in vitro* on nitrogen-free Burk's agar. **(A–C)** Shown is luminescence imaging of three replicates: **(A)** replicate 1, **(B)** replicate 2, and **(C)** replicate 3 of endophytes from Parviglumis and Mixteco that could secrete Gln on N-free agar as indicated by a luminescence signal. **(D–F)** Corresponding light images of these plates: **(D)** replicate 1, **(E)** replicate 2, and **(F)** replicate 3. Some strains did not grow on this N-free agar as expected.

the retention of beneficial plant bacteria that assist wild maize to grow in the absence of human-derived nitrogen. Therefore, in this study we tested root and shoot microbes from Parviglumis. We also tested endophytes from an ancient cultivated landrace from Mexico called Mixteco (Johnston-Monje and Raizada, 2011a). Parviglumis and Mixteco both originate from southern

TABLE 2 | Summary of maize endophytes that secrete Gln *in vitro* on nitrogen-free Burk's GlnLux agar.

Root/shoot endophytes					
Position	**Host**	**Tissue**	**Soil**	**Genus**	**Positive reps**
4A1	Mixteco	Shoot	Sand	*Microbacterium*	3
4A12	Mixteco	Shoot	Sand	*Stenotrophomonas*	3
4A3	Mixteco	Shoot	Sand	*Microbacterium*	3
4A7	Mixteco	Shoot	Sand	*Bacillus*	3
4A8	Mixteco	Shoot	Sand	*Cellulosimicrobium*	3
4A9	Mixteco	Shoot	Sand	*Stenotrophomonas*	3
4B5	Mixteco	Root	Sand	*Microbacterium*	3
4B6	Mixteco	Root	Sand	*Enterobacter*	3
4B7	Mixteco	Root	Sand	*Stenotrophomonas*	3
4B9	Mixteco	Shoot	Canada	*Stenotrophomonas*	3
4B10	Mixteco	Shoot	Canada	*Paenibacillus*	3
4B11	Mixteco	Shoot	Canada	*Stenotrophomonas*	3
4B12	Mixteco	Root	Canada	*Stenotrophomonas*	3
4C7	Mixteco	Root	Mexico	*Agrobacterium*	3
4C9	Mixteco	Root	Mexico	*Xanthomonas*	3
4D5	Mixteco	Root	Mexico	*Paenibacillus*	3
4D9	Parviglumis	Root	Sand	*Bacillus*	3
4E9	Parviglumis	Root	Sand	*Enterobacter*	3
4E12AB	Parviglumis	Shoot	Canada	*Bacillus*(A), *Pantoea*(B)	3
4F1	Parviglumis	Root	Canada	*Klebsiella*	3
4F5	Parviglumis	Root	Canada	*Microbacterium*	3
4F6	Parviglumis	Root	Canada	*Paenibacillus*	3
4F8	Parviglumis	Root	Canada	*Enterobacter*	3
4F11	Parviglumis	Root	Canada	*Microbacterium*	3
4F12	Parviglumis	Root	Canada	*Stenotrophomonas*	3
4G7	Parviglumis	Shoot	Mexico	*Paenibacillus*	3
4A10	Mixteco	Root	Sand	*Bacillus*	2
4A11	Mixteco	Root	Sand	*Stenotrophomonas*	2
4B4	Mixteco	Root	Sand	*Microbacterium*	2
4E1AB	Parviglumis	Root	Sand	*Bacillus*(A), *Klebsiella*(B)	2
4E2	Parviglumis	Root	Sand	*Bacillus*	2
4B8	Mixteco	Root	Sand	*Enterobacter*	2
4C6	Mixteco	Root	Mexico	*Enterobacter*	2
4C8	Mixteco	Root	Mexico	*Stenotrophomonas*	1
4D8	Parviglumis	Shoot	Sand	*Stenotrophomonas*	1
4D10	Parviglumis	Root	Sand	*Pantoea*	1

Mexico: Parviglumis was domesticated in the Balsas River Valley (Matsuoka et al., 2002; Piperno et al., 2009; Ranere et al., 2009), while the Mixteco accession used in this study (CIMMYT: OAX 569) was collected from the Nochixtlán District in the Mixteca Region of the nearby state of Oaxaca, the region of early maize diversification (Matsuoka et al., 2002). Critically for this study, in the Mixteca region, maize is cultivated by the indigenous Mixtec peoples on acidic, low-nitrogen soils, often on steep, depleted hillsides (Vergara-Sánchez et al., 2005; Bautista-Cruz et al., 2014), and despite such nitrogen limitation, Mixteco maize is giant in stature. Acidic soils prevent plant availability of most macronutrient fertilizers including nitrogen. The Nochixtlán Valley has been in continuous settlement since 2000 BC, and was the geographic center of the Mixtec peoples from 700 BC to 1600 AD, having a population estimated to be 50,000 people when the Spaniards arrived (Spores, 1969). The Nochixtlán Valley is only 50 km away from where another maize landrace called Sierra Mixe was collected (selected by the adjacent indigenous Mixe-speaking people) and recently reported to host potent nitrogen fixation in mucilage secreted from its above ground brace roots (Van Deynze et al., 2018). We thus hypothesized that the nitrogen stressed conditions of these ancient maize relatives may have driven natural and human selection, in Parviglumis and Mixteco respectively, for beneficial relationships with endophytic bacteria that provide tolerance to nitrogen starvation. We used three complementary approaches, two *in vitro* and one *in planta*:

Initially we tested the ability of 92 root and shoot endophytes of Parviglumis and Mixteco to grow in N-free liquid media, suggestive of N-fixation *in vitro*. Many endophytes showed minor

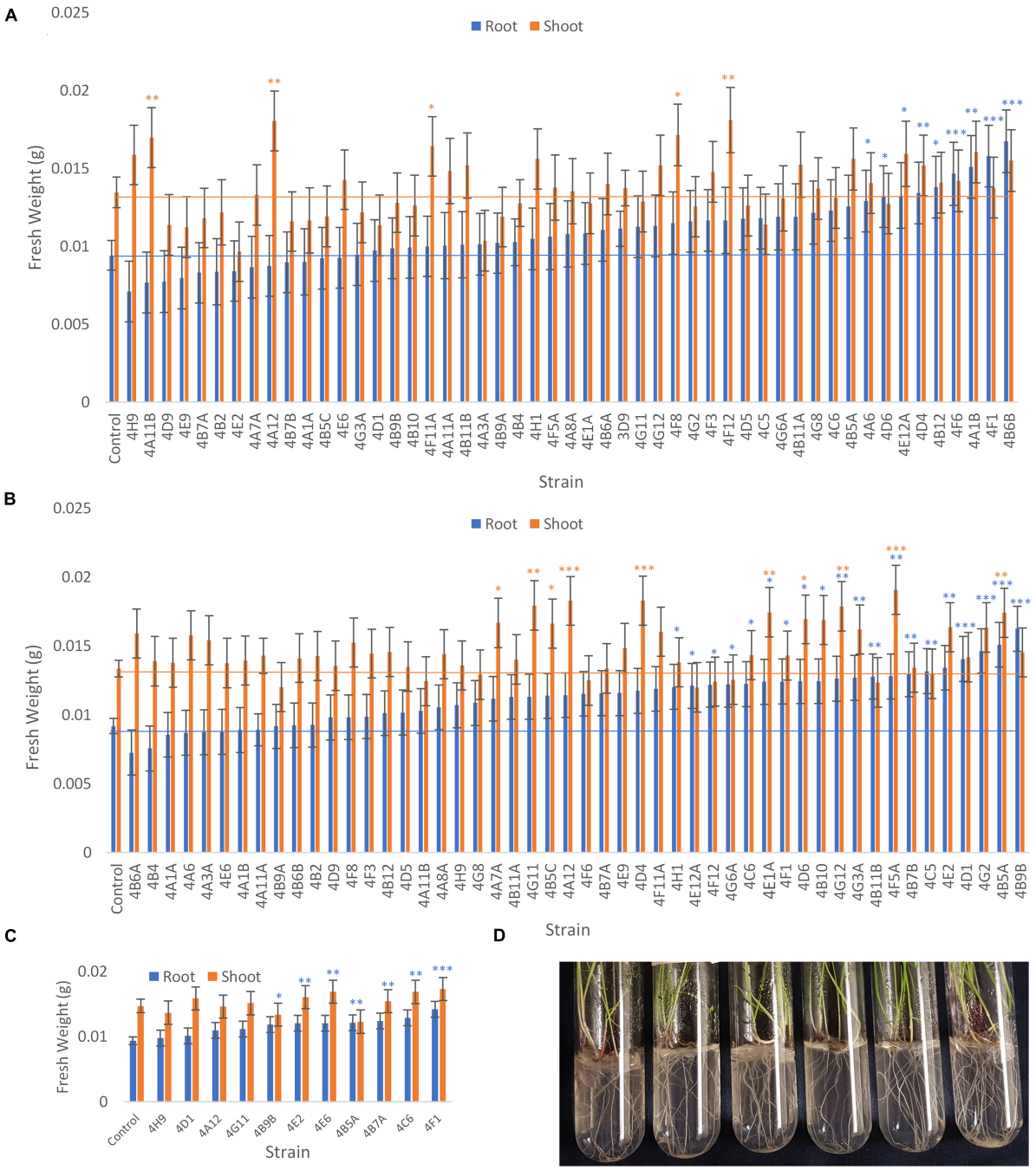

FIGURE 4 | Effect of endophyte seed coating on root and shoot fresh weight of annual ryegrass. Shown are the mean fresh weights of annual ryegrass, seed-coated with endophyte strains, and grown in N-free media for 4 weeks: **(A)** Trial 1, **(B)** Trial 2, and a subset in **(C)** Trial 3. Data is ordered by increasing root biomass (blue bars), and values significantly greater than the uninoculated control are denoted by $^{*}P < 0.1$, $^{**}P < 0.05$, and $^{***}P < 0.01$ as determined by *F*-tests. Error bars represent the standard error. **(D)** Images of the assay system: annual ryegrass growing in glass tubes in Phytagel based medium.

levels of growth, however, there were six endophyte species that consistently grew to the highest OD_{600} values in both trials (**Figure 2**). Though 55% of the root and shoot endophyte library was comprised of endophytes isolated from Parviglumis, with 45% from Mixteco (Johnston-Monje et al., 2014), five out of the six robust growing diazotrophs were from the former (Parviglumis) (**Figure 2**). All six of the endophytes were isolated from surface-sterilized root tissue, which comprised 75% of the library (Johnston-Monje et al., 2014). All endophyte strains from Mixteco and Parviglumis that could grow in N-free liquid were added to the pool of candidates to be tested as biofertilizers in annual ryegrass.

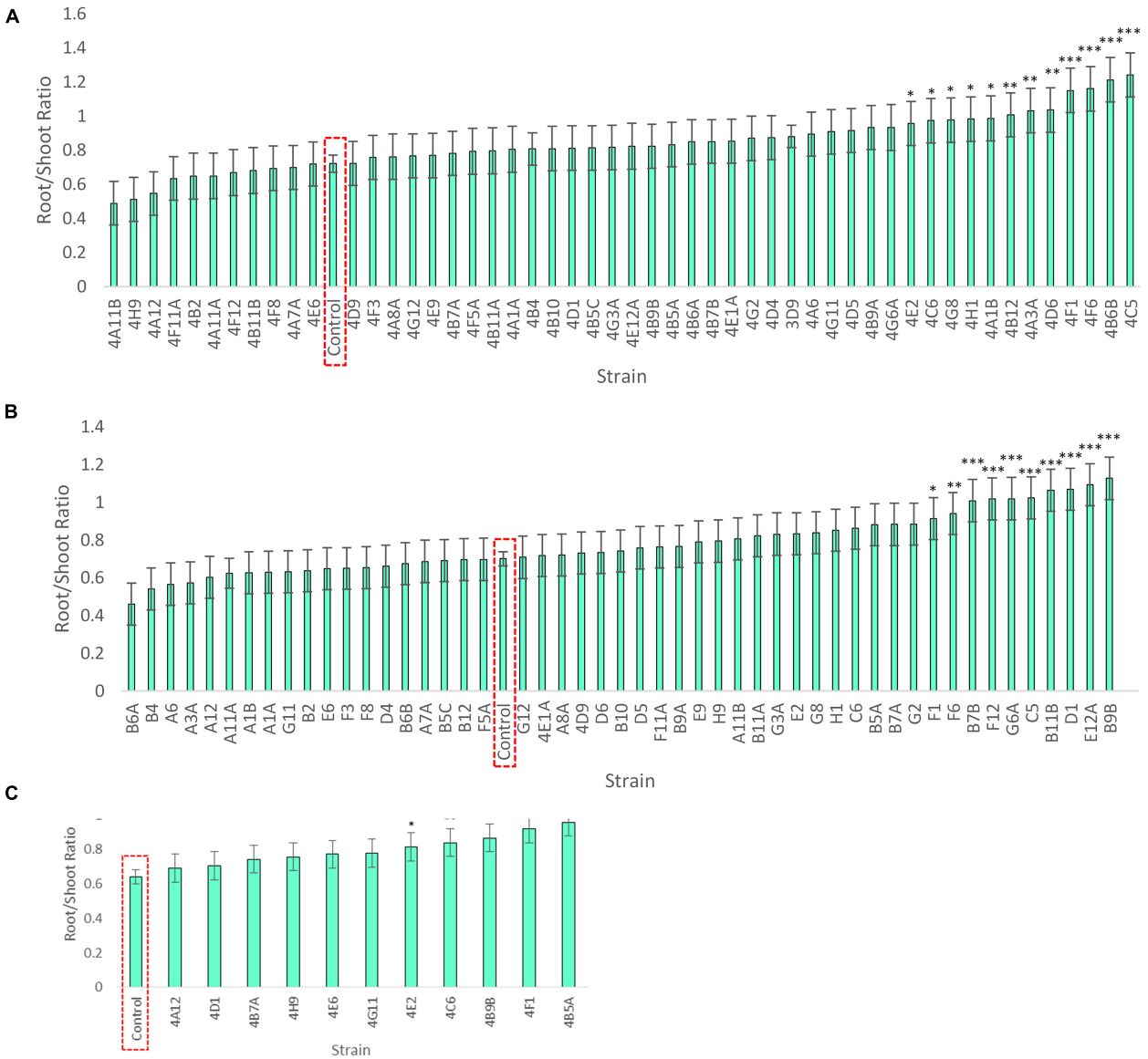

FIGURE 5 | Root/shoot biomass ratio of annual ryegrass following endophyte seed treatment. Shown is the data following 4 weeks of plant growth on N-free media for: **(A)** Trial 1, **(B)** Trial 2, and a subset in **(C)** Trial 3. The uninoculated control is outlined in red, and the data is ordered by increasing root/shoot ratio. Values significantly higher than the respective negative control are denoted by $^{*}P < 0.1$, $^{**}P < 0.05$, and $^{***}P < 0.01$ as determined by *F*-tests. Error bars represent the standard error.

Glutamine is the downstream assimilate of fixed nitrogen that also acts as a primary transport form of nitrogen in maize (Hirel et al., 2001; Näsholm et al., 2009; Tegeder and Rentsch, 2010), therefore, it has been previously hypothesized that, unlike rhizobia (in the legume symbiosis), Gln may be a nitrogen containing metabolite secreted by endophytes for cereal crops to utilize (Allaway et al., 2000). Therefore, in our second assay, we screened root and shoot endophytes from Mixteco and Parviglumis for their ability to grow on Burk's N-free agar and secrete Gln to support the growth of adjacent GlnLux biosensor cells and emit luminescence (**Figure 3**). The screen showed that 34/92 of the tested endophytes could grow and secrete Gln, which would be highly bioavailable to maize. These strains were also added to the pool of candidates to be tested as nitrogen biofertilizers in annual ryegrass.

Finally, in the third assay, the ability of the selected candidate endophytes to promote the growth of annual ryegrass in N-free media was tested. Annual ryegrass is a heterologous host, and hence the numbers might be greater in the native hosts. Four strains were found to significantly increase plant biomass in independent trials, and many more showed significant or borderline increases in at least one trial (**Figure 4** and **Table 3**). The 4 strains were 4F1, 4A12, 4E2, and 4B5A and originate

TABLE 3 | Summary of percent increases in annual ryegrass biomass following endophyte seed treatment compared to the respective uninoculated buffer control.

Strain ID	Genus	Host	Tissue	Soil	Root biomass % Increase vs. uninoculated						Shoot biomass % Increase vs. uninoculated					
					Trial 1		Trial 2		Trial 3		Trial 1		Trial 2		Trial 3	
4H9	*Microbacterium*	Pioneer	Shoot	Sand	−24.64		16.47		4.37		17.83		1.72		−6.9	
4A11B	*Stenotrophomonas*	Mixteco	Root	Sand	−18.5		12				26.08	*	−6.82			
4D9	*Bacillus*	Parviglumis	Root	Sand	−18		6.92				−15.45		1.57			
4E9	*Enterobacter*	Parviglumis	Root	Sand	−15.37		26.17				−16.57		11.17			
4B7A	*Stenotrophomonas*	Mixteco	Root	Sand	−11.89		25.95		32.73	**	−12.26		0.07		4.99	
4B2	*Pseudomonas*	Mixteco	Root	Sand	−11.24		0.77				−9.51		6.9			
4 E2	**Bacillus**	**Parviglumis**	**Root**	**Sand**	−10.72		46.13	**	28.66	**	−28.28	**	22.49		9.43	
4A7A	*Bacillus*	Mixteco	Shoot	Sand	−8.12		21.59				−1.26		24.89	*		
4A12	*Stenotrophomonas*	**Mixteco**	**Root**	**Sand**	−7.13		24.43		16.76		34.03	**	36.88	***	−0.55	
4B7B	*Stenotrophomonas*	Mixteco	Root	Sand	−4.64		41	**			−13.89		0.45			
4A1A	*Microbacterium*	Mixteco	Shoot	Sand	−4.3		−7.01				−13.45		3.22			
4B5C	*Bacillus*	Mixteco	Root	Sand	−1.97		23.99				−11.52		24.51	*		
4E6	*Delftia*	Parviglumis	Root	Sand	−1.67		−4.6		28.76	**	5.94		3		14.96	
4G3A	*Delftia*	Parviglumis	Root	Canada	0.68		38.17	**			−9.58		21.29			
4D1	*Bacillus*	Mixteco	Root	Mexico	3.18		53	***	8.19		−15.75		6.22		8.06	
4B9B	*Stenotrophomonas*	Mixteco	Shoot	Canada	4.78		77.1	***	26.51	*	−4.98		8.77		−9.15	
4B10	*Stenotrophomonas*	Mixteco	Shoot	Canada	5.53		35.44	*			−6.24		26.39			
4F11A	*Microbacterium*	Parviglumis	Root	Canada	6.19		29.44				21.92	*	20.01			
4A11A	*Stenotrophomonas*	Mixteco	Root	Sand	6.55		−3.11				10.25		7.05			
4B11B	*Bacillus*	Mixteco	Shoot	Canada	7.3		39.15	**			12.85		−7.5			
4A3A	*Microbacterium*	Mixteco	Shoot	Sand	7.51		−4.86				−23.03	*	15.37			
4B9A	*Cellulosimicrobium*	Mixteco	Shoot	Canada	8.36		−0.28				−11.74		−10.04			
4B4	*Microbacterium*	Mixteco	Root	Sand	9		−17.66				−5.2		4.05			
4H1	*Klebsiella*	Parviglumis	Root	Mexico	11.34		30.97	*			15.9		3.45			
4F5A	*Microbacterium*	Parviglumis	Root	Canada	12.82		39.26	**			2.23		42.88	***		
4A8A	*Cellulosimicrobium*	Mixteco	Shoot	Sand	14.73		14.83				0.52		7.8			
4E1A	*Bacillus*	Parviglumis	Root	Sand	15.05		35.01	*			−5.2		30.66	**		
4B6A	*Stenotrophomonas*	Mixteco	Root	Sand	17.39		−21.06				4.01		19.04			
3D9	*Enterobacter*	*Zea nicaraguensis*	Seed	N/A	18.24*		ND		ND		2.08		ND		ND	
4G11	*Enterobacter*	Parviglumis	Root	Mexico	19.52		23.12		19.34		−4.38		34.33	**	3.07	
4G12	*Pantoea*	Parviglumis	Root	Mexico	20.26		37.51	**			12.78		33.88	**		
4F8	*Enterobacter*	Parviglumis	Root	Canada	22.07		6.97				27.34	*	14.17			
4G2	*Klebsiella*	Parviglumis	Root	Canada	23.34		59.32	***			−6.84		22.34			
4F3	*Enterobacter*	Parviglumis	Root	Canada	23.77		7.48				9.81		8.17			
4F12	*Stenotrophomonas*	Parviglumis	Root	Canada	23.98		32.72	*			34.4	**	−7.12			
4D5	*Paenibacillus*	Mixteco	Root	Mexico	24.93		10.69				−6.32		1.05			
4C5	*Enterobacter*	Mixteco	Root	Mexico	25.46		43.4	**			−15.23		−2.85			
4G6A	*Paenibacillus*	Parviglumis	Shoot	Mexico	26.21		32.82	*			−2.9		−6.07			
4B11A	*Stenotrophomonas*	Mixteco	Shoot	Canada	26.31		23.01				13.22		4.95			
4G8	*Enterobacter*	Parviglumis	Root	Mexico	29.18		18.32				1.78		−3.3			
4C6	*Enterobacter*	Mixteco	Root	Mexico	30.67		33.26	*	37.65	**	−2.67		7.35		14.82	
4B5A	**Microbacterium**	**Mixteco**	**Root**	**Sand**	33.22		64.34	***	29.73	**	15.9		30.36	**	−16.33	
4A6	*Bacillus*	Mixteco	Shoot	Sand	36.94	*	−5.4				4.46		18.14			
4D6	*Rhizobium*	Parviglumis	Shoot	Sand	40.02	*	35.33	*			−5.35		26.84	*		
4E12A	*Bacillus*	Parviglumis	Shoot	Canada	40.87	*	31.84	*			18.42		−10.19			
4D4	*Bacillus*	Mixteco	Root	Mexico	42.46	**	27.92				12.7		36.96	***		
4B12	*Stenotrophomonas*	Mixteco	Root	Canada	46.5	*	10.25				4.61		9.07			
4F6	*Paenibacillus*	Parviglumis	Root	Canada	55.64	***	25.52				5.42		−6.45			
4A1B	*Paenibacillus*	Mixteco	Shoot	Sand	60.31	**	−3.12				19.17		4.35			
4F1	**Klebsiella**	**Parviglumis**	**Root**	**Canada**	67.75	***	35.22	*	51.79	***	2.3		7.05		17.9	
4B6B	*Enterobacter*	Mixteco	Root	Sand	77.84	***	0.51				15.23		5.7			

**Percent increases vs. uninoculated control values are shown as a heat map from red to green as values increase. Significant changes were calculated using an F-test with P-values of: *P < 0.1, **P < 0.05, and ***P < 0.01. Bold values indicate strains that significantly improve biomass across trials.*

equally from Mixteco and Parviglumis. All 4 of these strains were isolated as root endophytes. As research has suggested that a substantial portion of the juvenile maize rhizosphere originates from the plant (Johnston-Monje et al., 2016) including Parviglumis and Mixteco (Johnston-Monje et al., 2014), it may be that some of the beneficial activities of the root endophytes may actually be taking place in the surrounding soil. Interestingly 3 of the 4 endophytes that confer tolerance to N-starvation were originally isolated from plants grown in sterile sand in a greenhouse, suggesting they originated from seeds rather than being acquired from the soil (Johnston-Monje et al., 2014) which would be consistent with our hypothesis of long term natural and human selection on maize. Indeed, nitrogen fixing *K. pneumoniae-variicola* has been previously isolated from the seed endosphere of Parviglumis (**Supplementary Table 4**), suggesting this maize hosts nitrogen fixing bacteria that are seed borne, supporting the hypothesis that the strains in this study may have been vertically inherited. Additionally, these *Klebsiella* species that demonstrated nitrogen fixation also secreted Gln as demonstrated by the GlnLux biosensor assay (**Supplementary Table 4**). Furthermore, our research has shown that (seed-derived) endophytes can be deposited into the rhizosphere and root surfaces (Johnston-Monje et al., 2016; Mousa et al., 2016; Shehata et al., 2017a) where some of the above activities would need to be localized. Combined, the results of the three complementary assays used in this study provide evidence that the wild ancestor of modern maize, and an ancient race of corn from the region of early maize diversification, possess endophytes with the potential to confer plant tolerance to nitrogen stress.

As noted, four endophyte strains consistently promoted the growth of annual ryegrass in the absence of nitrogen fertilizer. *K. pneumoniae-variicola* 4F1 was isolated from surface sterilized root tissue of Parviglumis and belongs to the genus *Klebsiella*. *K. pneumoniae-variicola* 4F1 significantly increased both root biomass and the root/shoot biomass ratio of annual ryegrass compared to the uninoculated control in trials 1 and 2 (**Figures 4**, **5**). An increase in the root to shoot ratio is a conserved adaptation among plant species in response to nitrogen stress, as plants limit resource allocation to shoot tissue to favor of roots which have the ability to scavenge soil for nitrogen – a trait that we have previously characterized in Parviglumis (Gaudin et al., 2011). Interestingly, Parviglumis increases its crown root length 285% in response to low nitrogen conditions compared to a 30% elongation seen in a modern inbred maize line (Gaudin et al., 2011). Such an adaptation likely contributes to the tolerance to nitrogen stress seen in Parviglumis, and perhaps may be, in part, attributed to beneficial root endophytes such as *K. pneumoniae-variicola* 4F1.This strain was also found to grow well in nitrogen free media (**Figure 2**) and possess 19 ORFs for nitrogen fixation in its genome (**Supplementary Figure 1**) which together suggest the capacity for nitrogen fixation. Our previous study showed that this strain will also produce acetoin *in vitro* (Johnston-Monje et al., 2014), a compound that can alter plant hormone production to increase growth (Hu et al., 2003), and so the observed effect on plant growth may be a combination of mechanisms.

Bacillus velezensis-amyloliquefaciens 4E2 increased root biomass in annual ryegrass in two out of three trials. As above, *Bacillus* 4E2 was originally isolated from surface sterilized Parviglumis roots grown in sterilized sand, suggesting this endophyte is vertically transmitted to the next generation of plants (Johnston-Monje et al., 2014). The genus *Bacillus* is a well-studied plant growth promoting bacteria and has been shown to promote plant growth by a variety of mechanisms (Hu et al., 2003). This genus of spore-forming bacteria has been commercialized into a wide variety of biostimulants and biopesticides, and so it is unsurprising to find a plant growth promoting strain endophytic strain in the roots of Parviglumis plants. Species of *Bacillus* have been shown to improve NUE in greenhouse and field conditions by improving nutrient uptake (Adesemoye et al., 2008, 2009). Interestingly, the whole genome sequence of *B. velezensis-amyloliquefaciens* 4E2 did not identify any nitrogenase genes, suggesting an alternative mechanism for the observed growth promotion under nitrogen starvation. The strain did however secrete Gln on N-free agar (**Figure 3**), suggesting potential nitrogen transfer to its plant host. It could be that this strain is secreting or regulating phytohormones that are stimulating root growth and improving nitrogen/nutrient use efficiency when its host plant is under nitrogen stress. Again, it is interesting to speculate whether favorable selection pressure has caused Bacillus species such as 4E2 to be passed down to the next generation of plants as a member of a healthy Parviglumis microbiome to confer some level of tolerance to nitrogen stress.

Stenotrophomonas indicatrix 4A12 was found to significantly increase shoot biomass in trials 1 and 2 (**Figure 4**). The strain did not grow in Burk's N-free liquid media in either trial (**Figure 2**), however, it did grow on Burk's N-free agar and secrete Gln (**Figure 3**). Whole genome sequencing of 4A12 did not identify any nitrogenase genes in the annotation. Taken together, it was surprising that the endophyte did not increase root biomass in any trial (**Figure 5**), since the standard response to nitrogen stress in plants, as already noted, is to reallocate carbon from the shoots to the roots to promote growth (Han et al., 2015). The mechanism by which 4A12 is stimulating shoot growth is not currently known. The previous study from where this isolate originates found that it solubilized phosphate and produced acetoin *in vitro* (Johnston-Monje et al., 2014). *S. indicatrix* 4A12 could be stimulating annual ryegrass shoot growth under nitrogen starvation by secreting bioavailable Gln, stimulating plant growth by phytohormone regulation, and/or improving uptake of nitrogen and other nutrients such as phosphate. It should be noted that the host genotype of this endophyte is Mixteco which possesses a giant shoot, but whether *S. indicatrix* 4A12 has any causal relationship to this gigantism awaits further testing. *S. indicatrix* 4A12 was isolated from surface sterilized root tissue that were grown in sterilized sand, meaning this endophyte was likely vertically transmitted from parental Mixteco plants rather than incorporated from the surrounding soil (Johnston-Monje et al., 2014). A previous study demonstrated that endophytes of the genus *Stenotrophomonas* were widespread in the seeds of a diversity of maize genotypes (unpublished data).

Microbacterium barkeri 4B5A significantly increased root biomass in 3 independent trials, and showed some of the largest biomass increases observed compared to uninoculated control plants (**Figure 4**). There have been reports of nitrogenase activity in *Microbacterium* (Gtari et al., 2012), but to our knowledge no reports on endophytic N-fixing *Microbacterium* species. *M. barkeri* 4B5A did not show growth in N-free media (**Figure 2**), and nitrogenase genes were not found in the whole genome sequence. However a past study demonstrated that this endophyte secretes acetoin (Johnston-Monje et al., 2014), and so is likely promoting plant growth under nitrogen starvation by regulating plant hormones, and/or improving nutrient and NUE rather than through nitrogen fixation. As with *S. indicatrix* 4A12, this endophyte was isolated from surface sterilized Mixteco roots grown in sterile sand, again suggesting vertical transmission.

As this study was an exploratory high throughput screen looking at biomass increases, the underlying mechanisms were not examined and likely vary between endophytes. The main conclusion that can be drawn from the *in planta* experiments is that under nitrogen stress, these seed coated endophytes have biofertilizer activity and will increase root or shoot biomass of their heterologous annual ryegrass host (**Figure 5**). The endophytes may increase host NUE as discussed earlier or contribute nitrogen and/or other nutrients to their plant host. The data supports the hypothesis that Parviglumis and Mixteco possess endophytes that can act as nitrogen biofertilizers to promote plant growth under nitrogen starvation. Future experiments involving bacterial gene knockouts are needed to clarify the underlying mechanisms for the increase in plant biomass under N starvation conditions. Additionally, these bacterial isolates should be tested in larger scale experiments using maize.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://www.ncbi.nlm.nih.gov/genbank/, JF776463–JF776567.

AUTHOR CONTRIBUTIONS

CD and MR designed the study. CD, JM, and JG conducted the *in planta* assays. CD conducted all the *in vitro* assays in the manuscript and performed the statistical analysis. AS performed the 16S sequencing to confirm bacterial genus taxonomy. OH facilitated the bacterial whole genome sequencing. 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.2021.660673/full#supplementary-material

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