Variations of Phyllosphere and Rhizosphere Microbial Communities of Pinus Koraiensis Infected by Bursaphelenchus Xylophilus

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Research Article

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Abstract

Pinewood nematode, *Bursaphelenchus xylophilus*, as one of the greatest threats to pine trees, is spreading all over the world. During the nematode's pathogenesis, plant microorganisms play important roles.The phyllosphere and rhizosphere bacterial and fungal communities associated with healthy *P. koraiensis* (PKa) and *P. koraiensis* infected by *B. xylophilus* at the early (PKb) and last (PKc) stages were analyzed. Pine wood nematode (PWD) could increase the phyllosphere and rhizosphere microbial community diversity, and slight shifts of the microbial diversity were observed at the early stage of infection. Generally speaking, an increase in microbial diversity with more serious symptomatic phase was obvious and visible. The phyllosphere and rhizosphere microbial community potentially changed caused by *B. xylophilus* infection of *P. koraiensis*. With the infection of *B. xylophilus* in *P. koraiensis*, *Bradyrhizobium* (rhizosphere bacteria), *Massilia* (phyllosphere bacteria), *Phaeosphaeriaceae* (rhizosphere fungi) were the major contributors to the differences in community compositions among different treatments. With the infection of PWD, most of the bacterial groups tended to be co-excluding rather than co-occurring. These changes would correlate with microbial ability to suppress plant pathogen, enhancing the understanding of disease development and providing guidelines to pave the way for its possible management.

Introduction

With the acceleration of global climate change, terrestrial ecosystems face more and more abiotic and bio-disturbances. Among them, climate change has led to the emergence of plant diseases and insect pests in global forest systems, which has brought great ecological and economic challenges (Ghelardini et al., 2016), not only that, human activities also further aggravate the occurrence. In the forest ecosystem, leaf loss caused by diseases and pests leads to tree dieback, mortality and large-scale forest decline, resulting in changes of forest community structure (Metz et al., 2012), and thus affecting microbial community (Beule et al., 2017) and ecosystem function (Preston et al., 2016). Plant adaptation is the result of the integration of the plant itself and its microbiome, which is critical to the function of terrestrial ecosystem (Lu et al., 2018Chialva et al., 2018) and the response to global climate change (Van Der Heijden et al.2008; Cavicchioli et al.2019). Over the years, *Pinus* trees have been confronted with a severe and devastating disease, pine wilt disease (PWD) mainly caused by *Bursaphelenchus xylophilus* (Han et al., 2008), namely the pine wood nematode, which is one of the most serious conifer diseases worldwide, threatening several species of pine trees (Tóth, 2011), and resulting in profound economic losses and adverse ecological environmental threat worldwide (José and Rodrigues, 2008; Tóth, 2011; Vives-Peris et al., 2012; Zamora et al., 2015).

Trees infected with PWD appear photosynthesis decline, xylem deformity, resin duct disruption, cortex and cambium tissues damage, phytotoxin production, and water transportation and conduction impairment (Fukuda, 1997), resulting in discoloration, wilting, and consequent death of host trees (Futai, 2008). Given that, the biological characteristics of PWD (Zhou et al., 2017; Liu et al., 2019), the dispersing vector (Kawai et al., 2006; Vicente et al., 2012; Chen et al., 2020a), and the mechanism of PWD pathogenicity (Zhu et al., 2012; Qiu et al., 2016) have become research hot spots. In addition, there is growing evidences suggesting that during pathogenesis of *B. xylophilus*, plant microorganisms play important roles in host fitness (Guo et al., 2007; Tan et al., 2008; Han et al., 2010; Vicente et al., 2013; Vandenkoornhuyse et al., 2015). The growing amount of data demonstrated that plant-related bacteria have beneficial effects, promote plant growth and improve plant

stress and disease resistance (Hardoim et al., 2015; Rodriguez et al., 2019), particularly bacterial genera *Trichoderma*, *Serratia*, *Bacillus*, and *Esteya* which have nematicidal activity through mechanisms of parasitism or their production of toxic compounds (Maehara, 2008; Wang et al., 2017). In this perspective, there is a pressing need to illuminate the variations of plant microbiome during the disease development (Diogo et al., 2017), advancing our understanding of the relationship between plant compartments and the microbial communities after *B. xylophilus* infection, and paving the way for its possible management.

In recent years, the endophytic microbial community of several *B.xylophilus* host pine trees, such as *Pinus* flexilis (Carrell and Frank, 2014), *Pinus* contorta (Bal, 2003), *Pinus* pinaster (Proença et al., 2017a), and *Pinus* sylvestris (Izumi et al., 2008), have been well documented. It is well established that pine endophytic bacterial diversity and composition play an important role in regulating plant response to PWN (Proena et al., 2016; Alves et al., 2018, Mannaa et al., 2020). However, our understanding of the significant implications of phyllosphere and rhizosphere microorganisms remain limited. To the best of our konwledge, phyllosphere and rhizosphere microorganisms are two important components of plant microflora (Jia et al., 2018). Previous studies have shown that the decline in plant healthy status or changes in growth conditions caused by host pathogens could affect the microbial community in leaves and roots of the host (Douanla-Meli et al., 2013; Tian et al., 2015; Vives-Peris et al., 2018). The phyllosphere microbiome interact with the host plant affecting its health and function, and act as mutualists promoting plant growth and tolerance of environmental stressors (Stone et al., 2018). The plant healthy state also can affect the exudates of the plant root, which is the essential factor affecting the rhizosphere microbial community (Zhang et al., 2014; Hayat et al., 2017). The rhizosphere microbial community has excellent ability to synthesize diverse secondary metabolites in response to different abiotic and biotic stresses, which is fundamental to the healthy growth of plants (Bais et al., 2001; Mannaa et al., 2020). In addition, previous researches collected the samples were only at one time point at the last stage of the disease (Ma et al., 2020). Therefore, it is necessary to further investigate the microbial community at different stages of PWD after PWD occurrence and elucidate the relationship between the plant pathogen and host microbial community.

Pinus koraiensis is distributed in Northeast China, Japan, North Korea, South Korea, Russia and other countries, with a total area of about 300,000 km^2, among which, China possess the largest area, widely distributed in Liaoning, Jilin and Heilongjiang provinces (Pan et al., 2019). *P. koraiensis,* as a famous and valuable economic tree species, plays momentous ecological environmental value, and strongly contributes to the beauty of the landscape. However, plant microbiome, in *P. koraiensis*, a main host of *B. xylophilus* that is generally distributed in China, the information of PWD on the entire host microbial community under field conditions has been scarcely studied. Here, the phyllosphere and rhizosphere bacterial and fungal communities in healthy *P. koraiensis* (PKa) and *P. koraiensis* naturally infected by *B. xylophilus* at the early stage (PKb) and at the last stage (PKc) of the disease were analyzed by sequencing 16s rDNA and ITS rDNA (internal transcribed spacer) using the high-throughput Illumina NovaSeq PE250 to uncover the differences in host microbial community potentially caused by PWD. In this study, we hypothesized that (1) PWD could increase the diversity of phyllosphere and rhizosphere microbial community differed as the infection of *B. xylophilus* progressed; (2) the host bacterial and fungal community differed as the infection of *B. xylophilus* progressed; (3) with the infection of PWD, most of the microbial taxa tended to be co-excluding, besides, some microbial species unsuited towards living in infected pines disappeared and some species would present. The

main aim of the present study was to elucidate the shifts of the host microbial community caused by PWD and to better understand the relationships between pathogens and host microbial communities at different stages of PWD after PWD occurrence.

Materials And Methods

Overview of the research area

The study area is located at Dengta City, Liaoyang City, Liaoning Province, China (41°.17'44" N, 123°35'47" E). The climate in this area is characterized as north temperate continental climate with an annual average temperature of 8.8 ℃, annual average precipitation of 600 to 800 mm, and the annual average frost-free period of 140 to 160 days. The soil type is classified as Eutrochrepts soil (Deckers et al., 2016). Five fixed sites with the area of 1 ha were selected, eight healthy *P. koraiensis* trees (PKa), eight diseased *P. koraiensis* trees infected by B. xylophilus at early stage (PKb), and eight diseased *P. koraiensis* trees at the last stage (PKc) were selected for sampling in each site. The distance between diseased and adjacent healthy trees was less than 15 m. The selection of healthy and *P. koraiensis* trees infected by *B. xylophilus* was made in accordance with the method described by Millberg et al. (2015), in which the healthy trees were completely green needles and from which no B. xylophilus was isolated. The early stage of infection tree refers to needles that have become slightly wilted and browning of needles. The last stage of diseased trees were completely dead looking with brown needles (Fig. S1).

Sample collection

For the sampling, 24 needle samples from the tips of the shoot in the middle of the canopy from three directions from 8 trees, including 120◦ around the tree, were collected and mixed as one replicate at each site. A total of 15 leaf samples (3 types × 5 replicates) were collected from the study area. Rhizosphere soil samples were collected by removing the surface litter and tracing the root extension. The soil samples were collected after litter removal around each selected tree in each of the three directions (120◦ as the boundary). Rhizosphere soils were shaved off from the roots, and three direction for each tree were pooled together from 8 tress as one replication in each site, resulting in 15 rhizosphere soil samples (3 types × 5 replicates). All the samples were put in the icebox and transported to the laboratory for subsequent analysis.

To analyze the microbial community on the leaves according to Kembel and Mueller (2014), and Ren et al. (2015), 10 g of leaves samples from each replicate were cut into pieces and transferred to a sterile triangle flask. 1:20 (leaf weight/volume TE buffer =1:20) phosphate-buffered saline solution (20 mL, PBS, 0.01 M, pH 7.4) was added to each triangle flask. After sealed with a sterilized film, the samples were shaken on a shaker at 200 r/min for 30 min at room temperature, and the microbial cells were separated from the leaf surface. Vacuum filtration was used in a sterile environment, and microbes from the oscillating liquid were collected on a 0.22μm microporous membrane placed into 2ml sterile centrifuge tube, then stored -80 °C prior to DNA extraction. Fresh soil removed plant residues and stone was passed through a 2-mm sieve and immediately put into 2 ml sterile centrifuge tube and frozen at -80 °C for later DNA extraction and high-throughput sequencing.

DNA extraction, amplification, and NovaSeq PE250 sequencing

Genomic DNA was extracted from microporous membrane and 0.5 g soil using the FastDNA SPIN Kit for soil (MP Biomedicals, Santa Ana, CA, USA), in accordance with the manufacturer's instructions. The concentrations of DNA were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The V3-V4 regions of bacterial 16S rDNA gene were amplified and sequenced using the primer pairs 338F (5'-ACTCCTACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') with barcode sequence. And ITS1 regions of the fungal ITS rDNA gene were amplified using the primer pairs ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3') with barcode sequence (Deng et al., 2020). All the PCR reactions were carried out with 25 μl mixture, including 2 μl of dNTPs (2.5 mM); 2 μl DNA Template (40-50 ng); 0.25 μl (5 U/μl) of Q5 High-Fidelity DNA Polymerase; 8.75 μl of ddH_2O; 5 μl of Q5 High-Fidelity GC buffer (5×) and Q5 reaction buffer (5×), respectively; 1 μl (10 uM) of forward primer; 1 μl (10 uM) of reverse primer. The following PCR thermal cycling condition consisted of an initial denaturation step for 5 min at 98 °C, followed by 25 cycles of denaturation for 15 s at 98 °C, annealing for 30 s at 55 °C, and elongation of 72 °C for 30 s, with the final elongation step for 5 min at 72 °C. The PCR amplicons was further purified and quantified by using Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). PCR products for sequencing were carried out using an Illumina's NovaSeq PE250 platform at Shanghai Personal Biotechnology Co., Ltd, Shanghai, China. The high-throughput sequencing raw data of phyllosphere and rhizosphere microbe were uploaded in the NCBI database with the SRA accession numbers of PRJNA689361 and PRJNA689392.

Bioinformation analysis

After removing primers and barcode sequences with cutadapt, quality filter, denoise, joint and removal of chimeras, the high-quality sequences were finally obtained. Sequences with ≥97% similarity were assigned to the same OTU. The Silva Database for bacteria (Release132, http://www.arb-silva.de) (Quast et al., 2013) and Unite Database for fungi (Release 8.0 https://unite.ut.ee/) (Kõljalg et al., 2013) were used for each representative sequence.

Statistical analysis

One-way analysis of variance (ANOVA) with LSD test was used to identify differences in microbial community richness (Chao1 index, Observed species), diversity (Shannon index, Simpson index), and evenness (Pielou_e index) among different treatments. Venn diagrams were constructed using subsampled data to show the shared and unique OTUs in Rstudio with the "Venn" package. Linear discriminant analysis Effect Size (LEfSe) in Galaxy software was employed to identify microbial lineages (from the phylum to genus) responsible for the differentiation of the phyllosphere and rhizosphere microbial community caused by different treatments. Principal co-ordinates analysis (PCoA) based on Bray-Curtis distance matrix, as one of classical multidimensional scaling, was used to visualize the distinction of microbial community structure. Heatmap plots of phyllosphere and rhizosphere microbial community with the relative abundance of top 50 at the genus level were performed based on Bray-Curtis distance matrix using RStudio with the package of "Vegan". The co-occurrence patterns of OUTs from different treatments were evaluated by network analysis using the "psych" package in Rstudio based on the Spearman' rank correlation, and Gephi software was applied to visualize the networks with a Fruchterman-Reingold layout.

Results

Changes in phyllosphere and rhizosphere microbial community alpha diversity

A total of 1,367,262 and 1,174,114 high-quality phyllosphere and rhizosphere bacterial sequences were generated across all samples after sequence denoising and quality filtering with the average number of sequences per sample 91, 150 and 78, 274, severally, which were assigned into 11, 294 and 18, 175 OTUs. The number of shared phyllosphere bacterial OTUs among PPKa, PPKb, and PPKc was 1306, and the unique OTUs of PPKa, PPKb, and PPKc was 2,400, 3,028, and 2,862, respectively (Fig. 1A). The number of shared rhizosphere bacterial OTUs among RPKa, RPKb, and RPKc of was 2,297, and the unique OTUs of RPKa, RPKb, and RPKc was 3,416, 3,145, and 5,437, respectively (Fig. 1B). Moreover, the shared OTUs among RPKa, RPKb, RPKc, PPKa, PPKb, and PPKc was 13 (Fig. S2A).

The fungal communities were further explored by high-throughput amplicon sequencing. Across all samples, we obtained a total of 1, 318, 977 and 1, 316, 090 high-quality phyllosphere and rhizosphere fungal sequences after sequence denoising and quality filtering with the average number of sequences per sample 87, 739 and 87, 931, severally, which were respectively grouped into 1, 272 and 3, 190 OTUs. The number of shared phyllosphere fungal OTUs among PPKa, PPKb, and PPKc was 276, and the number of unique OTUs of PPKa, PPKb, and PPKc was 599, 846, and 997, respectively (Fig. 1C). The number of shared rhizosphere fungal OTUs among RPKa, RPKb, and RPKc was 269, and the number of unique OTUs of RPKa, RPKb, and RPKc was 272, 260, and 245, severally (Fig. 1D). In addition, the shared OTUs among RPKa, RPKb, RPKc, PPKa, PPKb, and PPKc was 23 (Fig. S2B).

As expected, there was considerable variation of phyllosphere bacterial Pielou_e (F= 12.639, P= 0.001), Shannon (F= 10.268, P= 0.003), and Simpson index (F = 5.882, P = 0.017) among PPKa, PPKb, and PPKc. Furthermore, relative to PPKa and PPKb, PPKc increased phyllosphere bacterial Pielou_e, Shannon, and Simpson index with 0.72, 7.83, and 0.97, respectively (Table 1). In addition to Goods_coverage (F = 3.533, P = 0.062) and Simpson index (F = 3.235, P = 0.075), rhizosphere bacterial Observed_species (F = 6.777, P = 0.011), Chao 1 (F = 4.655, P = 0.032), Pielou_e (F=47.496, P=0.0001), and Shannon index (F = 11.772, P = 0.002) were observed significant differences among RPKa, RPKb, and RPKc. What's more, RPKc hold the highest rhizosphere bacterial Observed_species, Chao 1, Pielou_e, Shannon, and Simpson index with 5384.50, 6916.43, 0.895, 11.09, 0.9987, separately (Table 2). With regard to fungi, phyllosphere fungal Chao 1 index (F= 56.306, P= 0.000), Goods_coverage (F= 14.509, P= 0.001), Observed_species (F= 56.689, P= 0.000) differed dramatically among PPKa, PPKb, and PPKc, and PPKc hold the highest Chao1 index with 597.46 (Table 1). Rhizosphere fungal Pielou_e (F= 12.639, P= 0.001), Shannon (F= 10.268, P= 0.003), and Simpson index (F= 5.882, P= 0.017) among RPKa, RPKb, RPKc also appeared obviously different. It is well established that RPKc owned highest rhizosphere fungal Pielou_e, Shannon, and Simpson index with 0.56, 4.66, and 0.89 (Table 2).

Variations in phyllosphere and rhizosphere microbial community beta diversity

It is well established that the microbial compositions from rhizosphere and phyllosphere samples formed distinct clusters (Fig. 2), which indicated that the plant compartment is a major selective force for the formation of plant-related microbial composition. The unconstrained principle coordinate analysis (PCoA) of bray-curits distance from all phyllosphere and rhizosphere samples based on the OTU data detected 64.9% of

the total variance among bacterial communities, with the first and second axes explaining 57.2% and 7.7% of the variance, respectively (Fig. 2A). PCoA analysis based on the OTU data detected 67.0% of the total variance of fungal communities, with the first and second axes explaining 55.7% and 11.3% of the variance, respectively (Fig. 2B). As expected, the infection of *B. xylophilus* had a profound effect on plant microbe. The results demonstrated that rhizosphere bacterial community (Fig. S3A), rhizosphere fungal community (Fig. S3C), and phyllosphere fungal community (Fig. S3D) from PKa, PKb, and PKc formed three distinct clusters, especially along the PCoA1.

Comparative analysis of phyllosphere and rhizosphere microbial community composition

For bacteria, at the phylum level, 36 rhizosphere bacterial groups were obtained, and 8 bacterial communities with the relative abundance more than 1% were detected, including Proteobacteria, Actinobacteria, Acidobacteria, Verrucomicrobia, Chloroflexi, Gemmatimonadetes, Patescibacteria, Bacteroidetes, and Firmicutes, accounting for 94.75% (Fig. 3A). 27 phyllosphere bacterial groups were obtained, and 4 bacterial communities with the relative abundance more than 1% were obtained, including Proteobacteria, Actinobacteria, Bacteroidetes, and Cyanobacteria (Fig. 3B). As for fungi, at the phylum level, 7 rhizosphere fugal groups were obtained, and 2 fungal communities with the relative abundance more than 1% were detected, including Ascomycota and Basidiomycota (Fig. 4A). 14 phyllosphere fungal groups were obtained, and 4 fungal communities with the relative abundance more than 1% were detected, including Basidiomycota, Ascomycota, Mortierellomycota, and Mucoromycota (Fig. 4B).

At the genus level, 851 rhizosphere bacterial communities were obtained, of which, the average relative abundance of *Candidatus_Udaeobacter*, *Mycobacterium*, *Acidothermus*, *AD3*, *Subgroup_6*, *KD4-96*, *Saccharimonadales*, *Subgroup_2*, *Bradyrhizobium*, *Pseudolabrys*, *Ellin6067*, *Burkholderia-Caballeronia-Paraburkholderia*, *Gaiella*, *Bryobacter*, *IMCC26256*, and *67-14* was more than 1% (Fig. S4A). 606 phyllosphere bacterial community were obtained, and the relative abundance of *Methylobacterium*, *Pantoea*, *Sphingomonas*, *1174-901-12*, *Hymenobacter*, *Amnibacterium*, *Massilia*, *Pseudomonas*, *Chloroplast*, *Enterobacter*, *P3OB-42*, *Rosenbergiella*, and *Endobacter* was more than 1% (Fig. S4B). Heatmap demonstrated that rhizosphere (Fig. 5A) and phyllosphere (Fig. 5B) bacteria from RPKa (PPKa) and RPKb (PPKb) formed a cluster, clearly distinguished from those of RPKc (PPKc). For fungi, 321 rhizosphere fungal communities were obtained, among which, the groups with the relative abundance more than 1% were *Didymella*, *Alternaria*, *Selenophoma*, *Septoria*, *Aureobasidium*, *Genolevuria*, *Phialemoniopsis*, and *Taphrina* (Fig. S4C). 492 phyllosphere fungal community were obtained, and the groups with the relative abundance more than 1% were *Mortierella*, *Russula*, *Sebacina*, *Saitozyma*, *Suillus*, *Phialocephala*, *Chalara*, *Trechispora*, *Ilyonectria*, *Solicoccozyma*, *Trichocladium*, *Amphinema*, *Penicillium*, *Fusarium*, *Umbelopsis*, *Tomentella*, and *Exophiala* (Fig. S4D). Heatmap demonstrated that rhizosphere (Fig. 5C) and phyllosphere (Fig. 5D) fungi from RPKb (PPKb) and RPKc (PPKc) formed a cluster, clear distinguished from those of RPKa (PPKa).

Furthermore, we conducted LEfSe analysis to identify which microbial taxa (from phylum to genus level) were major contributors to the differences in rhizosphere and phyllosphere community compositions among different samples (Fig. 6). At the phylum level, the larger groups of rhizosphere bacteria in RPKa were Actinobacteria, Gemmatimonadetes, and Patescibacteria, while in RPKb were Chloroflexi, Rokubacteria, and Verrucomicrobia, in RPKc were Acidobacteria, Bacteroidetes, and Proteobacteria (Krus-kall-Wallis test, P <

0.05) (Fig. 6A). The larger group of phyllosphere bacterial group in PPKa was Proteobacteria, while in PPKb were Acidobacteria, Chloroflexi, and Cyanobacteria, in PPKc were Actinobacteria, Armatimonadetes, Bacteroidetes, and Planctomycetes ($P < 0.05$) (Fig. 6B). Additionally, as for fungi, the RPKa contained a significantly higher abundance of Phialemoniopsis than RPKb and RPKc samples ($P < 0.05$). While, RPKb owned higher abundances of Ascomycota and Pseudovirgaria. Curvibasidium, Neophaeococcomyces, Selenophoma, and Symmetrosporaceae presented higher in RPKc (Fig. 6C). For phyllosphere, the fungi group of Chalara, Basidiomycota, Mucoromycota, and Staphylotrichum were significantly enriched in PPKa, while Rozellomycota, and Arthrocatena were more enriched in PPKb as compared to PPKa and PPKc ($P < 0.05$). The phyla Ascomycota was more abundant in PPKc than PPKa and PPKb ($P < 0.05$) (Fig. 6D).

Microbiological information network and co-occurrence analysis

In order to a deeper comparative look into the community structure of the microbial taxa, we created association networks of phyllosphere and rhizosphere bacterial and fungal communities from OTU data (Fig.7; Table S1). Total nodes of phyllosphere and rhizosphere bacterial community association network in PKc existed the highest, followed by PKb and PKa, and total nodes of phyllosphere and rhizosphere fungal community association network in PKb existed the highest, followed by PKc and PKa, both indicating that the OTUs of the ecological network increased after infection (Fig.7; Table S1). Graph density in the network of PKb, a key topological property to describe how well a node is connected with its neighbours, showed higher than PKa and PKc, suggestive of more intensive microbial coupling at the early stage of infection (Fig. 7; Table S1). Except for phyllosphere fungi, positive links showed decreased with the infection of PWD, and at the last stage, positive links existed the lowest (Fig. 7; Table S2), demonstrating that most of the microbial taxa tended to be co-excluding rather than co-occurring.

Discussion

Microbial community diversity response to different samples

Plants contain a variety of bacteria and fungi, which play critical roles in ecosystem functioning and in restoration and management of sustainability and health of many plant species (De Zelicourt et al., 2013; Toju et al., 2018; Naylor et al., 2020), and play positive or negative roles during the pathogenesis of plant pathogens (Kobayashi et al., 2009; Diogo et al., 2017). As climate change and human activity disrupt natural environments and microbial processes, there is essential to further explore the variations of microbe-microbe interactions (Goldford et al., 2018) and microbe–host interactions (David et al., 2019). We investigated the microbial community of phyllosphere and rhizosphere from healthy, diseased pine trees naturally infected by *B. xylophilus* at the different stages under field conditions. In our study, 11, 294 and 18, 175 phyllosphere and rhizosphere bacterial OTUs, and 1, 272 and 3, 190 phyllosphere and rhizosphere fungal OTUs of healthy and diseased pines were detected. In almost all samples, the rhizosphere bacterial Chao1 index, Pielou_e, Shannon, and Simpson index were much higher than the respective phyllopshere communities (Table 1), which was a common finding in similar studies of native and cultivated plants in different environments (Zhou et al., 2019; Dong et al., 2019; Bao et al., 2020). The differences in microbial community diversity between the two plant compartments might account for the direct influence of their surrounding environment, and their fundamental discrimination of physiology and function (Genitsaris et al., 2020). Mounting empirical

evidence have suggested that root exudates have a strong detrimental role in selecting the growth of specific bacteria (Bulgarelli et al., 2012; Wagner et al., 2016) through signal transmission of microbe-microbe and plant-microbe interactions (Venturi and Keel, 2016), ultimately promoting the differentiation of the bacterial assemblages (Bao et al., 2020). Additionally, phyllosphere exist generally lower bacterial richness and abundance due to the fluctuations in environmental pressures (Vorholt, 2012; Vacher et al., 2016). In regard to fungal community, phyllosphere fungal community diversity presented higher than rhizosphere fungal community diversity (Table 2), which was not in agreement with previous researches from Chen et al. (2020b) and Jia et al. (2020). Our results nicely demonstrated that the effects of root and leaf compartments on the α-diversity indices of fungal community were different from those of bacterial community.

What's more, there were significant differences of phyllosphere and rhizosphere microbial community diversity between healthy and infected *P. koraiensis*. In the early stages of the infection, rhizosphere bacterial Pielou_e and Simpson index, phyllosphere bacterial Pielou_e and Simpson index, rhizosphere fungal Shannon and Simpson index, phyllosphere fungal Chao1 index and Observed_species exhibited slightly higher than those of healthy *P. koraiensis* (Table 1; Table 2). At the last stage of the infection, phyllosphere bacterial Pielou_e, Shannon, and Simpson index, phyllosphere bacterial Observed_species, Chao 1, Pielou_e, Shannon, and Simpson index, rhizosphere fungal Pielou_e, Shannon, and Simpson index, phyllosphere fungal Chao1 index existed abundantly higher than those of healthy *P. koraiensis* and the early stage of the infection (Table 1; Table 2). Namely, at early stage of the disease slight shifts in the microbial diversity were observed. In general, an increase in microbial diversity with more severe symptomatic stage was visible. As have been seen in other finding from Proença et al. (2017b), namely, that the diversity of endophytic bacteria of *P. pinaster* trees infected by *B. xylophilus* at the late stages was the highest, while there was no difference in bacterial diversity at the early stage of the disease. However, opposite results were obtained from Ma et al. (2020) demonstrated that there were no significant differences of rhizospheric bacterial diversities between healthy pines and wilted pines. Besides, another investigation showed that *B. xylophilus* infection appeared to reduce soil bacterial diversity (Shi et al., 2015), similar findings were reported by Zhang et al. (2020) demonstrated that *B. xylophilus* infection likely decreased the richness and diversity of endophytic microbes. It thus appear that the inconsistent results might be due to different tree species and the sampling period after the infection of PWD. In present study, we revealed that PWD could increase the phyllosphere and rhizosphere bacterial and fungal diversity and the microbial community diversity differed as the disease progressed, suggesting the importance of disease development in the host microbial community. The differences might be caused by the growing abundance of the dominant microbial groups crowding out the weaker microbial groups or that microbial species unsuited towards living in infected pines disappeared.

Microbial community composition response to different treatments

As shown by a growing body of works (Tardif et al. 2016; Chen et al., 2020b; Xiang et al., 2020), we also observed that the microbial compositions from rhizosphere and phyllosphere samples formed distinct clusters. Collectively these studies suggested that although the assemblies of root-associated bacteria and fungi differ substantially from the phyllosphere microbial communities, both represent a subset of the microbe derived from soil communities and enriched in different plant-associated niches (Coleman-Derr et al., 2016; Hamonts et al., 2019). As previous findings indicated that the infection of plant pathogens could affect the host microbial community (Tian et al., 2015; Ma et al., 2020), we also documented that the PWD had a

profound impact on the host rhizosphere bacterial and fungal community and phyllosphere fungal community, which was not complete in line with previous work demonstrated that the community structure of healthy and diseased trees was only significantly different in the roots, and not in the needles and soil (Ma et al., 2020). It has become evident that root exudates are the essential factor determining the structure of the rhizosphere microbial community (Badri et al., 2013; Xu and Wu, 2016). The occurrence of pine wilt disease can lead to a decreased secretion of soluble sugar, total sugar, and protein in roots (Reva et al., 2012), which might have caused the observed difference in the microbial community structure in the rhizosphere.

Intriguingly, overall the bacterial community composition were similar (in terms of dominant groups) in all samples, different plant compartments at different stages of disease dominated mainly by Proteobacteria, followed by Actinobacteria, and this finding was consistent with several previous studies displayed that Proteobacteria and Actinobacteria were the dominant groups in rhizosphere bacterial communities (Alvarez-Lopez et al., 2016; Jorquera et al., 2016) and phyllosphere microorganisms (Delmotte et al., 2009; Vokou et al., 2019)In addition, these groups represent ubiquitous rhizosphere taxa were detected in various stressed environments (Yadav et al., 2018). While, the opposite observations from *P. massoniana* infected by *B. xylophilus* showed that Acidobacteria was predominate species in infected soils (Shi et al., 2015). Interestingly, due to *B. xylophilus* infection of *P. koraiensis*, the relative abundances of Acidobacteria, Bacteroidetes, and Proteobacteria were significantly higher in diseased pine roots, and the shifts of Proteobacteria have been observed in previous finding (Ma et al., 2020), which collectively demonstrated that Proteobacteria might be phytopathogens and parasites in plant tissues and cause a variety of diseases (Kersters et al., 2006). The roots and leaves metabolism of diseased trees was weakened relative to the healthy roots and leaves, resulting in a decreased ability of the root and leaf to adapt to the environment condition and it being easily colonized by microbes. Other investigations indicated that Proteobacteria prefer to grow under nutrient-rich conditions (Fierer et al., 2007), which might explain the high content of Proteobacteria in the diseased roots and leaves. The rhizospheric microbial abundance of *Bryobacter*, *RB41*, *Bradyrhizobium* were richer in diseased pines. Our findings were similar to the results in rhizosphere bacterial studies on *P. thunbergii* where bacteria in the genus *Bradyrhizobium* were more abundant in soil of wilted trees than in soil of non-infected trees (Ma et al., 2020).

The abundances of the genus *Massilia*, *Sphingoaurantiacus*, *Acidiphilium*, *Acetobacteraceae*, *Singulisphaera*, *Phascolarctobacterium*, and *Hymenobacter* in diseased needles were significantly higher than those in healthy needles, suggesting an association of particular microbial abundances with the infection of *B. xylophilus* in *P. koraiensis*. What's more, the research from Ma et al. (2020) demonstrated that *Massilia* was the obviously higher in diseased pines, which supported our results to some extent. The genus *Massilia* belongs to the family *Oxalobacteraceae* of the class *Betaproteobacteria* in the phylum Proteobacteria (Altankhuu and Kim, 2017). Members of this genus are characterized as Gram-negative, aerobic, non-spore-forming bacteria (Zhao et al., 2017). Some *Massilia* can produce cell lysis enzymes that promote tissue lysis (Maya et al., 2012). This may be the reason for the presence of *Massilia* in a high abundance in diseased needles.

In our study, Ascomycota and Basidiomycota were the dominant fungal phyla with phyllosphere and rhizosphere samples, and this result was agreement with previous research (Jia et al., 2020). Similar results were obtained in Taxus rhizosphere communities (Hao et al., 2016) and in tropical grasslands (Lienhard et al., 2014). The major phyllosphere fungal genera in healthy *P.koraiensis* were *Penicillium*, and *Trichoderma*,

agreement with study from Zhang et al. (2020). Interestingly, *Trichoderma* is an important genus in biocontrol of nematodes because some species produce metabolites harmful to nematodes (Yang et al., 2012). The enriched rhizosphere fungal groups in RPKc were *Phaeosphaeriaceae*, *Wukcoxina*, *Pseudocosmospora* and so on. *Phaeosphaeriaceae* was commonly associated with plants as pathogens, though some are also saprotrophs and parasites on powdery mildews (Zhang et al., 2012). Thus it can be seen that plant-associated microbes could influence plant health and fitness (Zamioudis and Pieterse, 2012), resistance to pathogens (Awasthi et al., 2014), and ecosystem services.

Shifts of co-occurrence association network response to different treatments

In our study, the co-occurrence network of phyllosphere and rhizosphere microbial community demonstrated dynamical relationships between healthy *P.koraiensis* and the infection of *B. xylophilus* in *P. koraiensis*, which could provide momentous details of microbial community assembly and represent interactions among different populations that regulate ecological processes (Fuhrman, 2009). Total nodes of phyllosphere and rhizosphere microbial community association network increased after *B. xylophilus* infection, indicating that the populations of the ecological network increased after infection (Fig.7; Table S1), resulting the microbial diversity increased in some degree. The edges of phyllosphere and rhizosphere microbial community association network existed higher in *P. koraiensis* infected by *B. xylophilus* than healthy *P.koraiensis,* which depicted changes among nodes, reflecting their responses to environmental perturbations (Shi et al., 2016). Furthermore, the role of microbial co-occurrence networks is important in revealing the interactions (such as through parasitism, competition, and mutualism) that exist among different species (Zhou et al., 2011; Deng et al., 2012). In our study, except for phyllosphere fungi, positive links of phyllosphere bacteria, rhizosphere bacteria and fungi showed decrease with the infection of PWD, and at the last stage, positive links existed the lowest (Fig.7; Table S1), demonstrating that most of the microbial taxa tended to be competition rather than mutualism.

Conclusions

Overall, an increase in diversity with more severe symptomatic stage was visible. What' more, the microbial compositions from rhizosphere samples and phyllosphere samples formed distinct clusters. Rhizosphere bacterial and fungal community, and phyllosphere fungal community from PKa, PKb, and PKc formed three distinct clusters, which clearly separated along the PCoA1. These findings manifested that the phyllosphere and rhizosphere microbial community changed potentially caused by *B. xylophilus* infection of *P. koraiensis*. Furthermore, Lefse analysis demonstrated that variations of some microbial abundances were associated with the infection of *B. xylophilus* in *P. koraiensis*, including *Bradyrhizobium* (rhizosphere bacteria), *Massilia* (phyllosphere bacteria), Phaeosphaeriaceae (rhizosphere fungi). With the infection of PWD, most of the bacterial taxa tended to be co-excluding rather than co-occurring. Together, our results explored PWD could increase the phyllosphere and rhizosphere microbial community diversity and microbial community composition differed as the disease progressed, and these changes would correlate with microbial ability to suppress plant pathogen. This study expanded our knowledge of the ecology of plant-microbe interactions as well as the structure and assembly of microbial communities of healthy *P.koraiensis* and the infection of *B. xylophilus* in *P. koraiensis*, which lay the foundation for studies that aim at improving plant growth by altering the plant microbiome.

Declarations

Finding

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

Not applicable

Consent to participate

Not applicable.

Consent for publication

Not applicable

Availability of data and material

The high-throughput sequencing raw data of phyllosphere and rhizosphere microbe were uploaded in the NCBI database with the SRA accession numbers of PRJNA689361 and PRJNA689392.

Code Availability

R scripts are available in the supplementary materials.

Authors' contributions

Jiaojiao Deng and Wenxu Zhu conceived and designed the experiments. Jiaojiao Deng and Wangming Zhou performed the experiments, analyzed the data. Jiaojiao Deng and Li Zhou prepared figures and tables, wrote original draft preparation; Wenxu Zhu and Dapao Yu authored or reviewed drafts of the paper.

John Mark planned the study. Georgina Wilson conducted a survey. Joseph Wright submitted the study etc.

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Tables

Table 1

Phyllosphere microbial community diversity among PPKa, PPKb, and PPKc. Mean ± standard error. PPKa: The phyllosphere of health *Pinus koraiensis*; PPKb: The phyllosphere of *Pinus koraiensis* naturally infected by *Bursaphelenchus xylophilus* at the early stage; PPKc: The phyllosphere of *Pinus koraiensis* naturally infected by *Bursaphelenchus xylophilus* at the last stage.

Phyllosphere bacterial community diversity	**PPKa**	**PPKb**	**PPKc**	**F**	**P**
Chao1 index	2050.54±169.79aA	2171.61±272.80aA	2508.21±142.48aA	1.366	0.292
Goods_coverage	0.985±0.001aA	0.985±0.003aA	0.983±0.001aA	0.584	0.573
Observed_species	1,533.26±126.09aA	1,689.48±201.14aA	1,927.50±106.01aA	1.749	0.215
Pielou_e index	0.61±0.02bB	0.64±0.01bB	0.72±0.01aA	12.639	0.001
Shannon index	6.40±0.30bB	6.85±0.19bAB	7.83±0.19aA	10.268	0.003
Simpson index	0.93±0.01bB	0.95±0.01abAB	0.97±0.00aA	5.882	0.017
Phyllosphere fungal community diversity	**PPKa**	**PPKb**	**PPKc**	**F**	**P**
Chao1 index	451.26±19.57cC	548.89±20.17bB	597.46±26.21aA	56.306	0.000
Goods_coverage	0.9994±0.0002aA	0.9994±0.0001aA	0.9991±0.000bB	14.509	0.001
Observed_species	441.64±17.76cC	538.74±19.80bB	582.20±25.75aA	56.689	0.000
Pielou_e index	0.653±0.035aA	0.637±0.010aA	0.635±0.009aA	0.976	0.405
Shannon index	5.74±0.33aA	5.78±0.12aA	5.84±0.11aA	0.277	0.763
Simpson index	0.96±0.02aA	0.94±0.01bA	0.95±0.00abA	3.321	0.071

Table 2

Rhizosphere microbial community diversity among RPKa, RPKb, and RPKc. Mean ± standard error. RPKa: The rhizosphere of health *Pinus koraiensis*; RPKb: The rhizosphere of *Pinus koraiensis* naturally infected by

Bursaphelenchus xylophilus at the early stage; RPKc: The rhizosphere of *Pinus koraiensis* naturally infected by *Bursaphelenchus xylophilus* at the last stage.

Rhizosphere Bacterial community diversity	**RPKa**	**RPKb**	**RPKc**	**F**	**P**
Chao1 index	5653.84±174.84bA	5604.88±553.16bA	6916.43±140.37aA	4.655	0.032
Goods_coverage	0.961±0.002aA	0.962±0.006aA	0.951±0.002bA	3.533	0.062
Observed_species	4434.48±112.81bB	4445.78±332.54bB	5384.50±91.15aA	6.777	0.011
Pielou_e index	0.883±0.001cB	0.886±0.001bB	0.895±0.001aA	47.496	0.000
Shannon index	10.70±0.04bB	10.72±0.10bB	11.09±0.02aA	11.772	0.002
Simpson index	0.99846±0.0001bA	0.99852±0.0001abA	0.9987±0.0000aA	3.235	0.075
Rhizosphere fungal community diversity	**RPKa**	**RPKb**	**RPKc**	**F**	**P**
Chao1 index	287.32±24.81aA	311.38±20.81aA	325.84±11.91aA	0.954	0.412
Goods_coverage	0.9996±0.0000aA	0.9995±0.0001aA	0.9995±0.0001aA	0.346	0.714
Observed_species	279.30±24.08aA	300.10±18.09aA	315.14±10.71aA	0.951	0.414
Pielou_e index	0.45±0.02bB	0.47±0.03bB	0.56±0.01aA	8.837	0.004
Shannon index	3.63±0.20bB	3.85±0.26bAB	4.66±0.10aA	7.825	0.007
Simpson index	0.76±0.03bB	0.81±0.03abAB	0.89±0.01aA	5.889	0.017

Figures

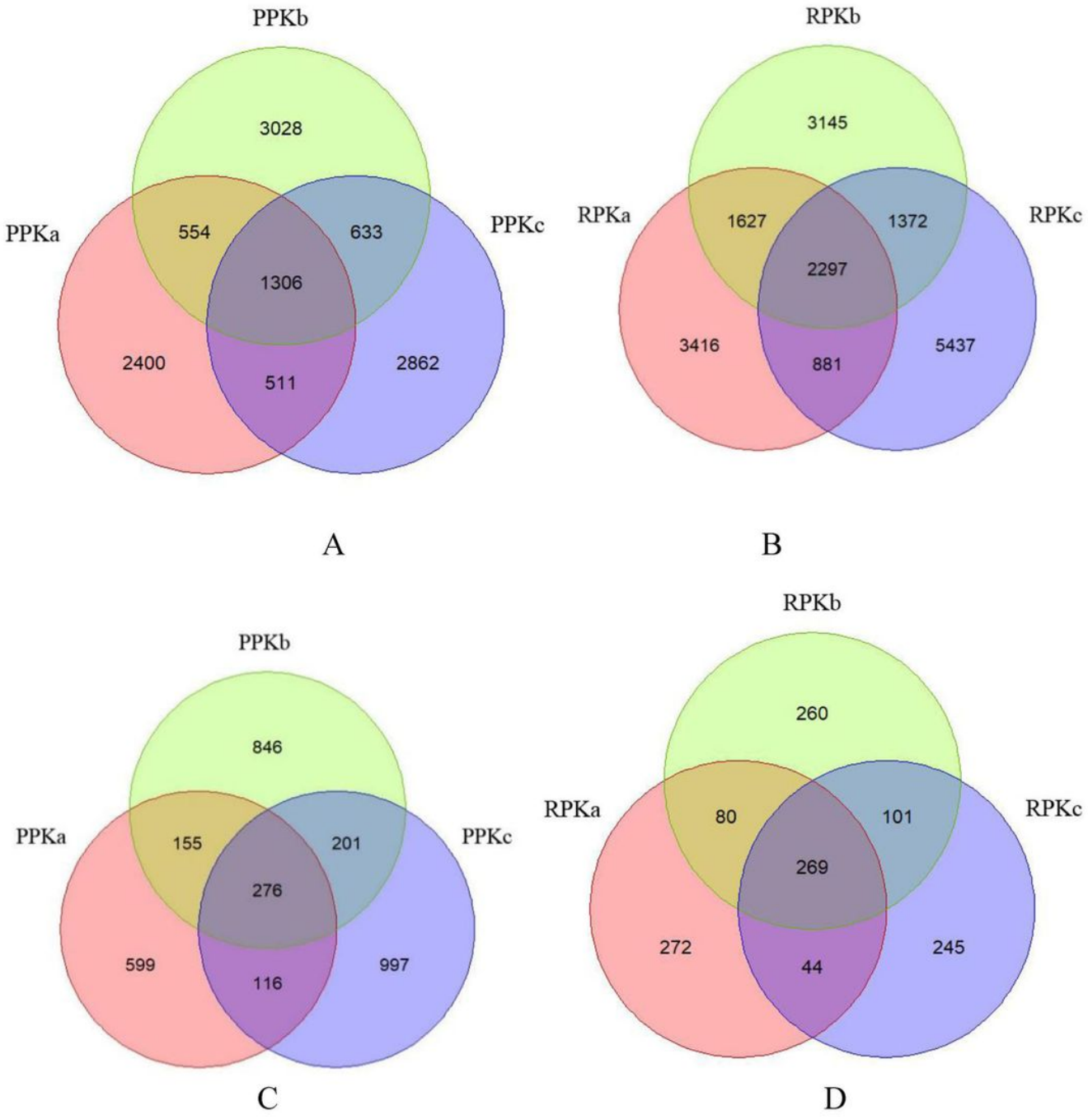

Figure 1

The venn diagrams of phyllosphere bacterial OTUs (A), rhizosphere bacterial OTUs (B), phyllosphere fungal OTUs (C), and rhizosphere fungal OTUs (D). PPKa: The phyllosphere of health Pinus koraiensis; PPKb: The phyllosphere of Pinus koraiensis naturally infected by Bursaphelenchus xylophilus at the early stage; PPKc: The phyllosphere of Pinus koraiensis naturally infected by Bursaphelenchus xylophilus at the last stage; RPKa: The rhizosphere of health Pinus koraiensis; RPKb: The rhizosphere of Pinus koraiensis naturally infected by Bursaphelenchus xylophilus at the early stage; RPKc: The rhizosphere of Pinus koraiensis naturally infected by Bursaphelenchus xylophilus at the last stage. The same below.

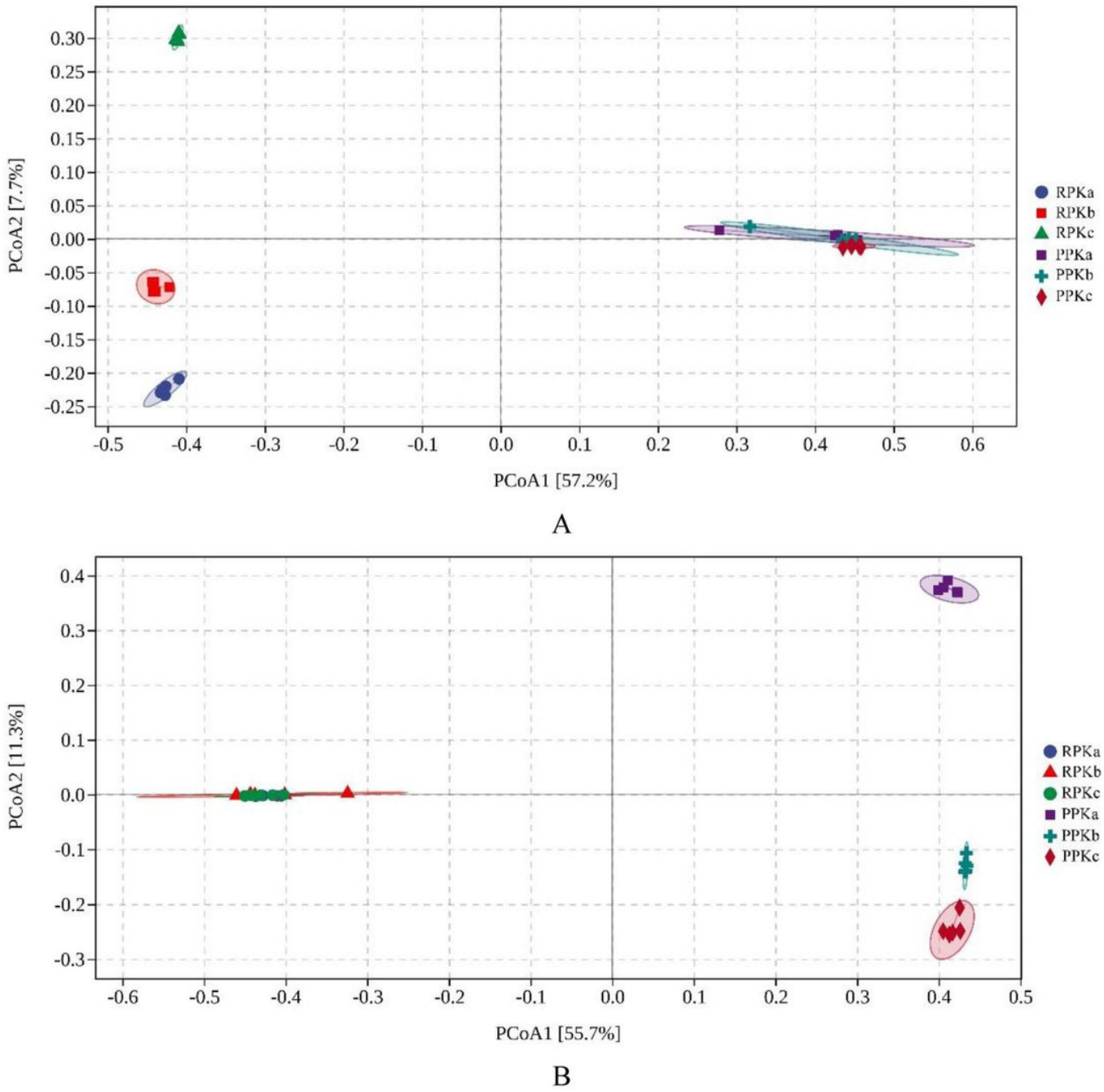

Figure 2

PCoA (principal coordinates analysis) based on Bray-Curits distance of bacterial (A) and fungal (B) community from phyllosphere and rhizosphere among different samples.

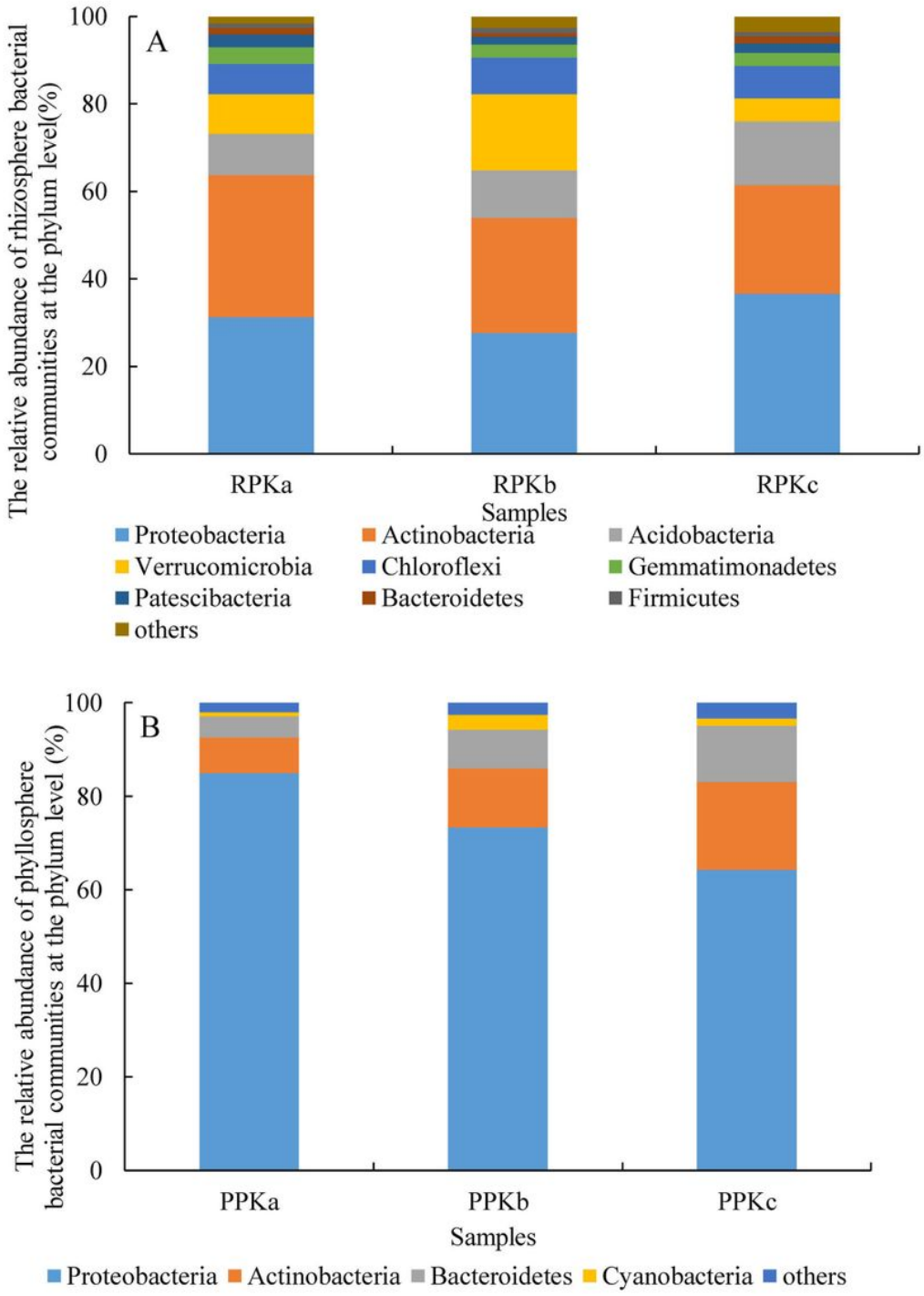

Figure 3

The relative abundance of rhizosphere (A) and phyllosphere (B) bacterial communities at the phylum level among different samples.

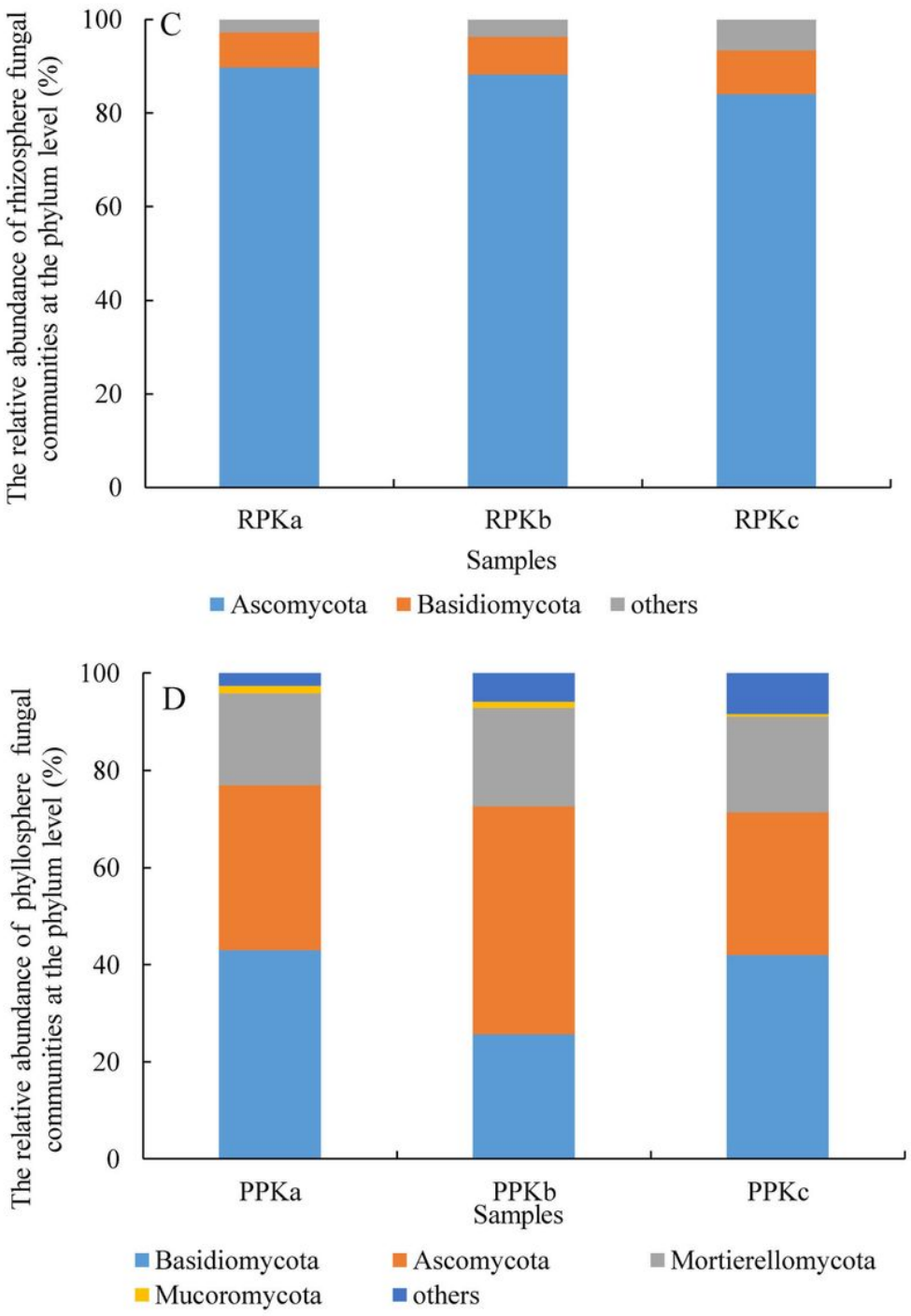

Figure 4

The relative abundance of rhizosphere (A) and phyllosphere (B) fungal communities at the phylum level among different samples.

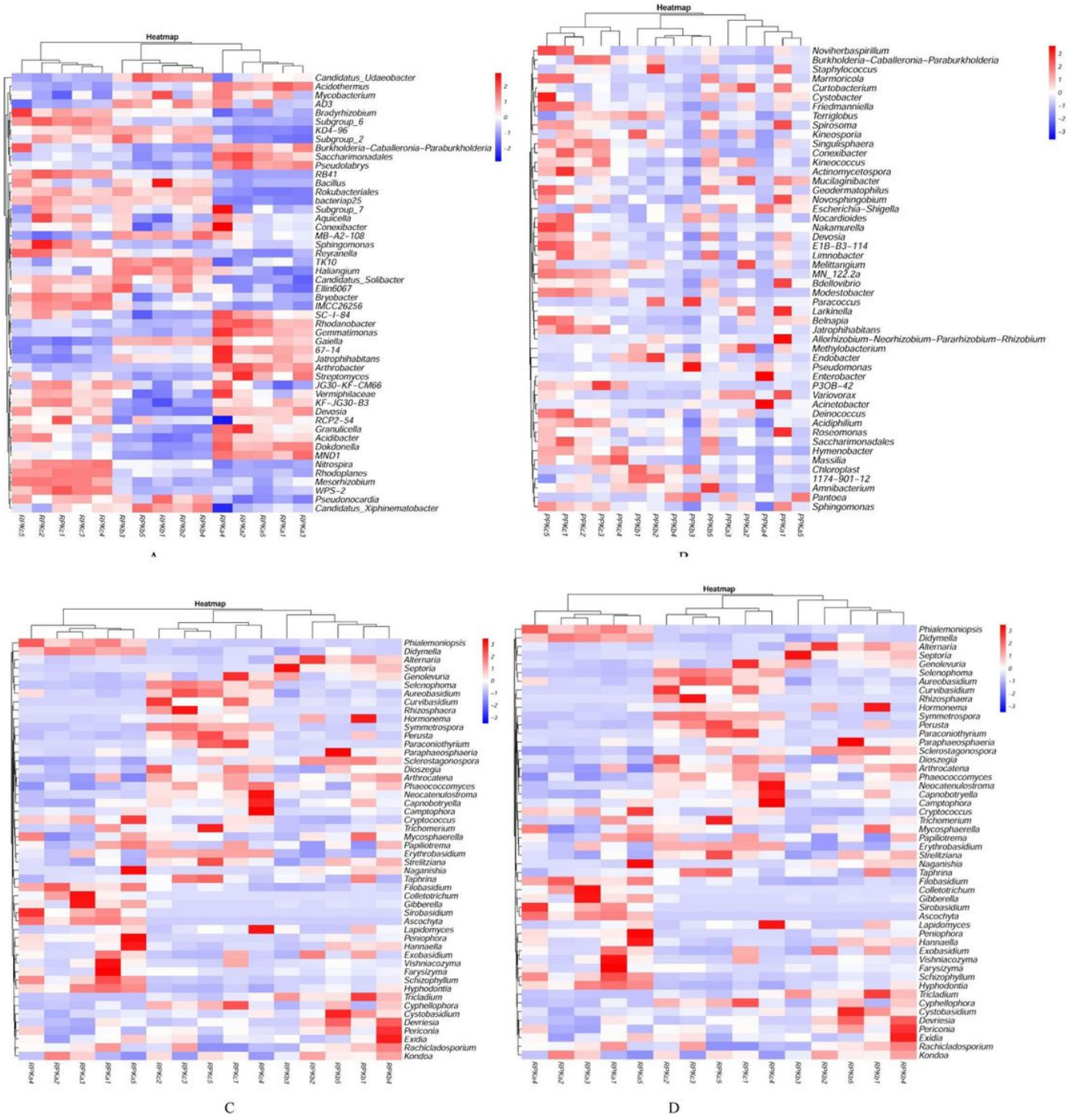

Figure 5

Heatmap of rhizosphere bacterial (A), phyllosphere acterial (B), rhizosphere fungal (C), and phyllosphere fungal (D) communities with the relative abundance at the top 50.

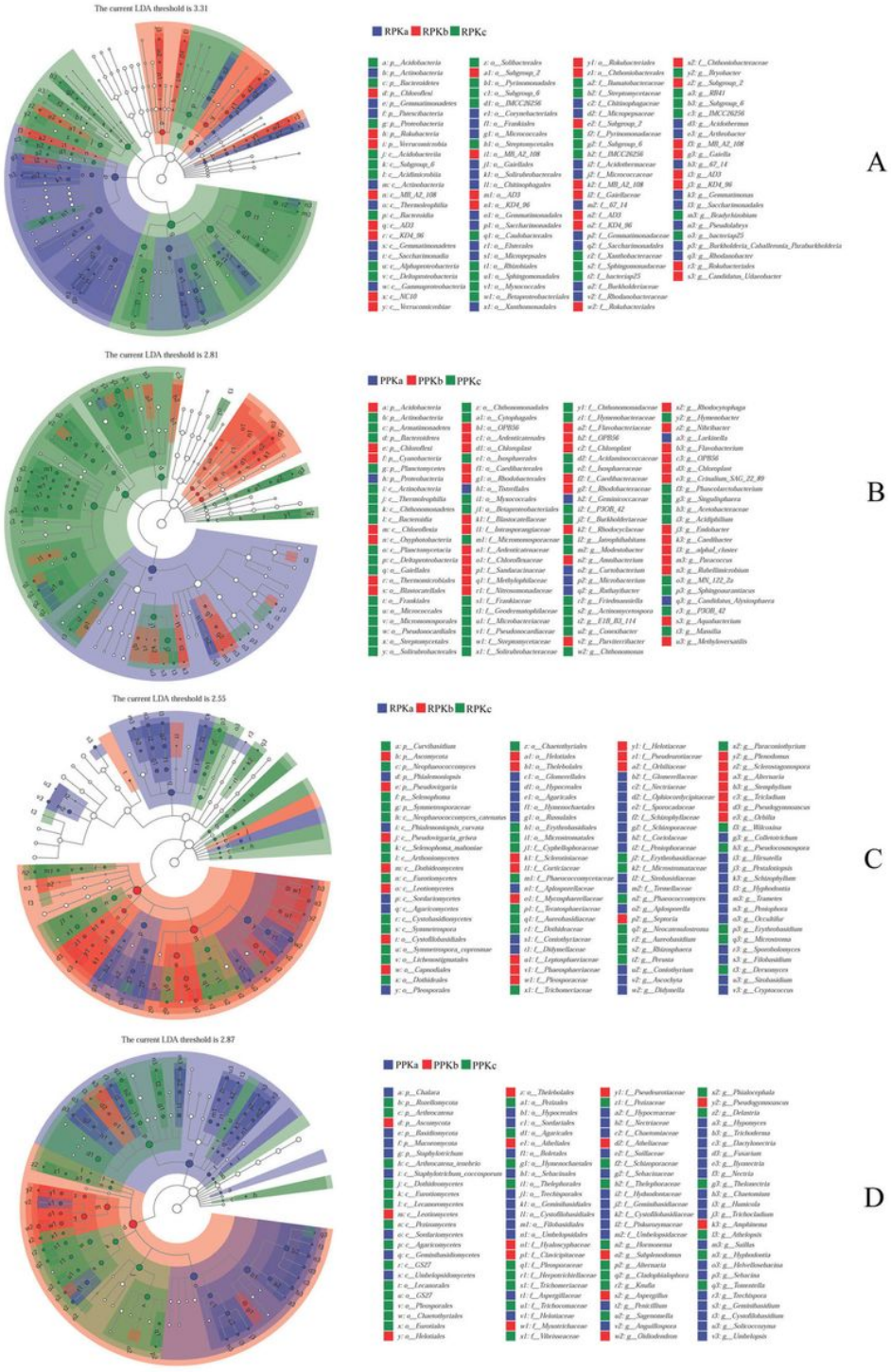

Figure 6

LEfSe analysis to identify which microbial taxa (from phylum to genus level) were major contributors to the differences in rhizosphere bacterial (A), phyllosphere acterial (B), rhizosphere fungal (C), and phyllosphere fungal (D) community compositions among different samples.

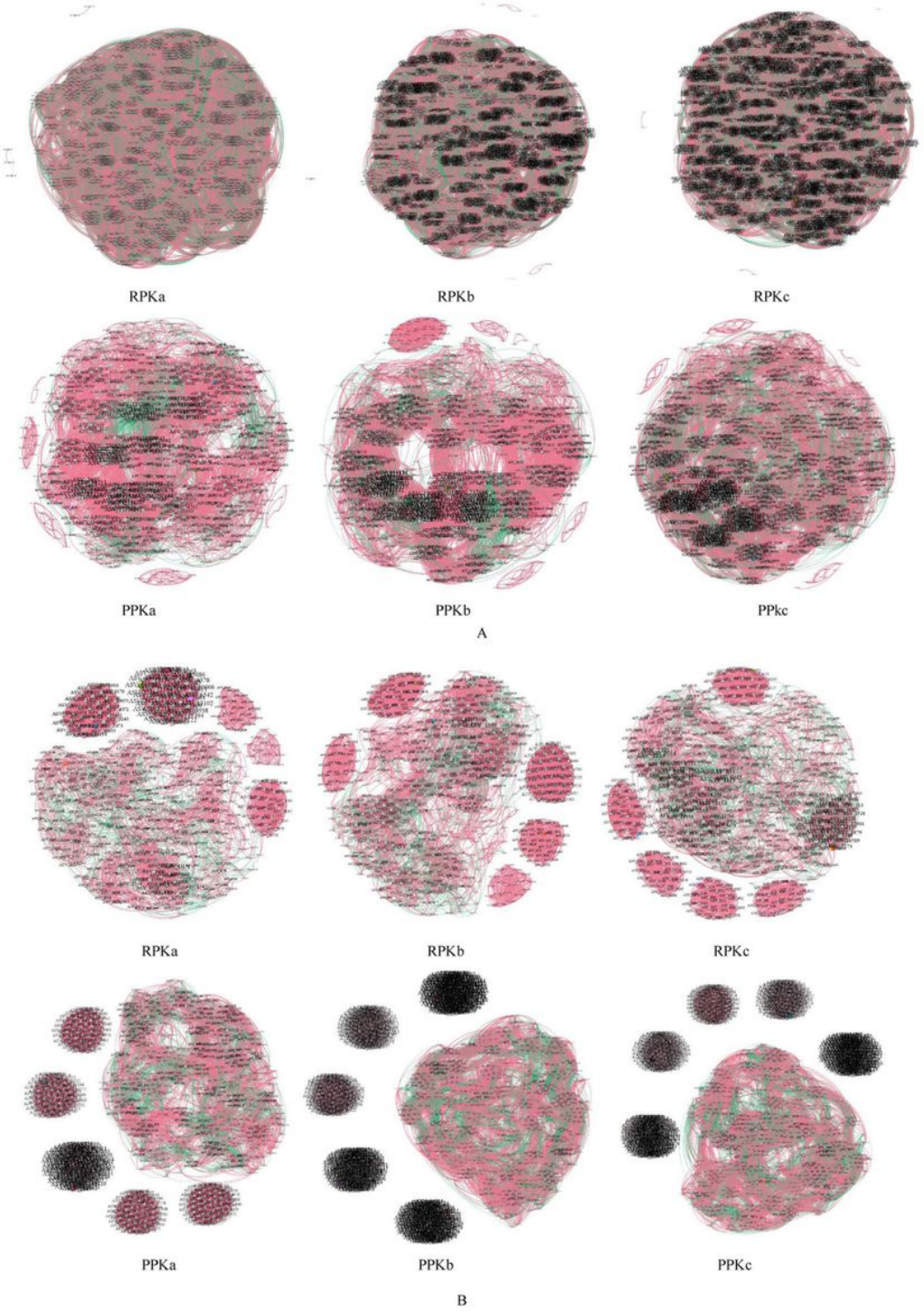

Figure 7

Network interactions of bacterial (A) and fungal (B) OTUs (OTUs with the abundance more than 5) from phyllosphere and rhizosphere. Each node represents an OTU, and colours of the nodes indicate different phyla. The OTUs were separated into different modules, shown as circles, by the greedy modularity optimization method.

Supplementary Files

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