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Fantastic plants and soil microorganisms: The secrets of interaction mechanisms in a warmer world

Edited by
Xin Sui, Jingqiu Liao and Tengxiang Lian

Coordinated by
Yunpeng Luo

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Fantastic plants and soil microorganisms: The secrets of interaction mechanisms in a warmer world

Topic editors
Xin Sui — Heilongjiang University, China
Jingqiu Liao — Virginia Tech, United States
Tengxiang Lian — South China Agricultural University, China

Topic coordinator
Yunpeng Luo — Swiss Federal Institute for Forest, Snow and Landscape Research (WSL), Switzerland

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EDITED BY
Tengxiang Lian,
South China Agricultural University, China

REVIEWED BY
Lukas Beule,
Julius Kühn-Institut,
Germany
Guozhuang Zhang,
China Academy of Chinese Medical Sciences,
China

*CORRESPONDENCE
Shu Wang
wangshu@swfu.edu.cn
Xiahong He
hxh@swfu.edu.cn

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Different factors drive the assembly of pine and *Panax notoginseng*-associated microbiomes in *Panax notoginseng*-pine agroforestry systems

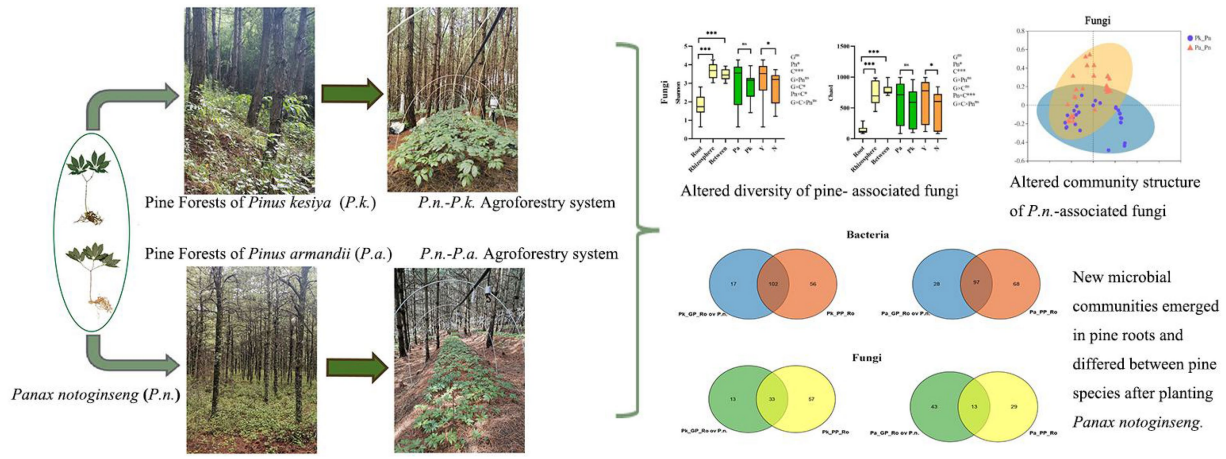
Weijia Jia[1], Shu Wang[2]*, Xiahong He[2]* and Xiaoyan Zhao[1]

[1]College of Landscape Architecture and Horticulture Sciences, Southwest Forestry University, Kunming, China, [2]Ministry of Education Key Laboratory for Forest Resources Conservation and Utilization in the Southwest Mountains of China, Southwest Forestry University, Kunming, China

Land-use conversion affects the composition and assembly of plant-associated microbiomes, which in turn affects plant growth, development, and ecosystem functioning. However, agroforestry systems, as sustainable land types, have received little attention regarding the dynamics of different plant-associated microbes. In this study, we used high-throughput sequencing technology to analyze the assembly mechanisms and the driving factors of pine- and *Panax notoginseng* (*P.n.*)-associated microbiomes during the conversion of different pine forests (*Pinus kesiya* var. *langbianensis* and *Pinus armandii*) into *P.n.*-pine agroforestry systems. The results showed that the conversion of pure pine forest into *P.n.*-pine agroforestry systems significantly altered the diversity of pine-associated fungi rather than the community structure, and the community structure of *P.n.*-associated fungi rather than the diversity. Additionally, plant-associated fungi were more responsive to land-use change than bacteria. Main effect analysis revealed that compartment rather than genotype was the driving factor of pine- and *P.n.*-associated microbiomes, but *P.n.* cultivation also significantly affected the assembly of pine-associated microbiomes. In addition, there was a transfer of *P.n.* endophytes to pine trees in agroforestry systems and the beneficial microbiomes (Massilia, Marmoricola, Herbaspirillum, etc.) were enlarged in pine roots. Therefore, the diversity of the assembly mechanisms of *P.n.*- and pine-associated microbiomes played an important role in the *P.n.*--pine agroforestry systems and were the basis for the sustainable development of the *P.n.*--pine agroforestry systems.

KEYWORDS

agroforestry system, land use conservation, rhizosphere, endophyte, microbial transmission



GRAPHICAL ABSTRACT

Highlights

- The conversion of pure pine forests into *Panax notoginseng*-pine agroforestry systems affected plant-associated microbiomes.
- The assembly of Pine- and *Panax notoginseng*-associated microbiomes had different influencing factors.
- Compartment rather than genotype was the driving factor of *Panax notoginseng* and pine-associated microbiomes, but *Panax notoginseng* cultivation also affected the assembly of pine associated microbiomes.
- There was a diffuse spread of *Panax notoginseng* endophytes into the pine roots, and beneficial microbiomes (Massilia, Marmoricola, Herbaspirillum, etc.) increased in pine roots.

Introduction

Plant-associated microbiomes include endophytes in plant tissue and rhizosphere soil microbiomes, mainly include bacterial and fungal taxa. These microbiomes play an important role in plant development and plant physiological state prediction (Montesino, 2003; Hardoim et al., 2015). Some endophytes have been proven to promote plant growth (Chandra et al., 2018) and accumulate beneficial components (Tiwari et al., 2010; Brader et al., 2014). Especially for medicinal plants, endophytes can regulate the synthesis of key secondary metabolites and increase the content of effective components, such as participating in the transformation and increasing the concentration of ginsenosides in ginseng (Song et al., 2017b; Fu, 2019) and promoting the accumulation of berberine in *Coptis teeta* (Liu et al., 2020). Similarly, many rhizosphere soil microbes (such as PGPB and PGPF) can enhance plant nutrient absorption and utilization (Van Der Heijden et al., 2008; Balsanelli et al., 2019) and enhance crop quality (Verginer et al., 2010; Nasopoulou et al., 2014). They are especially important for the formation of high-quality medicinal plants, such as improving the nutritional element enrichment of *Paris polyphylla* (Zhang H. Z. et al., 2019), and participating in the synthesis of indigo in *Baphicacanthus cusia* and artemisinin in *Artemisia annua* (Zeng et al., 2018; Zhai et al., 2019). Therefore, plant-associated microbiomes have a significant impact on the quality of plants, especially medicinal plants.

The assembly of plant-assiocated microbiomes follows the theory of microbial ecology and is affected by stochastic and deterministic assembly processes (Dini-Andreote et al., 2015). Previous studies have shown that in high microbial diversity communities, stochastic assembly processes are dominant for most cases, while in low microbial diversity communities, deterministic assembly processes are dominant (Kembel, 2009; Xun et al., 2019). And the potential and stability of ecosystems can be predicted by determining their relative contributions (Tilman et al., 1997; Duffy et al., 2017; Galand et al., 2018). In plant–microbe interactions, the assembly of plant-associated microbiomes is affected by specific driving factors, such as genotype (Bulgarelli et al., 2013; Xu et al., 2020), plant tissue (Dong et al., 2018; Alibrandi et al., 2020), soil conditions (Qiao et al., 2017; Long and Yao, 2020) and host-associated environments (Campisano et al., 2017; Cai et al., 2020). Fungi and bacteria are differentially affected due to their physiological and evolutionary differences. Bacteria, such as the rhizosphere

bacteria of maize (Ren et al., 2020), the endophytic bacteria of *Fagus sylvatica* (Coince et al., 2014) and rice (Feng et al., 2019), and the root-related bacteria of the medicinal plant *Polygonum cuspidatum* (Zhang et al., 2020), are mainly shaped by environmental variables and soil factors, while fungi, such as the root-related fungus *Helianthus annuus* (Leff et al., 2017) and the rhizosphere fungi *Picea asperata* and *Abies faxoniana* (Li et al., 2021), are more sensitive to soil nutrient contents and host inheritance. In addition, plant interactions are also crucial to the shaping of plant-assiocated microbiomes. For example, mangroves have a potential impact on the colonization of root endophytic bacteria of *Spartina alterniflora* (Hong et al., 2015). However, either interspecific or intraspecific impact of plant interactions, especially the relationship between *Panax notoginseng* (*P.n.*) flora and pine flora, on plant-associated microbes (fungi and bacteria) is scarcely studied. Therefore, the study of plant–microbe–plant interactions is of more significance to the assembly mechanism of plant-related microbes (fungi and bacteria), especially the transmission of plant-related microbes.

Agroforestry, as one of the land use conversion practices, is not only an important factor in the change in microbial composition and structure, but also a sustainable strategy to alleviate the shortage of cultivated land resources and the environmental burden (Anderson and Sinclair, 1993; Montagnini and Nair, 2004). Previous studies on the variation in soil microbial communities under agroforestry management have yielded different results. Several studies have found that the soil microbial diversity of agroforestry systems is richer than that of forests (Edy et al., 2019; Beule et al., 2020), and, some studies have found that soil microbial diversity remains stable, but community composition is significantly altered when forests are converted to agroforestry systems (Banerjee et al., 2016; Wang et al., 2017), while some others have concluded that native forests can maintain microbial richness and diversity better than agroforestry systems (Belay et al., 2020). In addition, agroforestry is also one of the determinants of plant endophytes. For example, some studies have shown that agroforestry increases the diversity of moso bamboo endophytes and significantly alters their community composition (Zhang H. Z. et al., 2019; Zhang X. et al., 2019). Similarly, studies have found that the richness and the colonization rate of arbuscular mycorrhizal fungi of crops increase under agroforestry management (Sousa et al., 2013; Edy et al., 2019). In fact, the changes in endophytic bacteria in crops are closely related to soil microbiomes. Studies have examined those endophytic fungi of trees and crops that are transmitted to the soil (Ingleby et al., 2007). Therefore, the combination of soil microbial changes and plant endophytic changes in agroforestry systems is of great significance for further understanding the dynamics of plant microbes within the systems.

Panax notoginseng (*P.n.*, Araliaceae) is a precious traditional medicinal herb in China (Yang et al., 2019; Wang et al., 2020). However, under traditional agricultural management, the serious continuous cropping obstacle of *P.n.* results in a shortage of arable land and a decline in yield and quality (Liu et al., 2011; Wang et al., 2019). The *P.n.*-pine agroforestry system, as an organic cultivation strategy for medicinal plants to restore their native habitat, is a necessary approach to ensuring the quality and pharmacological activity of *P. n.* and soil fertility. The success of this organic cultivation depends on the interplay between *P.n.*, pine trees, and the environment (Yu and Zhang, 2019; Wu et al., 2021). However, the relationship between plants and microbiomes in the *P.n.*-pine agroforestry systems is still unclear. Therefore, in this study, four land types including pure pine forests (*Pinus kesiya* var. *langbianensis* and *Pinus armandii*) and agroforestry systems (*Pinus kesiya* var. *langbianensis* - *Panax notoginseng* and *Pinus armandii* - *Panax notoginseng*) were targeted to analyze the changes and influencing factors of microbiomes associated with *P.k.*, *P.a.* and *P.n.* using 16S amplicons and fungal ITS sequencing techniques. Based on the experimental design, the following hypotheses were made: (I) *P.n.* cultivation in agroforestry systems can alter pine-related microbiomes; (II) different agroforestry systems (Lancang and Xundian) would drive different assemblies of *P.n.*-related microbiomes; (III) microbial transmission would exist within the agroforestry systems between pine and *P.n.* This studyaim to reveal the characteristics and driving mechanisms of plant microbial variation in the *P.n.*-pine agroforestry systems.

Materials and methods

Study site

The research was mainly carried out in the pure forest of *Pinus kesiya* var. *Langbianensis* (*P.k.*) in Lancang Lahu Autonomous County, Pu′er City, Kunming, Yunnan Province (99.82°E, altitude of 1457.39 m, mean annual temperature of 19.2°C, mean annual precipitation of 1008.6 mm) and the pure forest of *Pinus armandii* (*P.a.*) in Dadishui Village, Xundian Hui Autonomous County, Kunming, Yunnan Province (103.21°E, 25.47°N, altitude of 2247.81 m, mean annual temperature of 15.5°C, mean annual precipitation of 1624.0 mm). Both forests are located in the central and western parts of the Yunnan-Guizhou Plateau and are fallow forest stands with a regular plant spacing of about 3 m. The vegetation types are shown in the following table (Supplementary Table S1).

Experimental design and sample collection

Forested areas of *P.k.* and *P.a.* were selected as mentioned in 2.1 respectively, for *P.n.* understory cultivation. Before the

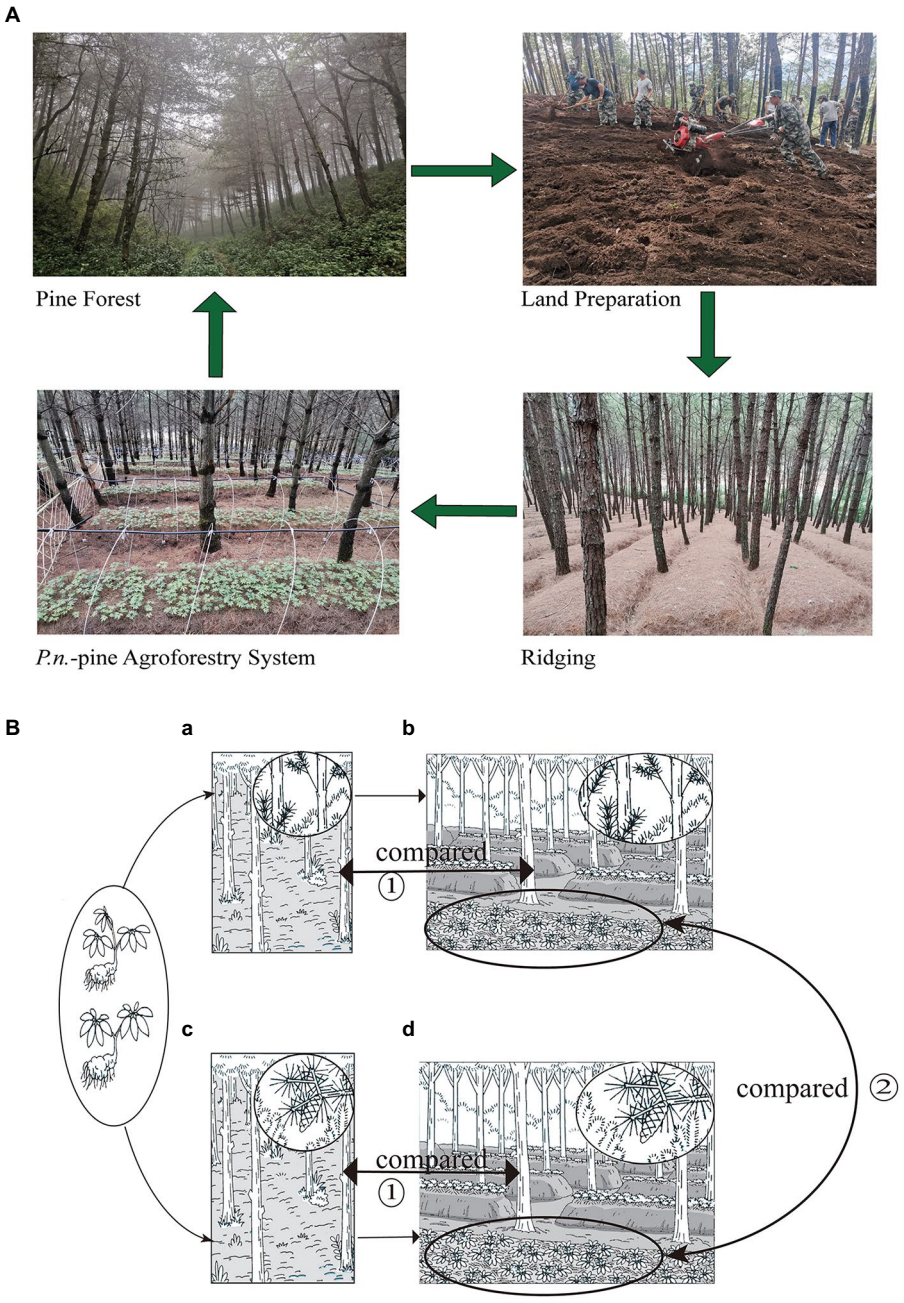


FIGURE 1
Specific planting process of the *Panax notoginseng* - pine agroforestry systems **(A)** and experiment design **(B)**. Experiment one (①) compared the pure forests of *P.k.* (a) and *P.a.* (c) with the corresponding *P.n.*-pine agroforestry systems (b,d) for the difference of pine-associated microbes. Experiment two (②) compared two *P.n.* -pine agroforestry systems (b,d) for the differentence of *P.n.* -associated microbes.

cultivation., weeds, small shrubs, and dead leaves were removed from the understory planting area. *P.n.* were planted in a ridge along the contour of each forest, with a height/bottom/top size of the ridge of 40 cm x 120 cm x 80 cm. The ridges were covered with 3 cm pine needles to retain water and heat (the specific planting process is shown in Figure 1). *P.n.* seedlings (two genotypes: trifurcated five-leaf and bifurcated seven-leaf) had been obtained after greenhouse cultivation in November 2018 before they were transferred to the forests in November 2019, with a plant spacing of 10 cm × 10 cm. The *P.n.*-pine agroforestry systems were constructed using a typical low-input "eco-agriculture" model, by which the use of pesticides and fertilizers was prohibited and only daily irrigation (a small amount) was provided to avoid drought. In addition, shelters were built during the rain season to prevent flooding.

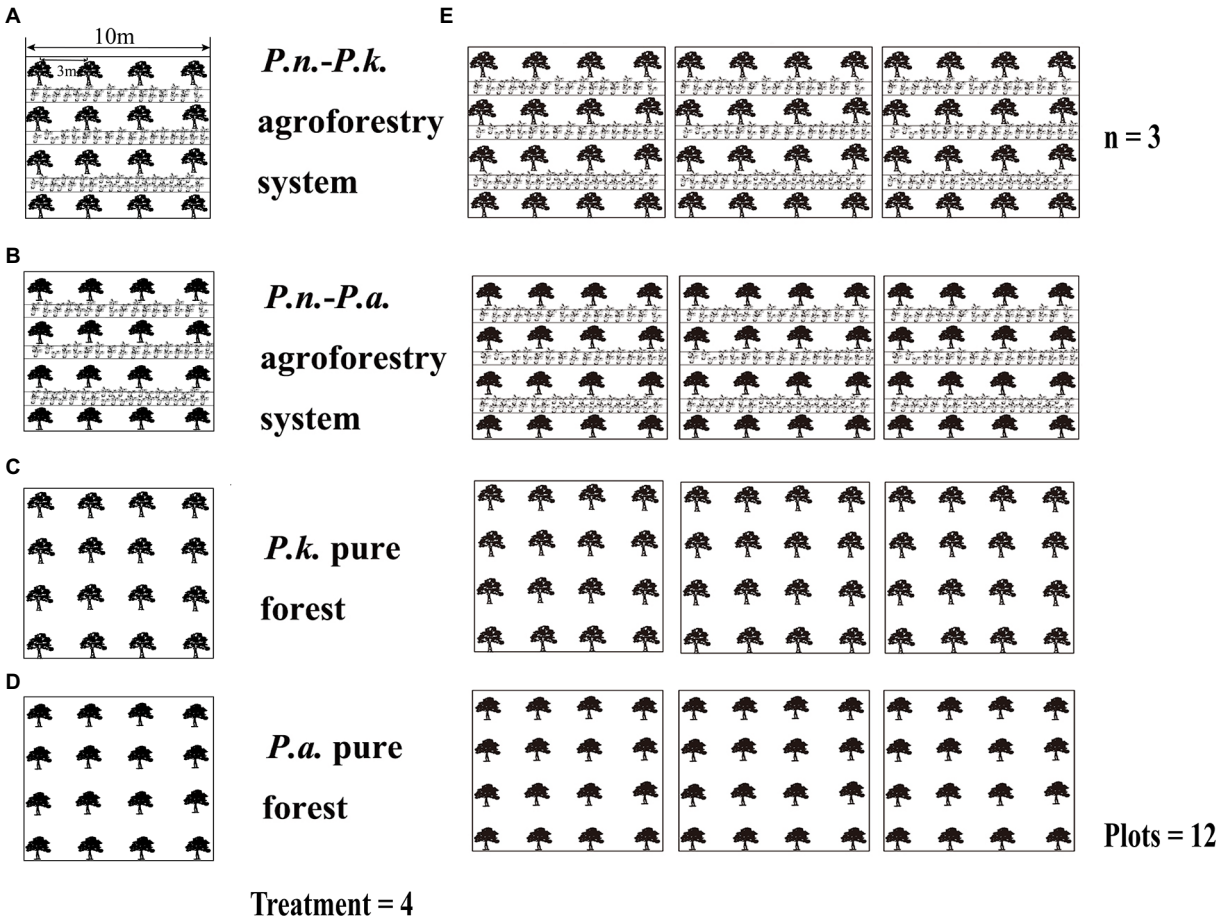


FIGURE 2
Four types of sampling plots and sampling plot design. [sample plots of *P.n.-P.k.* agroforestry system **(A)**, *P.n.-P.a.* agroforestry system **(C)**, and two types of pine pure forest **(B,D)** and sample plots design **(E)**].

The experiments were divided into two parts, Experiment One compared pure pine forests with the corresponding *P.n.*-pine agroforestry systems to explore the effect of land conversion on pine-associated microbiomes. Experiment Two compared *P.n.*-pine agroforestry systems with different pine species to explore the effect of different pine species on the assembly of *P.n.*-associated microbiomes (Figure 2). Therefore, four types of sampling plots (10 m × 10 m, plot spacing is more than 5 m) were set up in Lancang (L) and Xundian (X), i.e., *P.n.-P.k.* plots, pure *P.k.* forest plots, *P.n.-P.a.* plots and pure *P.a.* forest plots, and were replicated three times, totaling 12 plots (Figure 2; the samples within the pure forest plots were noted as PP, and the samples of *P.n.*-pine plots were noted as GP or PG). In Experiment One, pine root samples (named L_GP_Ro, L_PP_Ro, X_GP_Ro, and X_PP_Ro, respectively) and pine rhizosphere soil (named L_GP_Rh, L_PP_Rh, X_GP_Rh, X_ PP_Rh, respectively) in *P.n.-P.k.* plots, pure *P.k.* forest plots, *P.n.-P.a.* plots and pure *P.a.* forest plots; soil at the locations where the pine trees and the ridges meet (named L_GP_Be, X_GP_Be, respectively) in the *P.n.*-pine plots (*P.k.*, *P.a.*) and bulk soil of pine trees at the corresponding locations (named L_PP_Bu, X_PP_Bu, respectively) in the pure forest plots (*P.k.*, *P.a.*) were collected. In Experiment Two, roots, stems, and leaves of bifurcated seven-leaf and trifurcated five-leaf *P.n.* (named L_SL_Ro, L_SL_St, L_SL_Le, L_FL_Ro, L_FL_St, L_FL_Le, X_SL_Ro, X_SL_St, X_SL_Le, X_FL_Ro, X_FL_St, X_FL_Le, respectively), rhizosphere soil of bifurcated seven-leaf and trifurcated five-leaved *P.n.* (named L_FL_Rh, L_SL_R, X_FL_Rh, X_SL_Rh, respectively) *in P.n.*-pine (*P.k.*, *P.a.*) plots, and bulk soil of *P.n.* (named L_PG_Bu, X_PG_Bu, respectively) *in P.n.*-pine (*P.k.*, *P.a.*) plots and soil at the locations where the pine trees and ridges meet (L_PG_Be/X_GP_Be) *in P.n.*-pine (*P.k.*, *P.a.*) plots were collected.

P.n. is planted in the forest for 1 year and then stalked and regrown for another year, so sampling was conducted before the harvest of *P.n.* on November 20, 2020. The collection of plant samples in each sampling plot was divided into two parts: the collection of pine roots and *P.n.* plants. Pine root sampling: Five pine trees more than 50 cm away from the four sides of a sampling plot were randomly selected, and 15–20 young healthy roots (root diameter < 2 mm) of each pine tree were collected and mixed as a

plant sample. *P.n.* sampling: Ten *P.n.* Plants of two genotypes with similar growth performance were collected from the ridges adjacent to the selected pine trees in a *P.n.*-pine sampling plot, and the roots, stems, and leaves were separated. Soil sampling adopted the five-point method, which removed plant litter from the soil surface, collecting four soil cores (sampling depth 0–20 cm) at approximately 50 cm from the four corners of the sampling plot, collected one soil core at the center of the sampling plot, and thoroughly mixed them into one soil sample. The rhizosphere soil was the soil immediately attached to the plant roots (0–2 mm from the root surface). The sample size of pure forest plots in this experiment was 18 (3 samples of the individual plot × 2 (two pine species) × 3 replicates), and the sample size of *P.n.*-pine plots was 72 (12 samples of the individual plot × 2 (two agroforestry systems) × 3 replicates), which added up a total of 90. Plant and soil samples were stored in liquid nitrogen and dry ice, respectively, and then immediately transported back to the laboratory and stored at −80°C for further analysis.

Plant and soil physicochemical properties

The fresh soil samples were passed through a 0.298 mm sieve to determine the physical and chemical properties. The soil water content was measured by drying the soil at 85°C for 48 h to constant weight, and the soil pH and EC were determined by a 5:1 water/soil suspension with a pH meter and a conductivity meter, respectively. Total nitrogen (TN), total phosphorus (TP), NH_4^+−N, and NO_3^-−N were measured by a SmartChem200 analyzer using standard methods. TK was analyzed by atomic emission spectrometry on an AA-6300C flame photometer.

After the plants were collected, they were rinsed with pure water, dried with filter papers, and measured for fresh weight. They were then dried at 70° to constant weight, and measured for dry weight and water content. Afterwards, they were milled and 0.5 g of the milled plant samples was digested by Kjeldahl decoction (10 ml 95.5%H_2SO_4 and 3%H_2O_2), and the digestion solution was used to determine the TN and TP of plants (Lv et al., 2004; Wang et al., 2022) by a SmartChem200 analyzer.

DNA extraction, PCR amplification, and sequencing

The plant samples for endophyte collection were soaked and rinsed with sterile water, 70% ethanol, and 2.5% NaClO for 1 min, 90 s, and 30 s, respectively. Then sonication procedures were performed twice with phosphate-buffered saline and observed under a scanning electron microscope to ensure that all microbes were removed from the plant surface. DNA extraction from soil samples (0.5 g each) used No, 12888.100 Qiagen DNeasy PowerSoil Kit (MP Biomedicals, Solon, CA, United States) and from 0.5 g of the plant tissue samples (the pine roots and the roots, stems, and leaves of *P.n.*) used the Qiangen DNeasy Plant Kit following the manufacturer's instructions. The PCR amplification procedure and sequencing process are detailed in the Supplementary material. The bacterial 16S rRNA and fungal ITS gene sequencing was performed on the Illumina MiSeq PE300 platform. We filtered the OTUs assigned to chloroplasts and mitochondria from the OTU table before further analysis. Sequences have been deposited in the National Center for Biotechnology Information Sequence Read Archive under Accession No. PRJNA821648 (16S RNA data) and No. PRJNA821834 (ITS data).

Statistical analysis

Differences in the physicochemical properties of the plants (*P.n.*, pine roots) and the soil, microbial alpha diversity, and the relative abundance of major phyla/genera in this study were analyzed using various ANOVA methods. For comparisons between two groups, either the one-way Student's t test (normally distributed variables) or the Mann–Whitney nonparametric test (other variables) was used. For comparisons between multiple groups, the one- to multi-way ANOVA with a p value less than 0.05 was considered to be a significant difference. The microbial diversity (Shannon index), richness (Chao1), and evenness (Shannon even index) were selected to characterize the microbial alpha diversity. Alpha diversity was assessed in relation to soil and plant physicochemical properties using Pearson correlation analysis. These analyses were all performed using SPSS 25.0 (IBM, Armonk, NY, United States).

Venn diagrams and stacked bar charts were based on microbial OTU Tables (97% similarity level)were implemented using R (version 3.3.1) to visualize microbial community composition and differences. Microbial beta diversity was calculated in QIIME (1.9.1) based on weighted UniFrac distance. PCoA plots made with the vegan and ggplot2 packages in R were used to visualize differences in community composition. Permutational multivariate analysis of variance (PERMANOVA, 999 permutations calculated) was used to examine the effects of planting *P.n.* (pine species), compartment, genotype, and their interactions effects on the community composition of pine (*P.n.*)-associated microbiomes. Pairings between planted *P.n.* (pine species) and genotypes were also calculated to compare effects between different compartments (bulk, between, rhizosphere, root, stem, leaf for *P.n.* and bulk, rhizosphere, root for pine). Detrended correspondence analysis (DCA) was performed with microbial sample OTU tables (from the first axis of the length of the gradient) to determine RDA/CCA (redundancy analysis/Canonical correspondence analysis) and was used to further assess the effect of plant soil physicochemical properties on bacterial and fungal communities. These were analyzed and plotted using the Vegan package in R.

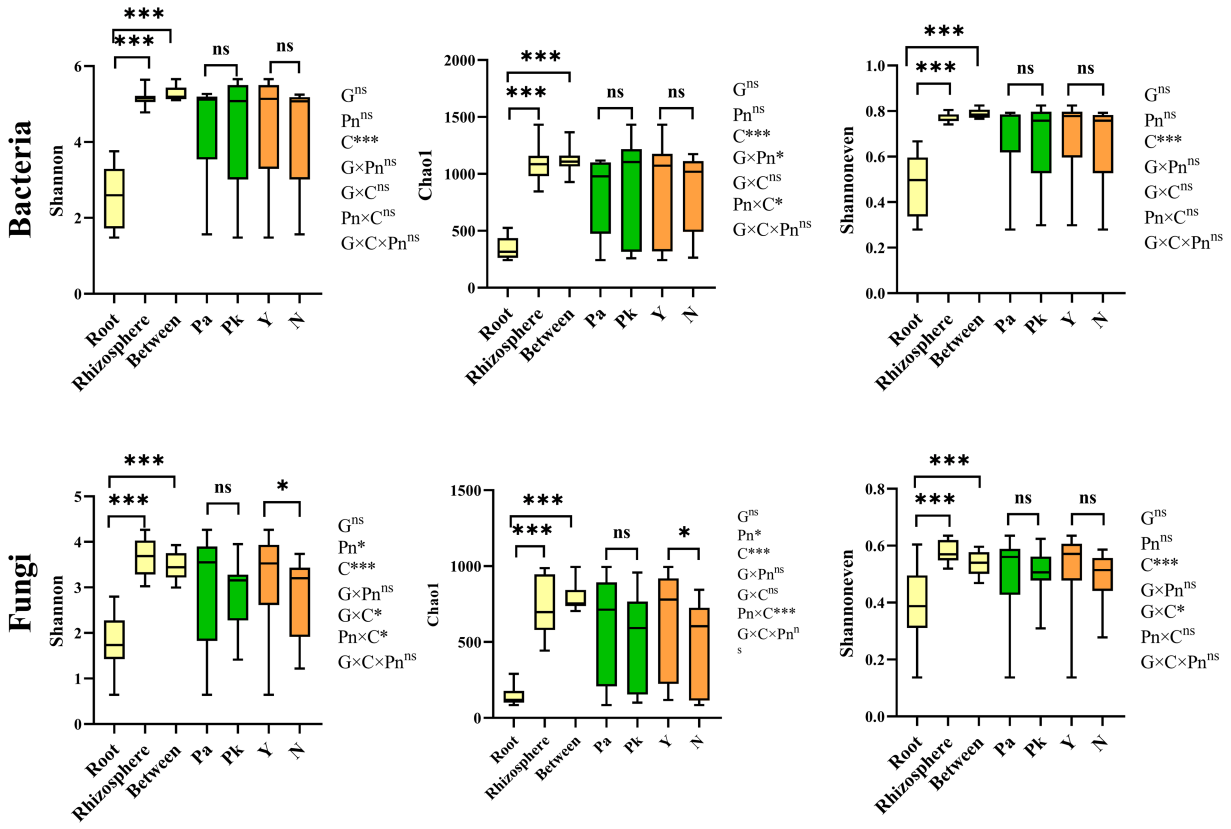


FIGURE 3
Boxplots of indices of bacterial and fungal alpha diversity of microbes in samples from different compartments (between (bulk) and rhizosphere soils, and root of pine trees), genotype and planting *P.n.* or not. Different letters showed significant difference ($p<0.05$). The Significance of effects of genotype (G), compartment (C) and planting *P.n.* or not (Pn) and their interactions on microbial diversity was evaluated by multi-way ANOVA. *, p<0.0 5, and ***, $p<0.001$, ns, no significance.

Results

Variations in plant-assoicated microbial richness and diversity

Compared to pine forests, the α-diversity of pine-associated fungi in agroforestry systems increased, while there was no significant change in bacteria. Main effect analysis showed that compartment and *P.n.* cultivation rather than pine genotype significantly affected the α-diversity (Chao1 and Shannon index and Shannoneven index) of pine-associated microbiomes (Figure 3). In general, the α-diversity of pine-associated microbes showed a decreasing gradient from the soil (bulk and rhizosphere) to the roots. In addition, significant genotype and compartment (G × C) interactions were found in α-diversity of pine-associated fungi but not bacteria. The interaction between planting *P.n.* and compartment (Pn × C) increased the fungal α-diversity in the rhizosphere of pine, while it had no significant effect on the intraroot and bulk soil (Figure 3; Supplementary Table S2). Significant (G × Pn) interactionsexsited in bacteria (rhizosphere and bulk soil) but not in fungi. The indicators that were most closely related to the microbial diversity of the rhizosphere soil of pine were total nitrogen, pH and soil water content. However, the correlations between root endophytes (*P.k.*, *P.a.*) and environmental indicators were not significant, except for the correlation between root endophytes and root nitrogen content (Supplementary Table S3).

In agroforestry systems, different pine tree species had no significant effect on the α-diversity of *P.n.*associated microbiomes (bacteria and fungi). Main effect analysis showed that compartment, rather than genotype or pine species, significantly affected the α-diversity of *P.n.*- associated microbiomes (Figure 4). In general, the α-diversity of *P.n.*-associated microbes exhibited a decreasing gradient from the soil (bulk and rhizosphere) to intraplant. As for endophytes, endophytic bacteria were the most abundant in roots, and endophytic fungi were the most abundant in leaves. The interaction effect (Pn × C) between planting *P.n.* and compartment was reflected by the species richness (Chao1) in the rhizosphere soil and leaves of *P.n.* but not in its roots and stems (Supplementary Table S4). Among the morphological and physiological indicators of plants, fresh weight was most closely related to microbiomes (Supplementary Table S5) and soil nitrate nitrogen was more closely related to the α-diversity of *P.n.*-associated microbes. In addition, bacterial α-diversity was also

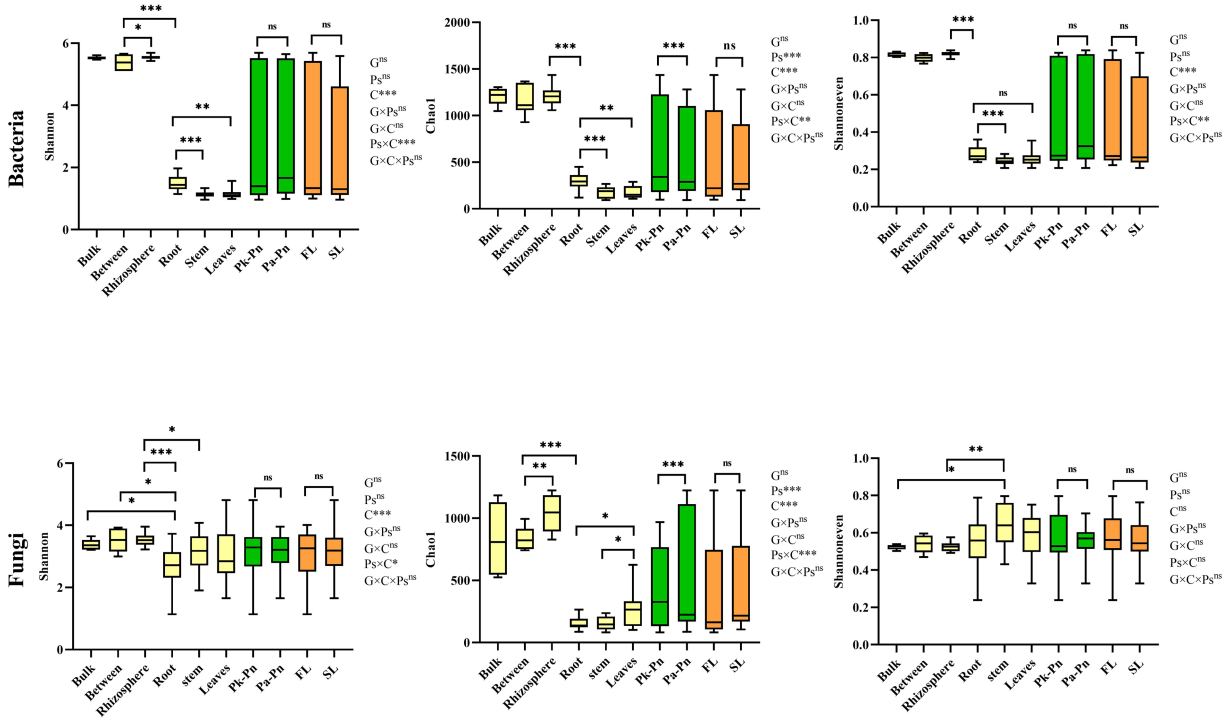


FIGURE 4
Boxplots of indices of bacterial and fungal alpha diversity of microbes in samples from different compartments (bulk, between, rhizosphere soils, and roots, stems, leaves of *P.n.*), genotype and pine trees species. Different letters showed significant difference (p<0.05). The Significance of effects of genotype (G), compartment (C) and pine trees species (Ps) and their interactions on microbial diversity was evaluated by multiway ANOVA. *, p<0.0 5, **, p<0.01, ***, p<0.001, ns, no significance.

significantly related to the total phosphorus of the soil, and fungal α-diversity was closely related to the TN and EC of the soil.

Changes in the composition of plant-related microbial communities

Land conversion did not change the community composition of pine and *P.n.*-associated microbiomes (Figures 5, 6).

The dominant phyla of pine-associated bacteria (bulk soil, rhizosphere, root of *P.k.* and *P.a.*) were Proteobacteria, Actinobacteria, Acidobacteria, and Chloroflexi (Figure 5). The main groups of bacteria in roots were similar to those in the soil, but the proportion of Proteobacteria and Actinobacteria in pine roots was larger (more than 90%). The fungal community of pine (bulk soil, rhizosphere, root) was mainly composed of Basidiomycota, Ascomycota, and Mortierella. Land conversion increased the proportion of Ascomycota and Mortierella, and decreased that of Basidiomycota.

At the genus level, the endophytic bacteria of pine roots were mainly composed of Pseudomonas and bacterial genera in the family Alcaligenes, which accounted for more than 40% of bacteria. The rhizosphere bacteria were mainly composed of the genera in the families of Xanthobacteraceae, Bradyrhizobium, Burkholderia-Caballeronia-Paraburkholderia, Acidobacter and genera in the phylum of Chloroflexi, which accounted for 45% above. The endophytic fungi of pine were dominated by Russula, Sabacina, Thozetella, and Lactarius. Soil fungi were dominated by Saitozuma, Sebacina, and Mortierella, accounting for 30%.

In all sequenced samples, the majority of the pine root endophytic microbes were also existed in the soil (rhizosphere and bulk soil), including bacteria (93.82%) and fungi (88.73%), and the number of OTUs of the roots shared by the rhizosphere soil was higher (93.07% of bacteria and 85.5% of fungi) compared with the bulk soil. This result indicated that most of the root endophytic microbes were recovered from the soil environment. Land conversion. Increased the proportion of OTUs specific to pine roots, and the proportion of OTUs specific to fungi was greater than that of bacteria (bacteria increased by 2.2%, fungi increased by 6.84%).

The main composition of *P.n.*-associated soil bacteria was largely similar to that of pine-associated soil bacteria. Proteobacteria (more than 90%) dominated the endophytic bacteria (roots, stems, leaves) of *P.n.* The fungal communities of *P.n.* (soil and root) were mainly composed of Basidiomycota and Ascomycota (accounting for more than 80%). Basidiomycota dominated in the soil, and Ascomycota dominated in the endosphere of *P.n.*

At the genus level, soil bacteria were dominated by the genera in the family Xanthobacteraceae, genera in the phyla

FIGURE 5
The relative abundance of the most abundant bacterial **(A,C)** and fungal **(B,D)** communities of pine at the phylum and genus level (means, n=6), Venn diagram showing the number of shared and unique bacterial **(E)** and fungal **(F)** communities at the OTU level.

Chloroflexi, Bradyrhizobium and Acidothermus, and endophytic bacteria of *P.n.* are dominated by Pseudomonas and the genera in the family Alcaligenaceae. Soil fungi were dominated by Saitozyma, Cladophialophora, Mortierella, and unclassified fungi. Unclassified fungi accounted for the largest proportion of endophytic fungi of *P.n.* A Venn diagram showed that the root endophytic microbial OTUs of *P.n.* overlapped with those of the rhizosphere the most (fungi 28.48%, bacteria 47.99%), while leaf endophytic fungi had more unique OTUs (45.63%).

The assembly of pine-related microbes and *P.n.*-related microbes is driven by different factors

Pine-associated microbial community assembly

There were no significant changes in the community structure of pine microbiomes (bacteria, fungi) in agroforestry systems compared to pure forests (Figure 7). PERMANOVA showed that compartment significantly affected the community structure of

FIGURE 6
The relative abundance of the most abundant bacterial **(A,C)** and fungal **(B,D)** communities of *P.n.* at the phylum and genus level (means, n=3), Venn diagram showing the number of shared and unique bacterial **(E)** and fungal **(F)** communities at the OTU level.

pine microbiomes, and the pine genotype significantly affected the community structure of pine fungi, and planting *P.n.* had limited effects (Supplementary Table S6).

PCoA showed that soil microbes and pine root endophytes were clustered into two groups along principal coordinate 1, indicating that the recruitment of pine endophytes was distinctive. However, both rhizosphere fungi and bulk soil bacteria had a noted distinction between land use types (*P.n.*-pine agroforestry systems vs. pure pine forests), indicating that planting *P.n.* was one of the sources of the β-diversity of the rhizosphere fungal and bulk soil bacterial communities. In addition, the fungal composition in pine roots was only affected by genotype.

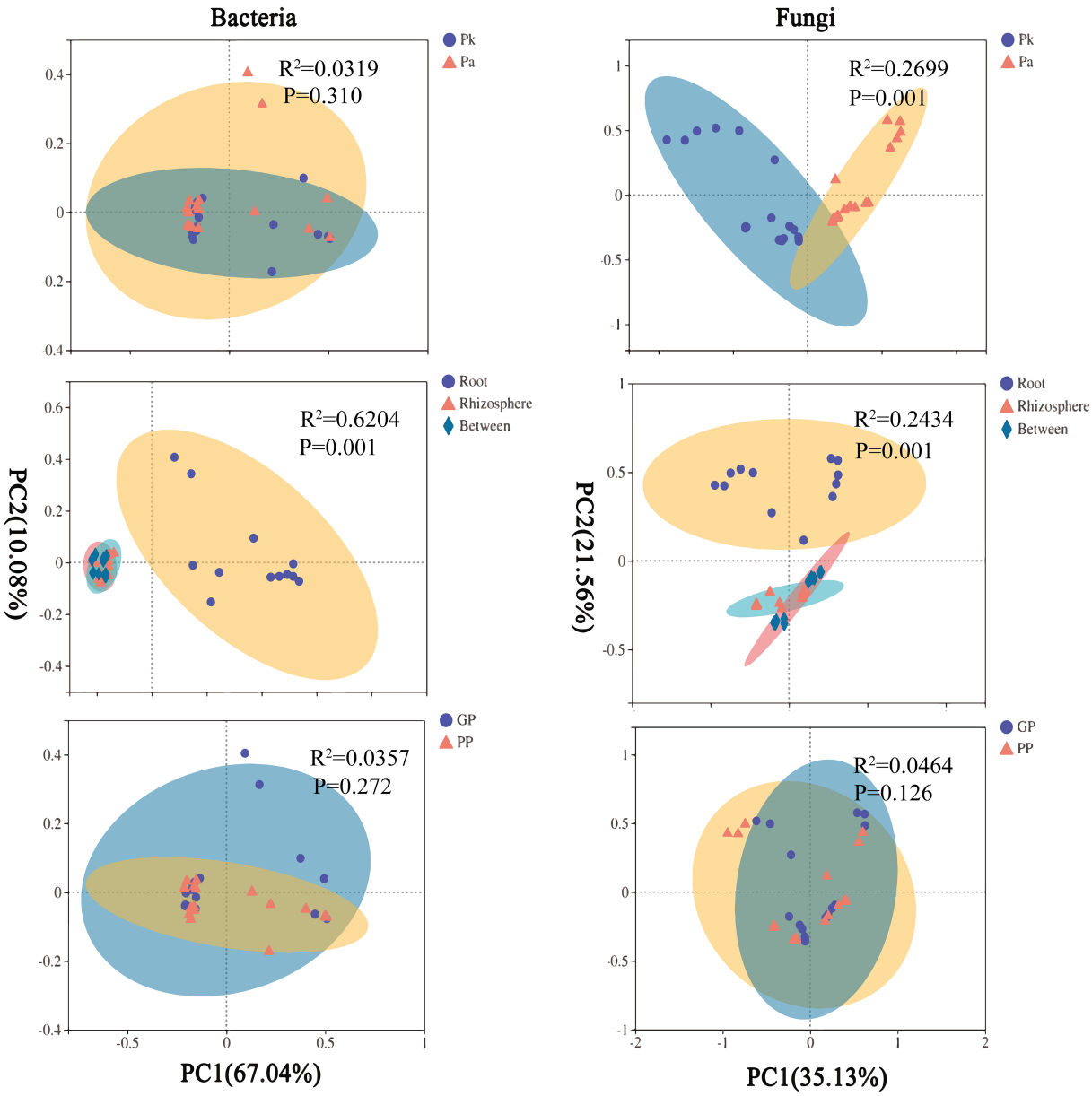


FIGURE 7
Principal co-ordinates analysis (PCoA) plots based on the weighted UniFrac distance (WUF) of pine-asscioated microbes. Pk, samples of *Pinus kesiya* var. *Langbianensis*, Pa, samples of *Pinus armandii* Franch., GP, *P.n.*-pine agroforestry systems, PP, pure pine forests.

When determining the effects of biotic and abiotic factors on community composition, the longest lengths for the DCA (detrended correspondence analysis) on the 16S rDNA and ITS datasets were 1.47 and 3.23, respectively. Therefore, RDA was chosen for the analysis of bacterial and fungal communities. Overall, bacterial RDA1 and RDA2 (total explanatory variance: 96.19%) explained more variance in community composition than fungi (total explanatory variance: 58.49%). RDA indicated that ammonium nitrogen, soil water content and total potassium were more correlated with bacterial community composition (Figure 8) and soil water content, nitrate nitrogen and root nitrogen were more correlated with to fungal community composition.

Assembly of *P.n.*-related microbial communities

The community structure of *P.n.* fungi was significantly altered in the two agroforestry systems with different pine species (Figure 9). PERMANOVA showed that compartment and pine species, rather than P.n. genotype, significantly affected the community structure of *P.n.*-associated microbiomes (Supplementary Table S7).

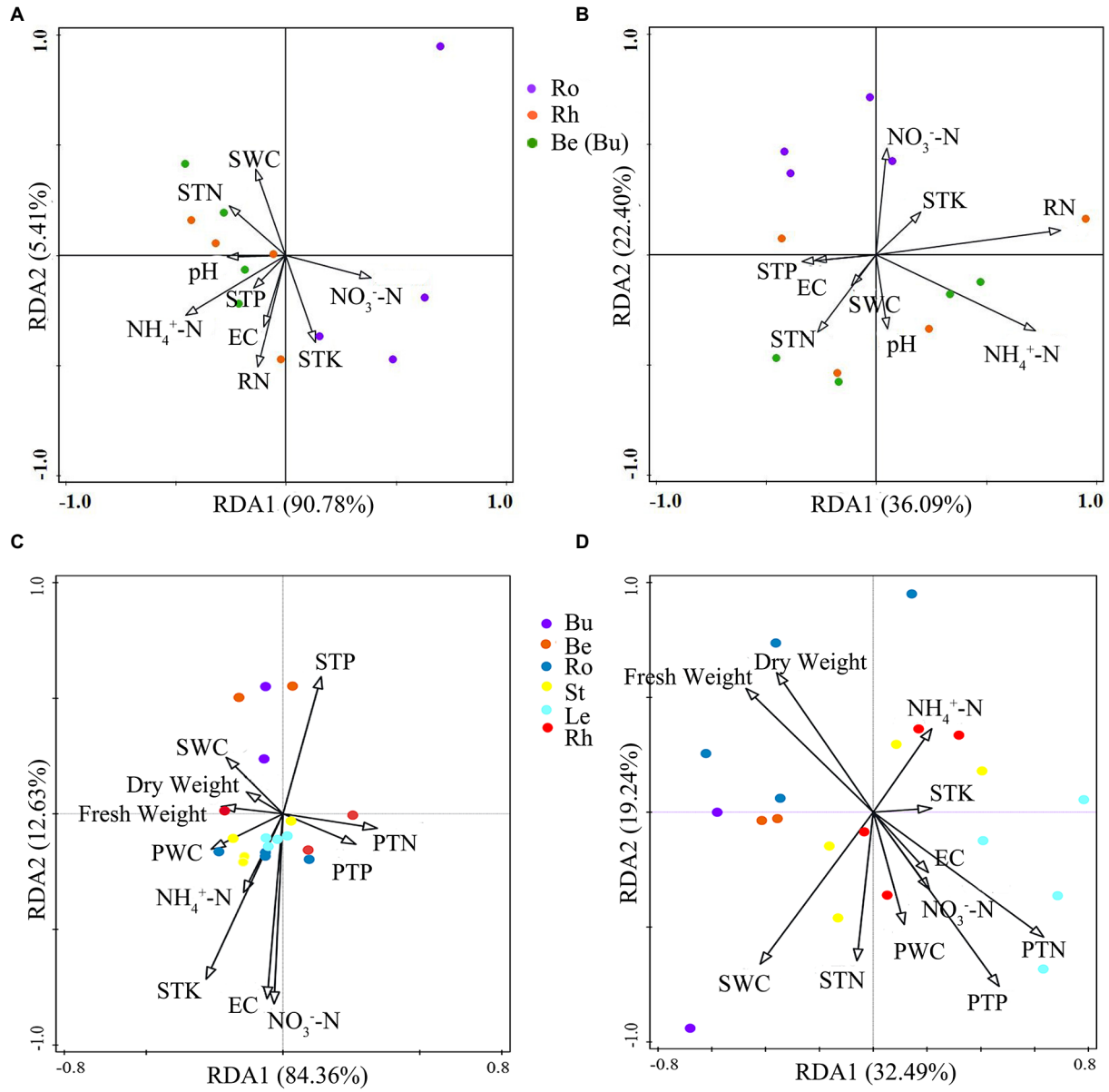


FIGURE 8
The RDA analysis plots showing the relationship between environmental factors and pine associated bacterial **(A)** and fungal **(B)** communities at the OTU level and the relationship between environmental factors and *P.n.*-associated bacterial **(C)** and fungal communities **(D)** at the OTU level. Soil traits: SWC, soil water content, STN, soil total nitrogen, STP, soil total phosphorous, STK, soil total potassium, NH_4^+-N, soil ammonium nitrogen, NO_3^--N, soil nitrate nitrogen, EC, soil conductivity. Plant traits: RN, Root nitrogen, Dry weight, plant dry weight, Fresh weight, plant fresh weight, PWC, plant water content, PTP, plant total phosphorous, PTN, plant total nitrogen.

PCoA showed that soil microbes and plant endophytes were grouped separately along the principal coordinate 1, indicating that the recruitment of *P.n.* endophytes was specific. In addition, the response of *P.n.*-associated fungi to pine species were different among compartments, and those in the rhizosphere and the root were the most significant (Supplementary Figures S1, S2).

The longest gradients for the DCA of the 16S rDNA and ITS datasets were 1.32 and 2.04, respectively. Therefore, RDA was chosen for the analysis of the microbial communities. Overall, RDA1 and RDA2 of bacteria (total explanatory variance: 96.99%) explained more variance in community composition than fungi (total explanatory variance: 51.73%). RDA showed that the indicators with greater correlations with bacterial community composition were total soil potassium and plant water content (Figure 8), and, plant TN and soil water content were more closely related to fungal composition.

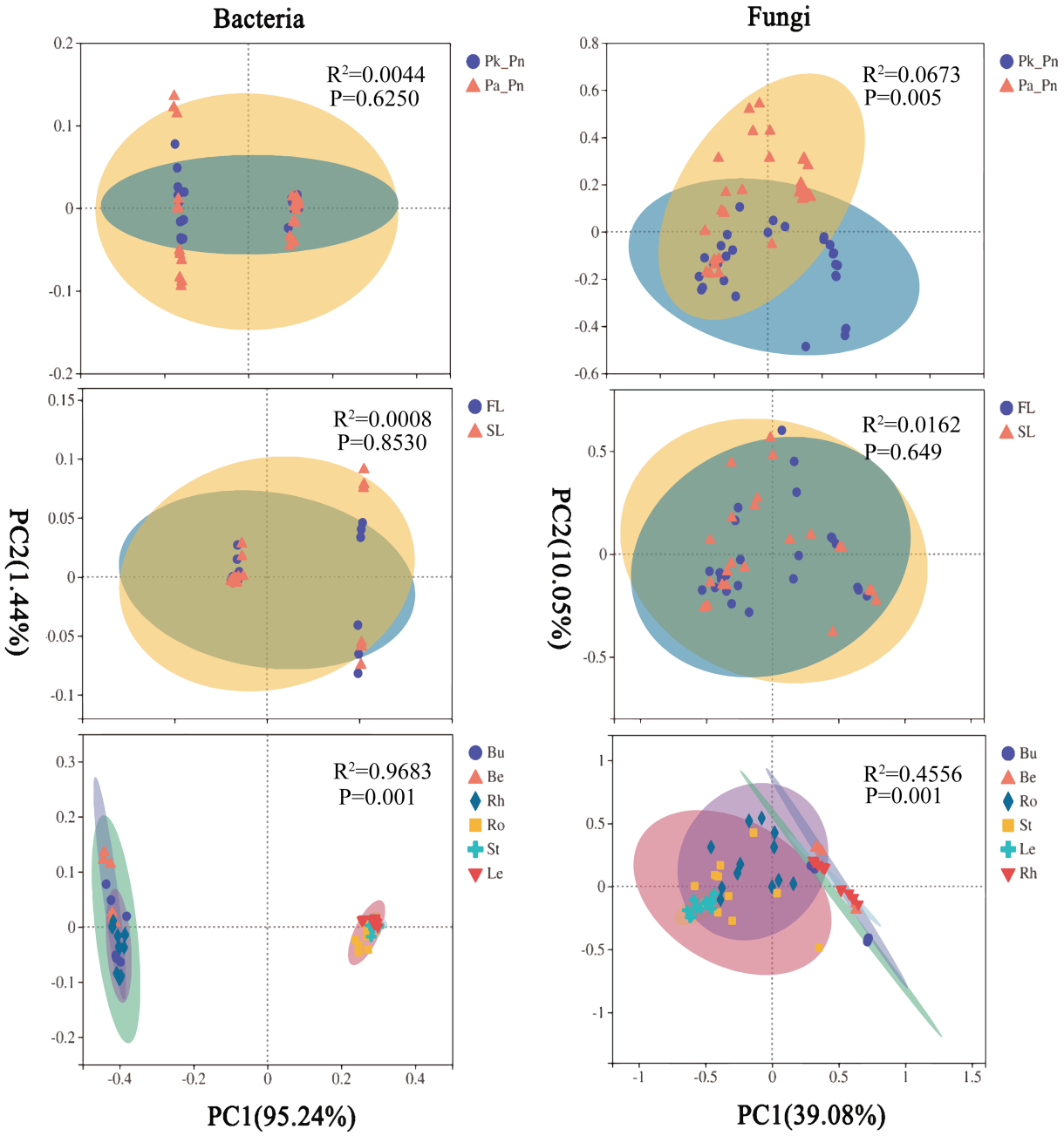


FIGURE 9
PCoA plots based on the WUF of *P.n.*-associated microbes. Pk_Pn, samples of *P.n.* under *P.n.-P.k.* agroforestry system, Pa_Pn, samples of *P.n.* under *P.n.-P.a.* agroforestry system, FL, *P.n.* of three palmately compound leaves and five leaflets, SL, *P.n.* of two palmately compound leaves and seven leaflets.

Partial overlap and differential microbial analysis of pine roots and *P.n* roots in different *P.n.*-pine agroforestry systems

There were variations in microbial taxa within pine roots in agroforestry systems compared to pure forests, and the taxa originated from endophytic bacteria of *P.n.* (Figure 10; Supplementary Figure S3). Fungal taxa varied more than bacteria in the rhizosphere of pine and bacterial taxa varied more than fungi in pine roots (Supplementary Figures S4–S7).

In the roots of *P.k.*, the bacteria increased by 23 genera and decreased by 48 genera; the fungi increased by 48 genera and decreased by 31 genera. In the root of *P.a.*, the bacteria increased by 30 genera and decreased by 66 genera, the fungi increased by 82 new genera and decreased by 27 genera. Interestingly, 73.9% of the new bacterial genera and 68.75% of the new fungal genera in

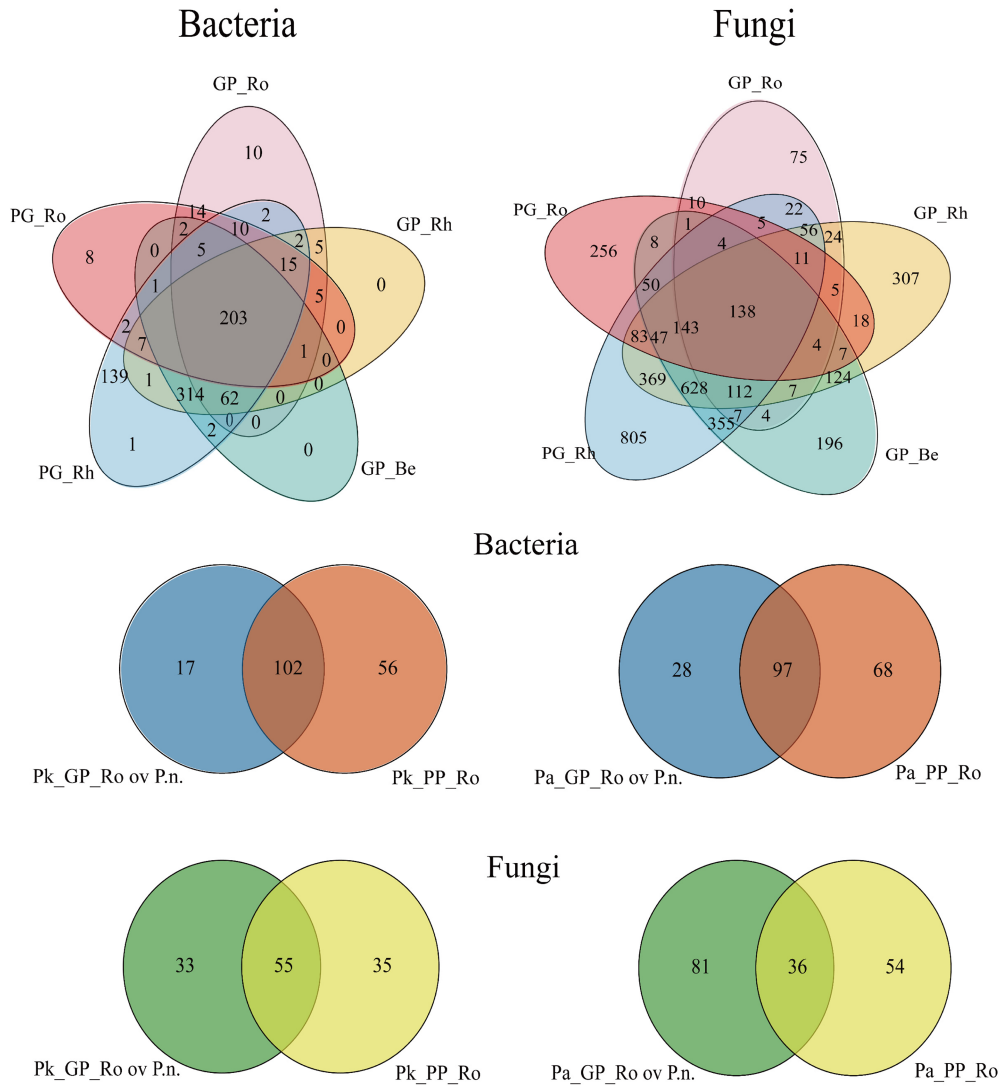


FIGURE 10
Venn diagram showing the overlap and possible transmission of colonies in the *P.n.*-pine agroforestry system. (Pk/Pa) GP_Ro, the pine root microbes of *P.n.*-(*P.k.*/*P.a*) agroforestry system, (Pk/Pa) GP_Rh, the rhizosphere microbes of *P.n.*-(*P.k.*/*P.a*) agroforestry system, GP_Be, the middle of the planting *P.n.* and adjoining pine tree, PG_Rh, the *P.n.* rhizosphere microbes, PG_Ro, the endosphyte of *P.n.* root. Pk(Pa)_GP_Ro ov *P.n.*, Overlap of the endophytic microbes of *P.k.* (*P.a.*) roots with the endophyte of *P.n.*

the roots of *P.k.*, and 93.3% of the new bacteria and 93.9% of the new fungi in the root of *P.a.* overlapped with the endophytes of *Panax notoginseng* (roots, stems and leaves).

Compared with the pure forests, the microbial changes among each compartment of the pine trees in the *P.n.*-pine agroforestry systems, are shown in the figure (Supplementary Figures S5–S8). The proportions of Acidobacteriales and Acidimicrobiia in the *P.k.* roots were significantly decreased, Biastococcus was significantly increased. However, in the rhizosphere bacteria, the proportion of Acidobacteriaceae was significantly decreased, and norank_c_AD3 and Acidothermus were significantly increased. For the *P.k.* rhizosphere fungi, the proportion of Amphinema and Cenococcum increased significantly, and Tricholoma, Clavulinaceae, and Helvellosebacina genera decreased significantly. Correspondingly, after planting *P.n.*, the proportions of Rhodanobacter and Methylovirgula were significantly increased for endosphere bacteria in the *P.a.* roots. In *P.a.* rhizosphere bacteria, the genera norank_o_TK10 and ADurb_Bin063_1 were significantly increased, and Mycobacterium, Granulicella, and Flavobacterium were significantly decreased. In *P.a.* root fungi, Venturia was significantly reduced. However, the proportions of the Mortierella, Thelephoraceae and Talaromyces genera increased significantly, while those of the Inocybe, Sagenomella, and Tomentella genera decreased significantly in the *P.a.* rhizosphere fungi.

In agroforestry systems with different pine species, the *P.n.*-associated fungi responded to a greater degree than bacteria, and

rhizosphere microbes responded to a greater degree than endophytes (roots, stems, leaves of *P.n.*), while stem endophytes were the most stable (Supplementary Figures S8, S9).

Under *P.n.*-pine agroforestry systems of different pine species, Acidothermus, Conexibacter, Rhodanobacter, etc., changed significantlyamong the *P.n.* rhizosphere bacteria, Paraburkholderia, Rhodanobacter MND1, etc., were significantly changed among the *P.n.* root bacteria, Alorhizobium, Pararhizobium and Tardiphaga were significantly changed among the *P.n.* stems bacteria, and Soilbacter and Patulibacter were significantlychanged among the *P.n.* leaf bacteria.

The rhizosphere fungi of *P.n.* changed significantly in the genera Saitomyza and Cladophialophora. In the *P.n.* roots, the genera Trichophaea, Paraglomerales, Sebacina, Phialophora, etc., changed significantly. The unclassified_fungi of Teratosphaeriaceae, Aureobasidium, Papiliotrema, etc., changed significantly in the *P.n.* stems. In the *P.n.* leaves, Aureobasidium, Lapidomyces and Genolevuria changed significantly.

Discussion

Plant-associated microbiomes influence the health and yield of crops and the functioning of agroforestry ecosystems. The community diversity and composition of plant-associated microbiomes are important indicators of the stability of agroforestry ecosystems. Additionally, tissue type (Dong et al., 2018; Alibrandi et al., 2020), plant introduction (Zhang X. et al., 2019; Beule et al., 2020), plant genotype (Bonito et al., 2014; David et al., 2016), and soil conditions (Qiao et al., 2017; Long and Yao, 2020) are important factors for the assembly of plant-associated microbiomes. In this study, a systematic investigation of the variation and assembly of plant-associated microbiomes under the *P.n.*-pine agroforestry systems has revealed differences in the microbial driving factors associated with *P.n.* and pine.

Compartment, cultivation of *P.n.*, and pine genotype drive the diversity and community structure of pine-related microbiomes

Compared to pure forests, the α-diversity of pine-associated fungi in the agroforestry systems showed an increasing trend, while bacteria did not change significantly. In addition, there were no significant changes in community structure (β-diversity) and community composition (bacteria and fungi). This is consistent with the results of *Manihot glaziovii-Gliricidia sepium* agroforestry system (Sousa et al., 2013). Main effects analysis showed that compartment, *P.n.* cultivation and pine genotype significantly affected the diversity of pine-related microbiomes (α-diversity and β-diversity). The effect of compartment on plant microbial diversity has been demonstrated by many studies (Dong et al., 2018; Alibrandi et al., 2020), and we have found that compartment is the most important factor in the microbial assembly of pine trees in agroforestry systems. Microbial diversity of different compartments (root, rhizosphere, bulk) of pine trees showed that the soil (bulk, rhizosphere) microbes were greater than endophytes (root), which is similar to the results obtained in previous studies on poplar systems (Gottel et al., 2011). This suggested a hierarchical filtering effect (Chen et al., 2016) on the assembly of pine root-associated microbiomes. The root epidermis constituted a natural barrier that creates a filtering effect on the microbiomes that spread to the plant (Bulgarelli et al., 2013). Additionally, the critical influence of compartment might arise from the complex interactions of microbiomes and the ecological differentiation due to the different proportions of substrate and genotype drivers further leading to the formation of different structures of microbiomes in different compartments of pine (Bulgarelli et al., 2013). Planting *P.n.* increased the diversity of fungi associated with pine trees but had no significant effect on bacteria (associated with pine).

As the second important factor, *P.n.* cultivation significantly affected the α-diversity of pine fungi, but not the community structure (bacteria, fungi). Land use conservation, such as forest conservation in the cacao agroforestry system, can significantly affect fungal diversity but not bacterial diversity (Edy et al., 2019). Previous studies have shown that the effect of land conservation on fungi is mainly due to variations in soil organic matter content and plant root vigor (Beule et al., 2020). The introduction of *P.n.* and factors such as tillage and covering with plant litter during the cultivation may have changed the soil chemical and physical properties, thereby affecting the fungal diversity. In addition, there was very little effect of *P.n.* cultivation on the community structure of pine-associated microbiomes, which is different from the result that the invasive plant *Spartina alterniflora* (*S.a.*) significantly changed the root-related microbiomes of *Kandelia obovata* (Hong et al., 2015). This may be because *S.a.* as an invasive plant, was in competition with *K.o.* for nitrogen sources. Although *P.n.* replaced the original herbaceous plants in the pine forests, the nitrogen demand of the whole forest system did not increase, and at the same time, the coveringwith plant litter during the cultivation of *P.n.* also provided a certain nitrogen for *P.n.*

Pine genotype did not have a significant effect on the α-diversity of pine-associated microbiomes (bacteria and fungi), but significantly changed the community structure of fungi (associated with pine). The finding differed from some previous studies which have found a significant effect of host genotype on root-associated bacteria (Bonito et al., 2014), probably because both *P.k.* and *P.a.* belong to the family Pinus spp. and their difference at the taxonomic level is slight. In addition, the differences in fungal community structures may because pine trees, as typical trophic species with ectomycorrhizal symbiosis, are often symbiotic with different fungi and are largely influenced by genotype (Peršoh, 2013).

Compartment and pine species rather than *P.n.* genotype drive the changes in *P.n.*-associated microbiomes

In the agroforestry systems, different pine species did not significantly affect the α-diversity of *P.n.*-associated microbiomes (bacteria and fungi) but significantly altered the fungal community structure, which is partially consistent with the results obtained in poplar-based alley cropping systems (Beule and Karlovsky, 2021). Main effects analysis showed that compartment and pine species rather than *P.n.* genotype significantly affected the diversity of *P.n.* related microbiomes (α-diversity and β-diversity). Compartments significantly affected the α-diversity of *P.n.*-associated microbiomes, which is consistent with the result for pine trees in this study, suggesting that compartment is an important influencing factors of plant-associated microbial diversity in both herbaceous and woody species. The α-diversity in different compartments of *P.n.* showed that soil microbiomes were greater than endophytes, and endophytes showed *P.n.* root bacteria were more abundant and leaf fungi were more abundant. Moreover, the hierarchical filtering effect of plants was significant in *P.n.*-associated bacteria but not in fungi, and it is possible that endophytic fungi are more relevant to bioactive metabolites in medicinal plants (Jalgaonwala et al., 2011; Jia et al., 2016). In addition, compartment significantly affected the community structure of *P.n.*-associated microbiomes, which is consistent with previous results obtained in medicinal plants such as *Panax ginseng*, *Macleaya cordata,* and *Pseudowintera colorata* (Chowdhury et al., 2017; Purushotham et al., 2020; Lei et al., 2021) and may stem from the fact that the synthesis and transformation of secondary metabolites in different organs of medicinal plants are closely related to endophytes (Zhao et al., 2016; Song et al., 2017a).

Pine species, as a factor second to compartment had no significant effect on the diversity of microbiomes (bacteria and fungi) of *P.n.*, but changed the community structure of *P.n.* fungi. The differences in community richness (Chaos index) of *P.n.*-associated microbiomes in different pine species systems indicated that pine species changed rare populations rather than abundant microbial species (Shannon, 1948; Chao and Yang, 1993). Previous studies showed that tree rows in agroforestry systems significantly affected fungi rather than bacteria (Beule and Karlovsky, 2021), which is consistent with this study. This may be because soil fungi are more sensitive to changes in plant litter than bacteria (Yang et al., 2017), and bacteria are more resistant to disturbance than fungi in terms of structure, diversity, and biomass (Uroz et al., 2016). Additionally, endophytic bacteria are more easily influenced by the host plants than the soil source, and therefore plant-associated bacteria are more stable (Bonito et al., 2014).

P.n. genotype did not significantly affect the diversity and community structure of *P.n.*-associated microbiomes, which is consistent with the results obtained from previous studies on the assembly of quinoa-associated microbiomes (Cai et al., 2020). Some studies showed that the effect of host genotype on microbial community structure was more pronounced when plants have distant phylogenetic affiliations (Brassicaceae and Poaceae, for example) (Glynou et al., 2018), whereas the two *P.n.* genotypes in this study differ slightly, so the effect of genotype was limited.

Pine and *P.n.*-associated microbial assembly driven by different factors

Microbial assembly associated with *P.n.* and pine were influenced by different factors. Correlation analysis and RDA analysis showed that the main influencing factor for both pine and *P.n.* bacterial assembly was total potassium in the soil, while those for fungal community assembly of pine and *P.n.* were plant N and soil water content. Water content affects the community structure of fungi and has been verified in many studies (Kaisermann et al., 2015; Supramaniam et al., 2016), and some microbiomes involved in the potassium cycle related to potassium may play an important role in plant potassium uptake (Meena et al., 2014). Additionally, plant microbial community composition was significantly corrlated not only with soil N content (Harrison et al., 2007; Lagomarsino et al., 2007; Farrer and Suding, 2016), but also with plant N, because fungi can assist plants in accessing soil N (Adesemoye et al., 2008; Hardoim et al., 2015). However, bacterial community assembly of pine was also affected by ammonium nitrogen and soil water content, while *P.n.* bacterial assembly was influenced by nitrate nitrogen and plant water content. Previous studies showed that variations in soil water content were more likely to affect the bacterial community associated with oaks than with grasses (Fierer et al., 2003), which is consistent with this study. This is probably because bacteria associated with woody plants are less exposed to drought stress (Fierer et al., 2003), while *P.n.* as an understory herb itself is less susceptible to water loss (Chen and Cao, 2014), and the associated bacteria are more sensitive to the water content. The nitrogen preferences of pine- and *P.n.*-associated bacteria may stem from the different choices of woody and herbaceous plants in decomposing and utilizing nitrogen sources. Pine, as a coniferous species, has a large accumulation of lignin and secondary metabolites in the understory layer, which limits nitrogen nitrification (Peng et al., 2006), whereas herbaceous plants prefer to absorb nitrate nitrogen, a directly available nitrogen source (Bedell et al., 1999).

Composition of microbiomes associated with pine trees and *P.n.* under agroforestry systems

Land conversion did not change the community composition of pine- and *P.n.*-associated microbiomes. The pine-associated bacteria were mainly composed of Proteobacteria, Actinobacteria, Acidobacteria, and Chloroflexi, while the pine-associated fungi were mainly composed of Basidiomycota and Ascomycota, which was consistent with the results obtained by previous studies on

pine-dominated forest soil microbiomes (Jiang et al., 2021; Li et al., 2021). The different compartments of the pine trees presented a greater proportion of the Proteobacteria in the roots and Actinobacteria in the soil. Probably because Proteobacteria, as a fast-growing eutrophic group in bacteria, can survive sufficient instability and rapidly propagate in substrates, its relative dominance is particularly pronounced in the root, an important organ of plant nutrient uptake (Lundberg et al., 2012; Edwards et al., 2015; Zhang et al., 2016). In contrast, actinomycetes were able to assist in the decomposition of the more massive plant litter through hyphae, and soil is more conducive to their reproduction than the internal plant environment (Dang et al., 2017). Additionally, the Ascomycota abundance of the pine fungi increased, while the Basidiomycota abundance decreased. This may be because of an increase in the herbaceous component of the plant litter and a relative decrease in the low-quality litter components with high lignification during land conversion (Purahong et al., 2016).

In addition, bacteria associated with *P.n.* included Proteobacteria, Actinobacteria, and Acidobacteria, while fungi mainly included Basidiomycota and Ascomycota. Ascomycota was dominant in the endosphere of *P.n.*, and the results were consistent with previous studies in which the most abundant genera of endophytic fungi of 29 medicinal herbs were all Ascomycota. This may be because the synthesis of antioxidant and antimicrobial active metabolites in medicinal herbs is associated with some species of Ascomycota (Huang et al., 2008).

Possible transmission of endophytes between pine and *P.n.* in *P.n.*-pine agroforestry systems

There were new taxa of endophyte species added to pine trees in the *P.k./P.a.* -*P.n.* agroforestry systems. The increased species of endophytes of *P.k.* and *P.a.* share common populations with endophytes of *P.n.* (bacteria: 73.9, 93.3%, fungi: 68.75, 93.9%). The results showed that the endophytes (roots, stems, and leaves) of *P.n.* spread to the pine roots, which is consistent with the *Calliandra calothyrsus-Phaseolus vulgaris* agroforestry system and *Spartina alterniflora-Kandelia obovata* agroforestry system (Ingleby et al., 2007; Hong et al., 2015). This is because interplant interactions are critical for shaping the plant flora (Hong et al., 2015). Additionally, the existence of complex plant-microbial-plant networks within agroforestry systems formed by interplanting trees and crops can significantly reshape the composition of endophytes (Mei et al., 2022). We speculated that most of the transferred taxa are opportunistic endophytes, which have a certain probability of vertical transmission by factors such as plant internal factors (material transport, metabolism) and environmental factors (rain, wind) or horizontal diffusion (Tadych et al., 2007; Hardoim et al., 2015).

In addition, the endophyte that *P.n.* transferred to pine trees include multiple species of beneficial microbiomes. Some bacterial strains of Massilia and Marmoricola have the capacity to produce IAA, provide siderophores and antagonize pathogenic microbes *in vitro* (Ofek et al., 2012; Jiang et al., 2017), and strains of Herbaspirillum, Tardiphaga and Telmatospirllum are involved in biological nitrogen fixation (Monteiro et al., 2012) and slow-growing nitrogen fixation and participate in the sulfur cycle (Perley and Stowe, 1966; Hausmann et al., 2018), respectively. The increased beneficial fungi included Umbelopsis, Xylara, Geminibasidium, Inocybe, Alatospora, and Pleotricholadium, with the functions of participating in the transformation of lipids and polysaccharides (Dourou et al., 2017), synthesizing antioxidant active compounds (Liu et al., 2007), participating in the carbon and nitrogen cycle (Pulido-Chavez et al., 2021) and decomposing and utilizing functions of organic matter (Feckler et al., 2017; Wang et al., 2021). In summary, these beneficial microbiomes were delivered by *P.n.* to pine trees were favorable to the growth of pine trees. Therefore, the agroforestry organic economic cultivation of *P.n.*-pine trees is sustainable.

Conclusion

In conclusion, the conversion of the two pure pine forests (*P.k.* and *P.a.)* to the *P.n.*-pine agroforestry systems significantly changed the diversity, but not the community structure, of pine-associated fungi. The community structure, but not the diversity, of *P.n.*-associated fungi was significantly changed. Fungi were more sensitive to alterations in both plant-associated factors than bacteria. Main effect analysis showed that compartment but not genotype was the driving factor affecting *Panax notoginseng* and pine, but *Panax notoginseng* cultivation also significantly influenced the assembly of pine related microbiomes. In addition, a diffuse spread of *P.n.* endophytes into the pine roots, and beneficial microbiomes (Massilia, Marmoricola, Herbaspirillum, etc.) increased in pine roots. Therefore, the different assembly mechanisms of *Panax notoginseng* and pine microbiomes functioned as an important role in the *Panax notoginseng*-pine agroforestry systems and were the basis for the sustainable development of the *Panax notoginseng*-pine agroforestry systems.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/, PRJNA821648 and PRJNA821834.

Author contributions

WJ: methodology, formal analysis, investigation, data curation, writing - original draft, writing - review and editing, and visualization. SW: methodology, formal analysis, conceptualization, investigation, writing - original draft, and writing - review and editing. XH: project administration, funding acquisition, conceptualization, and writing - review and editing. XZ: methodology, investigation, and writing -review and editing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1018989/full#supplementary-material

SUPPLEMENTARY TABLE S1
Vegetation type of two different pine forests.

SUPPLEMENTARY TABLE S2
PerMANOVA (Permutational multivariate analysis) indicated the relative contributions of genotype (G), planting *P.n.* or not (Pn) and their interactions on bacterial and fungal alpha- diversity among different compartments (bulk and rhizosphere soils, and root of pine trees). Significant effects ($p<0.05$) are shown in bold. $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

SUPPLEMENTARY TABLE S3
Pearson correlations (r value) between bacterial and fungal α diversity of pine-associated microbes (Shannon, Chao 1, Shannoneven) and plant and soil variables among different compartments (bulk, rhizosphere soil and roots of pine trees). Significant effects ($p<0.05$) are shown in bold. Abbreviations: F-sha, shannon index of fungi; F-Chao1, Chao1 of fungi; F-even, shannoneven index of fungi; B-sha, shannon index of bacteria; B-Chao1, Chao1 of bacteria; B-even, shannoneven index of bacteria; STK, soil total potassium; STN, soil total nitrogen; STP, soil phosphorous; NH_4^+-N, soil ammonium nitrogen; NO_3^--N, soil nitrate nitrogen; SWC, soil water content; EC, soil conductivity.

SUPPLEMENTARY TABLE S4
PerMANOVA (Permutational multivariate analysis) indicated the relative contributions of genotype (G), pine tree species (Ps) and their interactions on bacterial and fungal alpha diversity among different compartments (rhizosphere soils, roots, stems, leaves of *P.n.*). $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

SUPPLEMENTARY TABLE S5
Pearson correlations (r value) between bacterial and fungal α-diversity of *P.n.*-associated microbes (Shannon, Chao 1, Shannoneven) and plant and soil variables. Significant effects ($p<0.05$) are shown in bold. Abbreviations of soil variables are as defined in **Supplementary Table S3**. Abbreviations: F-sha, shannon index of fungi; F-Chao1, Chao1 of fungi; F-even, shannoneven index of fungi; B-sha, shannon index of bacteria; B-Chao1, Chao1 of bacteria; B-even, shannoneven index of bacteria; PWC, plant water content; P_Fw, plant fresh weight; P_Dw, plant dry weight; PTN, plant total nitrogen; PTP, plant total phosphorous.

SUPPLEMENTARY TABLE S6
PerMANOVA based on WUF revealing the relative contributions of genotype (G), compartment (C) and planting *P.n.* or not (Pn) on bacterial and fungal variations across all samples and in each compartment of pine-associated microbes. Significant levels: $p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

SUPPLEMENTARY TABLE S7
PerMANOVA based on WUF revealing the relative contributions of genotype (G), compartment (C) and pine tree species (Ps) on bacterial and fungal variations across all samples and in each P.n.-asscioated compartment (rhizosphere, root, stem, leaves). Significant levels: $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$.

SUPPLEMENTARY FIGURE S1
PCoA plots based on WUF (weighted unifrac distance) of bacterial and fungal OTU showing the variation in each compartment of pine-associated microbes (root, rhizosphere, between).

SUPPLEMENTARY FIGURE S2
PCoA plots based on WUF of bacterial and fungal OTU showing the variation in each compartment of *P.n.*-associated microbes (rhizosphere, root, stem, leaves).

SUPPLEMENTARY FIGURE S3
Venn Diagram exhibiting the overlap among the *P.n.* endophyte and the pine root endophyte of *P.n.*-pine agroforestry systems and the root endophyte of pure pine forests.

SUPPLEMENTARY FIGURE S4
Student *t*-test bar plots for different compartments of bacterial community of *P.k.* on genus level. PP, pure pine forest, GP, agroforestry system.

SUPPLEMENTARY FIGURE S5
Student *t*-test bar plots for different compartments of bacterial community of *P.a.* on genus level. PP, pure pine forest, GP, agroforestry system.

SUPPLEMENTARY FIGURE S6
Student *t*-test bar plots for different compartments of fungal community of *P.k.* on genus level. PP, pure pine forest, GP, agroforestry system.

SUPPLEMENTARY FIGURE S7
Student *t*-test bar plots for different compartments of fungal community of *P.a.* on genus level. PP, pure pine forest, GP, agroforestry system.

SUPPLEMENTARY FIGURE S8
Student *t*-test bar plots for different compartments of fungal community of *P.n.* on genus level. Pk, *P.n.-P.k.* agroforestry system, Pa, *P.n.-P.a.* agroforestry system.

SUPPLEMENTARY FIGURE S9
Student *t*-test bar plots for different compartments of fungal community of *P.n.* on genus level. Pk, *P.n.-P.k.* agroforestry system, Pa, *P.n.-P.a.* agroforestry system.

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EDITED BY
Xin Sui,
Heilongjiang University, China

REVIEWED BY
Xinzhen Wang,
Institute of Genetics and Developmental Biology (CAS), China
Zhihuang Xie,
Guangdong Academy of Agricultural Sciences (GDAAS), China
Wenxuan Mai,
Xinjiang Institute of Ecology and Geography (CAS), China
Meiyan Yang,
South China Agricultural University, China

*CORRESPONDENCE
Zhao Yang
hljyangzhao@163.com

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Land use differentially affects fungal communities and network complexity in northeast China

Yanxia Xu, Zhao Yang*, Xiaolong Wang, Hua Chai, Shasha Li, Yue Wu and Ruoding Wang

Branch of Animal Husbandry and Veterinary of Heilongjiang Academy of Agricultural Sciences, Qiqihar, China

Background: The soil fungal community is one of the most important drivers of the soil nutrient cycling that sustains plant growth. However, little research has been done on the effects of different land uses on soil fungal communities in northeast China.

Methods: In this study, we conducted a field experiment to investigate the effects of continuous cropping of grass, maize, and alfalfa on their respective fungal communities and co-occurrence networks.

Results: We showed that the physicochemical properties of the soil, such as nitrate (NO_3^-N), available phosphorus, and soil pH, were the most important driving factors affecting the structure of the soil fungal community in different cropping systems. In addition, compared to the cultivation of grass and maize, the continuous cropping of alfalfa increased the abundance of several beneficial as well as pathogenic species, such as Mortierella and Gaiellales. In addition, the networks differed among plant species and according to the number of years of continuous cultivation.

Conclusion: This suggests that the continuous cropping of alfalfa results in greater cooperation among fungi, which may be beneficial to the soil as well as to the development of the alfalfa.

KEYWORDS

land use, continuous cropping and alfalfa, grass, maize, fungal network

Introduction

Alfalfa (*Medicago sativa L.*) is a leguminous, perennial plant of great importance for livestock and agriculture and is therefore widely grown in many countries and regions (Han et al., 2005; Raiesi, 2007). The arid regions of northeast China are the main areas where alfalfa is grown. Due to the climatic specificity of the long winters in northeast China, livestock in the region rely heavily on summer pasture storage for forage (Chen et al., 2013). Alfalfa has a high yield and a comprehensive range of nutrients and can therefore reduce forage shortages for herbivores in winter (Su, 2007; Chen et al., 2013). As a result, perennial alfalfa is grown year after year in the region to meet the winter demand for fodder and to increase livestock productivity (Dong et al., 2003). However, this type of agricultural intensification has led to a loss of biodiversity

(Sala et al., 2000; Romdhane et al., 2022). Moreover, the number of pathogenic microorganisms increases with the continuous planting of alfalfa, eventually leading to a decrease in yield, a phenomenon that is closely linked to soil microorganisms (Yan et al., 2012; Yao et al., 2019; Liu et al., 2020).

The number of years that alfalfa is grown is related to its productivity. Generally, alfalfa yields increase with the number of years planted; however, yields begin to decline when they reach a certain critical year, usually considered to be the 9th year (Jiang et al., 2007; Li and Huang, 2008). In addition, continuous alfalfa cultivation significantly alters the physicochemical properties of the soil, which is significantly associated with yields (Ren et al., 2011). Previous studies have shown that planting alfalfa increases the organic matter and nitrogen content of the soil compared to virgin sandy soils. In addition, soil nutrients such as organic matter, nitrogen, and phosphorus increase with the number of continuous planting years. However, previous research has shown that soil nutrients tend to decrease after 10 continuous years of alfalfa cultivation (Jiang et al., 2007; Dong et al., 2016; Luo et al., 2018).

Soil microorganisms serve a crucial function in the maintenance of plant health by interacting with plants, participating in nutrient uptake and resisting stress, and responding rapidly to changes in the physical and chemical characteristics of the soil (Song et al., 2021). Differences in tillage systems, soil types, crop species, and cropping systems greatly influence the structure of the soil's microbial community (Zhou et al., 2018; Yao et al., 2019; Yuan et al., 2021). For example, one study observed that soil microbial biomass declined in the short term, but ultimately increased over the long term, in a context of continuous alfalfa cultivation (Jiang et al., 2007). Another study reported that continuous cultivation of alfalfa changed the microbial diversity by altering the physicochemical properties of the soil (Luo et al., 2018). Some studies have shown that continuous alfalfa cultivation can increase the relative abundance of *Paecilomyces phaeomycocentrospora* and *Fusarium* sp. and decrease the relative abundance of *Penicillium* sp. (Xu et al., 1995; Yao et al., 2019). However, other studies have found no effect of continuous alfalfa cultivation on soil microbiota structure (Hu and Wang, 1996). These different results might be attributed to heterogeneity among the soil types, sample collection times, and tillage systems used in these studies. Therefore, there is a need for more in-depth studies to investigate the barriers to continuous alfalfa cultivation under intensive tillage patterns.

Co-occurrence network analysis is a useful tool for exploring microbial associations and obtaining key information on microbial co-abundance communities associated with soil functions (Banerjee et al., 2018; Fan et al., 2021). One recent study used symbiotic networks to demonstrate that members of network modules were significantly associated with the genes involved in nutrient cycling after long-term fertilization, and that the number of members (operational taxonomic units, OTUs) in each module, rather than overall microbial diversity, influenced soil function (Fan et al., 2021). This raises the question of whether different land use practices alter the topological structures of networks, and what potential effects an altered network structure may have on soil function.

Here, we examined the influence of continuously cultivating grass, maize, and alfalfa (for 6, 10, 14, 20, and 30 years) on soil microbes and soil characteristics. Because grass grows without human intervention, we hypothesized that alfalfa and maize would have higher microbial diversity than grass, and that continuous planting of alfalfa would increase the complexity of the co-occurrence. The aims of this study were to explore the various changes in the structures of soil fungal communities across different cropping systems, and to assess the association between the physical characteristics of soil and the characteristics of its fungal community.

Materials and methods

Experimental site and design

The experimental site was located in Qiqihar, Heilongjiang Province, China. The experiments were carried out on natural meadow (Me), maize (Ma), and alfalfa (AC) in consecutive cultivation periods of different lengths: 6, 10, 14, 20, and 30 years, labeled C6, C10, C14, C20, and C30, respectively. Each treatment area was approximately 900 m^2. Each summer, a compound fertilizer (N 18%, P_2O_5 18%, and K_2O 20%) of 18 kg mu^{-1} was applied to each treatment area, and the alfalfa was cut to the surface around July of each year.

Soil sampling and measurement of soil characteristics

In total, 42 soil samples were collected with the z-stamping method at the end of June 2019. The plant roots and stones were filtered out using a 2-mm sieve. Soil samples of about 2 g each were filled into centrifuge tubes and then stored at −80°C for the DNA extraction and follow-up process. The remaining soil was stored at 4°C for testing of its physical and chemical properties. The pH of the soil was measured using a pH meter in a soil–water suspension (1:5 w/v). The total soil carbon and nitrogen contents were determined using an elemental analyzer (Jones and Willett, 2006). Nitrate (NO_3^--N) and ammonium (NH_4^+-N) were extracted using 2.0 M potassium chloride and determined in a continuous flow analysis system. Furthermore, 0.5 M H_2SO_4-$HClO_4$ and $NaHCO_3$ were used to extract available and total phosphorus, respectively. HNO_3-$HClO_4$-HF and CH_3COONH_4 were used to extract total and available soil potassium, respectively, and two forms of potassium were determined using inductively coupled plasma emission spectrometry (ICPS-7500) (Lu, 1999).

DNA extraction and sequencing

The Fast DNA Spin Kit (MP Biomedicals, USA) was used to extract total soil DNA. Fungal genes were amplified using the primers of ITS1 and ITS2 (Shi et al., 2020). PCR was performed using a 25 ml PCR mixture containing 10 ng DNA template, 10 mM of each primer, and 22 ml Platinum PCR SuperMix. The PCR program was 94°C for 4 min; 94°C for 20 s, 56°C for 10 s, 72°C for 15 s for 28 cycles; and 75°C extension for 10 min (Liu et al., 2015). Sequencing was performed on the Illumina MiSeq platform at Majorbio BioPharm Technology. The raw sequencing data were deposited in the NCBI BioProject, under accession number PRJNA890435.

Raw sequence data were processed using QIIME, version 1.17 (http://qiime.org/). PCR primer sequences and low-quality reads (length $<$ 200 bp and mean quality score $<$ 30) were trimmed using preliminary analyses. Sequence chimeras were removed using the UCHIME algorithm (Edgar et al., 2011). The sequences were then classified as operational taxonomic units (OTUs) using CD-HIT, with 97% similarity. Moreover, the trimmed sequences were phylogenetically assigned based on sequence alignment using RDP taxonomy with the UNITE database, version 7 (Cole et al., 2009; Li and Godzik, 2015).

The Shannon and Chao1 indices were calculated in QIIME. In addition, the canonical correspondence analysis, principal coordinate analysis (PCoA), and adonis test were performed using the "vegan" package, version R4.2.3. Using GenStat 13, one-way ANOVA was performed to analyze the differences in physicochemical properties of the soil samples and the relative abundances of various fungal genera. Fungal co-occurrence network analysis was performed for the fields of Me, Ma, and AC and for the treatments of AC6, AC10–20, and AC30. The OTU data were analyzed statistically in R and visualized in Gephi using the "psych" package (Jiang et al., 2017). The correlation between any two OTUs had to have a $p < 0.05$, and a Spearman's correlation coefficient of 0.7 or greater (Shi et al., 2020).

Results

Physicochemical characteristics of the soil

Compared to Me and Ma, AC had higher levels of soil nitrate, total potassium, available potassium, and pH, and lower levels of total nitrogen, total phosphorus, available phosphorus, and ammonium. In terms of the continuously cropped alfalfa, the soil nitrate, total potassium, total carbon, and nitrogen increased along with the number of years of continuous cropping, while ammonium, total phosphorus, and available phosphorus showed the opposite trend.

Soil fungal diversity changed with different treatments

Based on the results of the Shannon and Chao1 indices, the Me and AC30 treatments had the lowest and the highest fungal diversity, respectively (Figures 1A,B). Regarding the beta diversity, different cropping systems showed significantly different fungal community structures, according to the PCoA

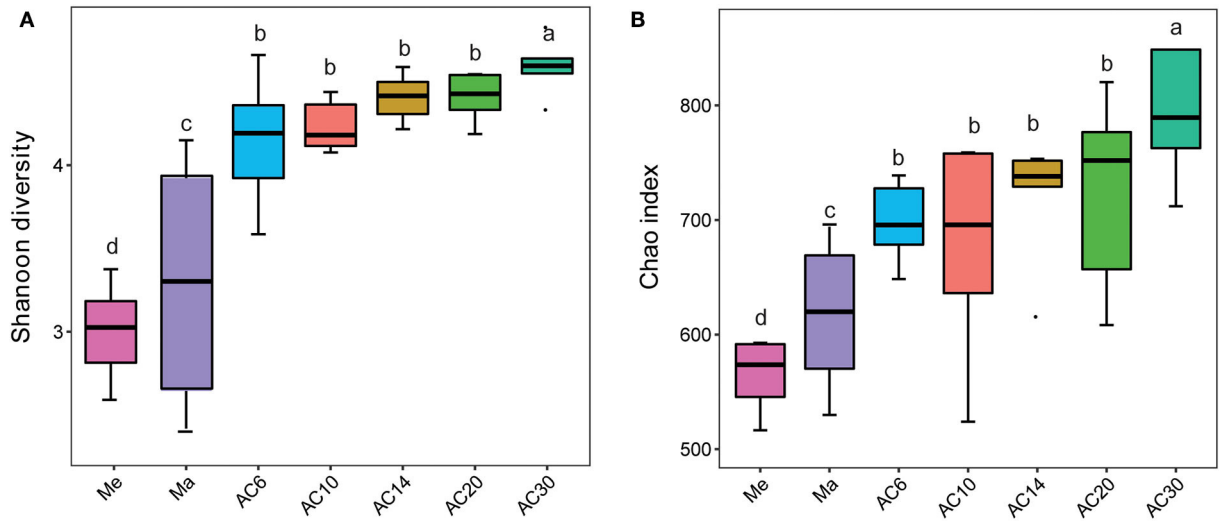

FIGURE 1
(A) Fungal Shannon diversity and **(B)** Chao index. The a–d letters indicate the significance of difference at $p < 0.05$ level.

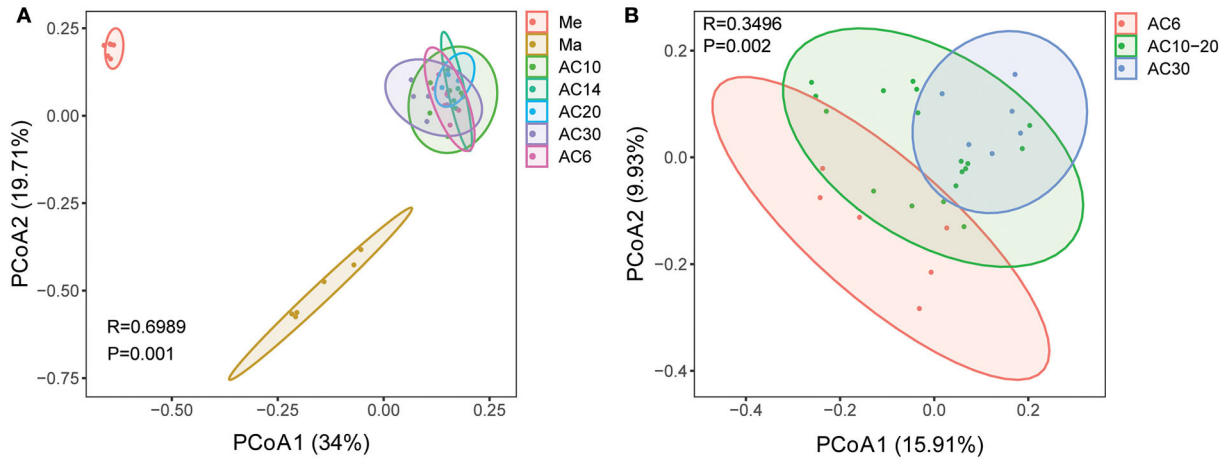

FIGURE 2
Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarities in the soil among different crop types **(A)** and different lengths of continuous cropping **(B)**.

(PERMANOVA, $p < 0.05$) and adonis analysis (Figure 2A, Table 1). Moreover, the continuous cropping of alfalfa also had a significant influence on the fungal community structure (PERMANOVA, $p < 0.05$) (Figure 2B). Based on the PCoA results, all treatments were able to be divided into three distinct groups, i.e., Me, Ma, and AC6–30 (PERMANOVA, $p < 0.05$) (Figure 2A). We further performed PCoA on the samples from the continuous alfalfa planting and found that these samples could also be divided into three distinct groups, i.e., AC6, AC10–20 (i.e., 10, 14, and 20 years of continuous planting), and AC30, respectively (Figure 2B, Table 2). Moreover, using CCA analysis, we found that there was a significant association between the fungal community composition and soil characteristics (Figure 6). In detail, pH ($r = 0.356$; $p = 0.03$), total carbon ($r = 0.553$; $p = 0.014$), carbon:nitrogen ratio ($r = 0.546$; $p = 0.03$), nitrate ($r = 0.691$; $p = 0.04$), total potassium ($r = 0.657$; $p = 0.03$), available potassium ($r = 0.564$; $p < 0.01$), and available phosphorus ($r = 0.543$; $p < 0.01$) showed statistically significant associations with the fungal community composition.

TABLE 1 Effects of crop types, alfalfa continuous cropping times, and their interaction on the structure of soil fungal communities, based on adonis analysis.

Factor	r	*p*
Crop type (CT)	0.785	0.001***
Continuous years (CY)	0.624	0.02*
CT × CY	0.751	0.021*
Pairwise comparison		
Me vs. Ma	0.865	0.001***
Me vs. AC	0.763	0.001***
Ma vs. AC	0.567	0.003**
AC6 vs. AC10–20	0.529	0.02*
AC6 vs. AC30	0.688	0.001***
AC10–20 vs. AC30	0.587	0.002**

The *, **, and *** indicates the values of $p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively.

Specific fungal taxa changed with different treatments

Across all the treatments, *Ascomycota*, *Basidiomycota*, and *Mortierellomycota* were the dominant phyla, accounting for 93.45–96.32% of the whole community (Figure 3). In general, the relative abundance of *Basidiomycota* was much higher in the Me field compared with that in the Ma and AC fields, while the abundance of *Ascomycota* and *Mortierellomycota* was much higher in the AC treatments than in the Me and Ma fields. We then used the Kruskal–Wallis H-test to analyze different taxa on the genus level. Some genera, such as *Tausonia*, *Mortierella*, *Talaromyces*, *Gibberella*, *Fusarium*, and *Schizothecium*, showed significant difference among the various treatments ($p < 0.05$). In addition, other genera, such as *Metarhizium*, *Phaeomycocentrospora*, *Lectera*, *Beauveria*, *Didymella*, and *Schizothecium*, showed significant differences according to the number of years of continuous alfalfa cropping (Figure 4). Furthermore, the AC fields had a significantly higher relative abundance of *Mortierella*, *Gibberella*, *Solicoccozyma*, *Metarhizium*, and *Phaeomycocentrospora* compared with the other two fields and a lower relative abundance of *Tausonia, Talaromyces, Fusarium, Schizothecium, and Pseudobrophila* (Figure 4A). The relative abundance of *Metarhizium, Phaeomycocentrospora,*

TABLE 2 Network topological characteristics of the different treatments.

Network metrics	Me	Ma	AC	AC6	AC10–20	AC30
Number of nodes	160	150	191	142	182	199
Number of edges	273	304	453	219	270	258
Number of positive correlations	243	291	369	208	246	230
Number of negative correlations	30	50	84	11	24	28
Average degree (avgK)	3.413	4.053	4.743	3.085	2.967	2.593
Average weighted degree	3.32	5.343	3.326	3.544	2.711	2.06
Network diameter	4	3	11	3	11	1
Graph density	0.021	0.027	0.025	0.022	0.016	0.013
Modularity (M)	0.967	1.256	1.371	1.033	1.16	3.556
Interconnecting piece	95	78	62	95	117	153
Average clustering coefficient (avgCC)	0.9	0.919	0.574	0.902	0.701	0.883
Average path length (APL)	1.291	1.222	4.255	1.215	4.336	1

Beauveria, and *Monocillium* decreased with the number of years of continuous alfalfa cropping, while *Chaetomium, Titeae, Schizothecium,* and *Microdochium* showed the opposite trend (Figure 4B).

Co-occurrence network in different treatments

The co-occurrence network displays the relationship between the fungi in various treatments based on the OTU level (Figure 5). Neither the average degree (avgK) nor the clustering coefficient (avgCC) showed a significant difference among the treatments. The network modularity and the number of negative correlations were ranked as AC > Ma > Me. In a comparison of the AC6, AC10–20, and AC30 treatments, the negative correlations, modularity, and avgCC increased with the number of years of continuous cropping.

Discussion

In the present study, fungal diversity was higher in the AC treatment than in the Me or Ma treatments, and the fungal diversity of alfalfa soils increased with subsequent years of planting. This result is in line with our first hypothesis, which suggests that alfalfa has more microbial species than grass and maize, and that continuous alfalfa planting is more beneficial to fungal diversity maintenance and soil sustainability, at least in terms of fungal diversity. Previous studies have found that continuously planted soybean has less soil microbial diversity than corn–soybean rotation systems (Liu et al., 2020). The number of years of continuous planting is also related to the microbial diversity of the soil (Liu et al., 2020). However, other studies have found no difference in microbial diversity between soils farmed continuously with soybean and soils farmed continuously with soybean–corn rotations (Li et al., 2010). These inconsistent results might be based on the soil types and the number of years of repeated harvests. Alterations in plant genotype may also be responsible for this result, given that microbial diversity has been shown to exhibit diverging trends in the context of successive cultivation of resistant and vulnerable cultivars (Yuan et al., 2021). Organic acids, phenols, and other compounds found in plant root exudates have an influence on microbial diversity in a range of agricultural situations (Tan et al., 2017; Lian et al., 2019; Liu et al., 2020; Shi et al., 2020). Furthermore, soil pH influences other soil characteristics that can directly or indirectly affect microbial diversity (Lian et al., 2019).

Regarding the beta diversity, the results from the principal coordinate analysis showed that crop types and continuous tillage time were the two most important factors affecting the structure of the soil fungal community ($p < 0.05$). Every species of plant releases a specific set of metabolites during growth, and this in turn allows its root system to provide a unique habitat for, and host different types of, soil fungal microorganisms. These microbes may also help plants absorb nutrients and resist stresses (Lian et al., 2019).

Moreover, our results were also consistent with previous studies that found that continuous crop planting also affects soil fungal community structure (Zhu et al., 2017; Yao et al., 2019; Yuan et al., 2021). This is mainly due to the effect of root exudates on soil microorganisms. For example, long-term continuous cultivation of soybean can lead to the accumulation of organic acids in the soil and ultimately cause soil acidification. This provides an ideal environment for pathogenic fungi to survive and alters the community structure of soil fungi, ultimately leading to reduced soil quality and crop yields (Zhu et al., 2017; Lian et al., 2019; Yuan et al., 2021).

Furthermore, according to the CCA results, the physicochemical characteristics of our soil samples, such

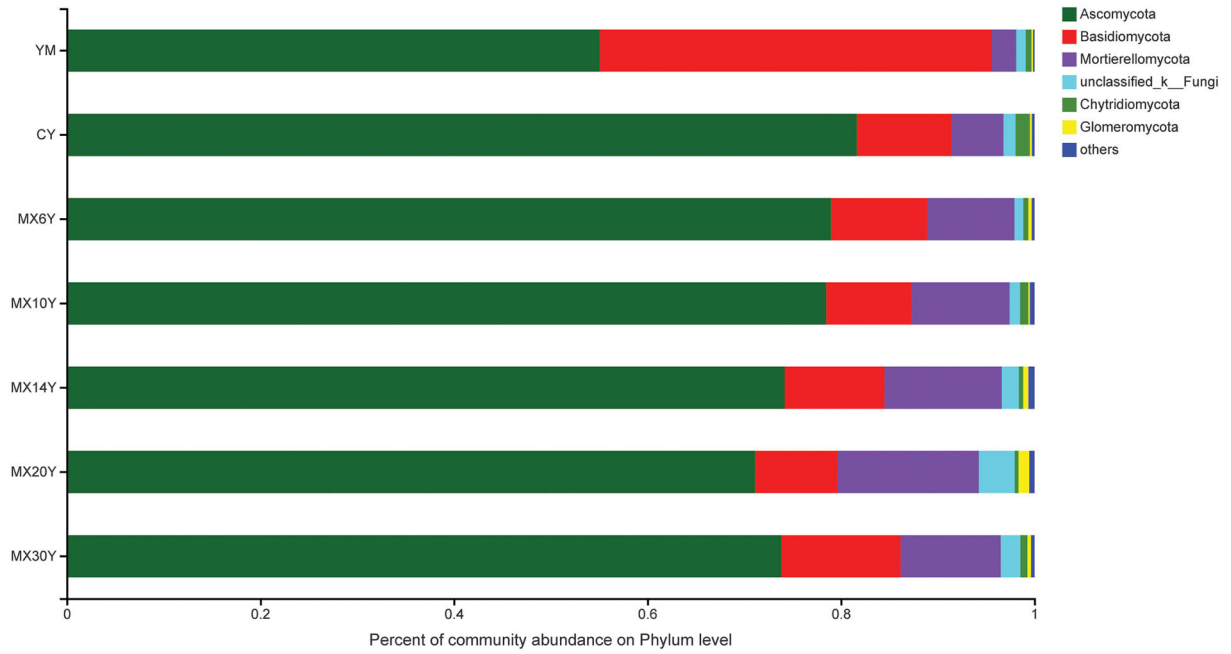

FIGURE 3
Composition of the fungal phylum level.

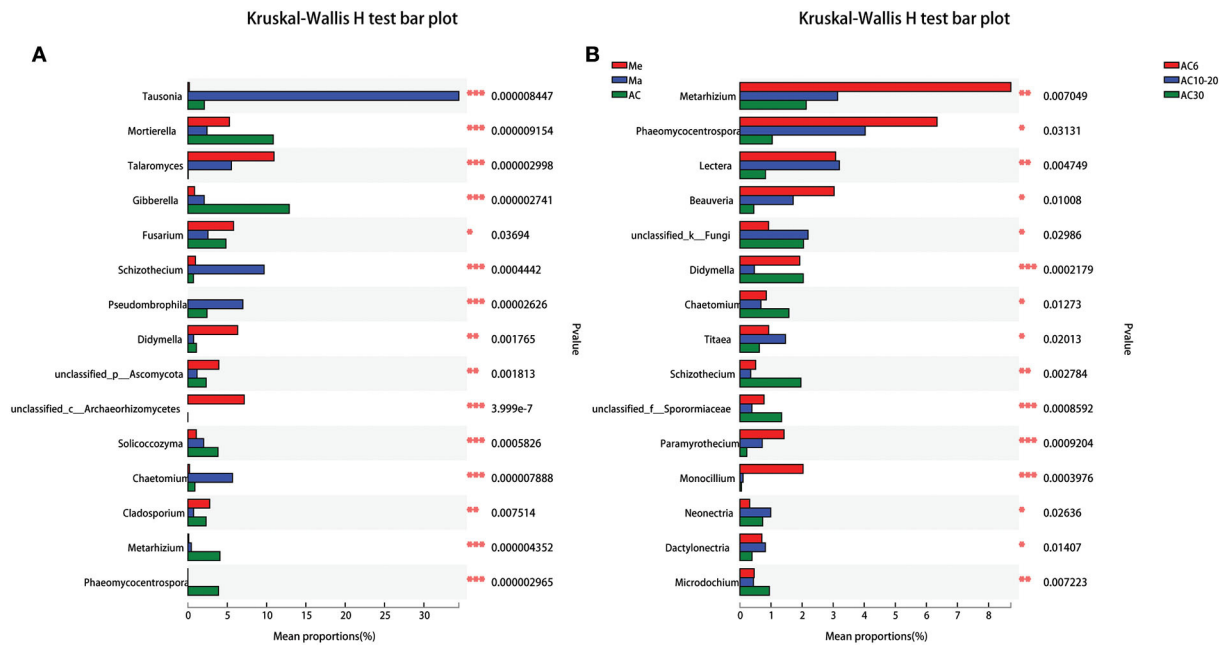

FIGURE 4
Effect of crop types **(A)** and continuous cropping years **(B)** on fungal genera.

as NO_3^-N, available phosphorus, and soil pH, were the main factors that altered the structure of the soil fungal community in the different treatments (Figure 6). This is similar to the findings of many previous studies, in which changes in tillage practices were shown to affect the microbial environment by altering the soil characteristics (Lian et al., 2019; Yao et al., 2019). Over all, our results also suggest that some important soil parameters changed significantly through

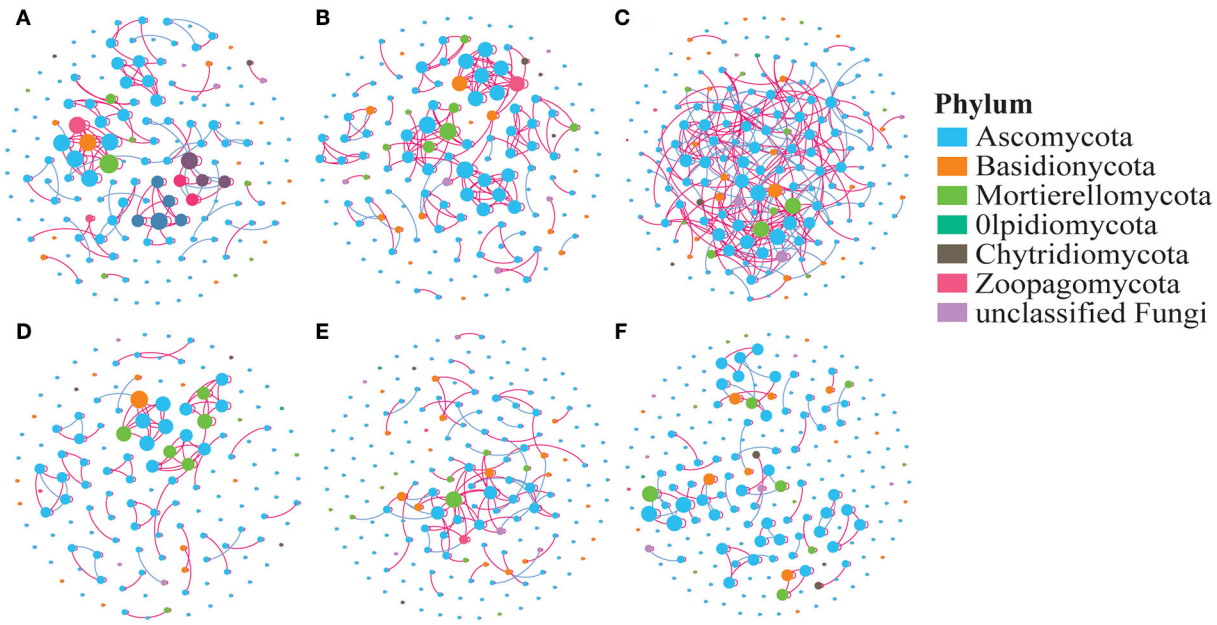

FIGURE 5
Co-occurrence network of the soil fungal community. **(A)** Me, **(B)** Ma, **(C)** AC, **(D)** AC6, **(E)** AC10-20, and **(F)** AC30. The nodes represent OTUs, and the edges represent the significant association between each OTU. The connection indicates a strong (Spearman's $r > 0.7$ or $r < -0.7$) and significant ($p < 0.05$) correlation. Red edges: positive connections; green edges: negative connections.

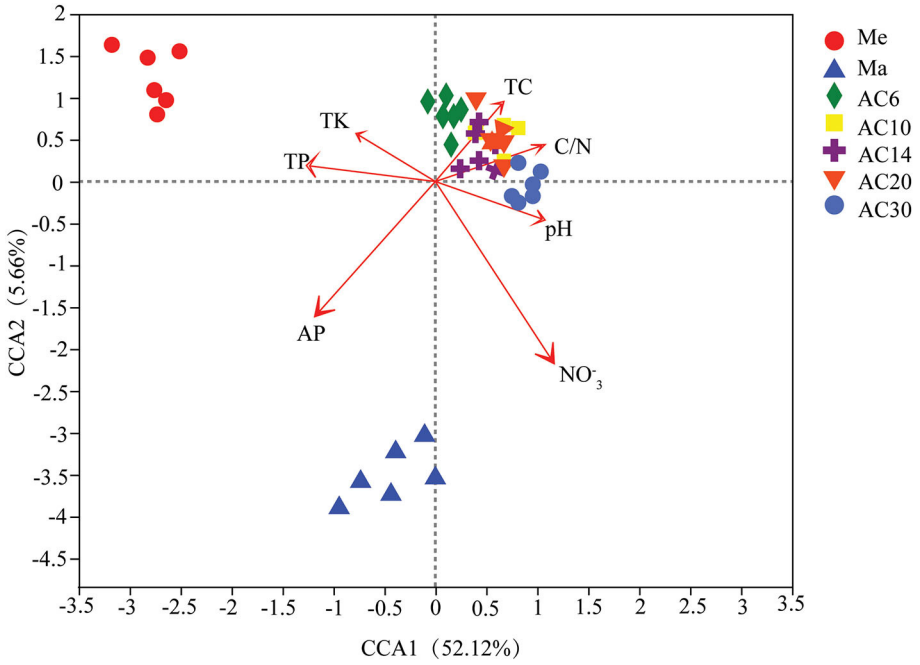

FIGURE 6
Relationship between soil properties and fungal community structure based on canonical correspondence analysis (CCA).

continuous cropping over time, leading to changes in the fungal community. However, this change was not unidirectional, and longer periods of continuous cropping will perhaps lead to more positive developments for the soil microorganisms, such as increased diversity and significant enrichment of the beneficial fungi.

In AC soils, the relative abundance of *Ascomycota* was substantially higher compared to that of soils from the Ma and Mc systems (Figure 4). Many ascomycetes are plant-pathogenic, such as rice blast, black knot, the ergot fungi, and the powdery mildews, which suggests that continuous planting of alfalfa may have increased the abundance of potential pathogens, and influence the growth of alfalfa (Yuan et al., 2021). The relative abundance of *Mortierella*, *Gibberella*, *Solicoccozyma*, *Metarhizium*, and *Phaeomycocentrospora* was increased in AC fields compared to Me and Ma fields. It has been reported that *Mortierella* can survive under very unfavorable environmental conditions and make efficient use of carbon sources contained in polymers such as cellulose, hemicellulose, and chitin, and that it can synthesize phytohormones and 1-aminocyclopropane-1-carboxylic acid deaminase through improved access to bioavailable forms of phosphorus and iron in the soil, thereby protecting agricultural plants from pathogens (Ozimek and Hanaka, 2020). However, some pathogenic microbial species, such as *Gaiellales*, with high relative abundance in the AC treatment, can cause sear rot, suggesting that these fungi may suppress soil diseases, suggesting that these fungi may suppress soil diseases (Gómez Expósito et al., 2015). Therefore, it is likely that the changes in these fungi caused by the different treatments are related to the soil nutrient structure and the antagonistic activity of the plant pathogens.

In the present study, co-occurrence networks have helped us explore the complex relationships between fungi in different treatments in greater depth (Xue et al., 2018; Xiong et al., 2021). Our results show that both negative network correlations and modularity were significantly higher for AC than for Ma and Mc, which is consistent with our second hypothesis. This suggests that successive plantings of alfalfa promoted cooperation between fungi, which may be beneficial for alfalfa survival (Yao et al., 2019; Liu et al., 2020). Moreover, the difference in network topology of these treatmentsmay be due to the fact that certain microbial species are enriched to help the host increase nutrient uptake or resist stress, causing the structure of the microbial community to deviate from its original equilibrium (Lian et al., 2019). However, the results for the fungal networks alone are one-sided, as functional bacteria are also present in the soil. Therefore, in future studies, combined bacterial and fungal network analysis may yield more comprehensive results and a fuller assessment of the effects of different tillage practices on soil microbes.

In conclusion, alfalfa crop cultivation increased the alpha-diversity of soil fungi compared to grass and maize cultivation, and alpha-diversity increased further in continuous cropping systems, which is of great interest to maintain soil microbial diversity. The physicochemical properties of the soil, such as NO_3^--N, soil Ph, and available phosphorus, were the most important driving factors affecting the soil fungal community structure across the different cropping systems. Compared to the cultivation of grass and maize, the continuous cropping of alfalfa increased the abundance of several beneficial as well as pathogenic fungal species, such as *Mortierella* and *Gaiellales*. In addition, the networks differed among plant species and also among different lengths of time of continuous alfalfa cultivation. This suggests that the continuous cropping of alfalfa results in greater cooperation among fungi, which may be beneficial to the soil as well as to the development of the alfalfa.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA890435.

Author contributions

YX and ZY conceived of the presented idea. YX wrote the manuscript. YX, XW, HC, YW, RW, and SL verified the analytical methods. ZY supervised the findings of this work. All authors discussed the results and contributed to the final manuscript.

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Conflict of interest

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

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EDITED BY
Xin Sui,
Heilongjiang University, China

REVIEWED BY
Changjiang Zhao,
Heilongjiang Bayi Agricultural University, China
Chunxiang Liu,
Shandong University of Technology, China
Jing Jin,
South China Agricultural University, China

*CORRESPONDENCE
Xinzhen Wang
xzwang@sjziam.ac.cn

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Application of microalgae *Chlamydomonas applanata* M9V and *Chlorella vulgaris* S3 for wheat growth promotion and as urea alternatives

Mekiso Yohannes Sido[1,2], Yinping Tian[1], Xiaogai Wang[3] and Xinzhen Wang[1]*

[1]Key Laboratory of Agricultural Water Resources, Hebei Key Laboratory of Soil Ecology, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, China, [2]College of Agriculture, Wachemo University, Hosaena, Ethiopia, [3]School of Life Science and Engineering, Handan University, Handan, China

Excessive use of chemical fertilizers to meet the global food demand has caused extensive environmental pollution. Microalgae can be used to enhance agricultural crop production as a potentially sustainable and eco-friendly alternative. In this study, *Chlamydomonas applanata* M9V and *Chlorella vulgaris* S3 were isolated from the soil and mass-cultured for use as microalgal fertilizers. The influence of microalgae M9V and S3 on the growth of wheat (*Triticum aestivum* L.) and soil properties was evaluated and compared with that of chemical urea fertilizer. A pot experiment was conducted with six treatments, i.e., living M9V (M9VL), dead M9V (M9VD), living S3 (S3L), dead S3 (S3D), urea fertilizer (urea), and control without fertilizer (control). M9VL was found to have the best effect on wheat growth promotion, followed by M9VD and S3D. In addition, M9VL resulted in the highest enhancement of shoot fresh weight (166.67 and 125.68%), root dry weight (188.89 and 77.35%), leaf length (26.88 and 14.56%), root length (46.04 and 43.93%), chlorophyll *a* (257.81 and 82.23%), and chlorophyll *b* contents (269.00 and 247.27%) comparing to the control and urea treatments, respectively. Moreover, all microalgal fertilizer treatments increased soil organic matter (SOM) by 1.77–23.10%, total carbon (TC) by 7.14–14.46%, and C:N ratio by 2.99–11.73% compared to the control and urea treatments. Overall, this study provided two microalgae strains, M9V and S3, that could promote wheat growth and improve soil properties, thus highlighting the use of microalgae as biofertilizers to reduce the use of chemical fertilizers and promoting sustainable agricultural production.

KEYWORDS

chemical fertilizer, microalgal fertilizer, *Chlamydomonas applanata* M9V, *Chlorella vulgaris* S3, crop growth promotion, urea alternative

Introduction

The global food demand has been increasing rapidly, with a predicted increase of 60–110% in the global crop demand by 2050, so huge are the environmental impacts expected from the increased agricultural production to meet this demand correspondingly (Bruinsma, 2009; Tilman et al., 2011; Odegard and van der Voet, 2014; Rockström et al., 2017; Nascimento et al., 2019). Chemical fertilizers have been used on crops to increase food production for a long time; this increases the input cost of farming, reduces the utilization rate of soil fertilizers, and causes soil agglomeration and hardening, biodiversity loss, and lower productivity. This will seriously affect the sustainable and stable development of agricultural production (Garcia-Gonzalez and Sommerfeld, 2016; Rahman and Zhang, 2018). In addition, the large amount of fertilizer accumulated in the soil causes large-scale soil and water pollution through surface runoff and leaching, which seriously endanger the natural environment and human health. Thus, in the coming decades, one fundamental challenge will be preventing food shortages without accelerating environmental pollution and ecological degradation (Godfray et al., 2010; Odegard and van der Voet, 2014; Garcia-Gonzalez and Sommerfeld, 2016).

Microalgae, autotrophic plants with fast photosynthesis, fast reproduction, and strong environmental adaptability, whose cell metabolism results in the production of fat, protein, pigment, and polysaccharides, are considered to have great potential for solving major practical challenges, such as the lack of healthy food, increasing greenhouse effect, pollution of the ecological environment, and energy crisis (Michalak et al., 2017; Chiaiese et al., 2018; Behera et al., 2021). They are widely used in fuel, food, medicine, cosmetics, animal feed, and sewage purification (Zhu et al., 2013; Yaakob et al., 2014; Michalak et al., 2017; Moreno-Garcia et al., 2017; Morais et al., 2021; Moreira et al., 2022), but their use is rarely recognized in agricultural production (Garcia-Gonzalez and Sommerfeld, 2016). Moreover, these studies on microalgal resources mainly focused on water environments, such as seawater and fresh water, while few have focused on more complex and diverse soil environments that contain rich biological resources. Many microalgal resources derived from the soil environment, especially those beneficial to agricultural production, are yet to be developed.

Some microalgae have been proven to have a robust effect on the root system of crops and enhance crop yield and quality by improving soil structure or fertility, promoting the activity of beneficial soil microorganisms, and balancing the soil micro-ecosystem (Ronga et al., 2019; Martini et al., 2021). In this study, we aimed to isolate and identify microalgal strains from soil environments and assess their prospects in agricultural crop production by analyzing the influence of microalgae on wheat growth and soil properties. This study will support the use of microalgae as biological fertilizers that have great potential to meet various needs, such as reducing the application of chemical fertilizers, increasing food production, and maintaining environmental and ecological health in agricultural production.

Materials and methods

Microalgae isolation and purification

Isolation and purification of the microalgal strains were conducted using saline (38°10′02″N, 117°33′49″E) and grassland soils (37°52′44″N, 114°15′49″E) in Hebei Province, China. Soil samples were taken from 0 to 10 cm of the topsoil, carefully transported to the laboratory, and stored at 4°C. To isolate the microalgae from the soil, 1 g fresh soil was mixed thoroughly with 10 mL sterilized distilled water by vortexing for 30 min. The mixture was supplied with the Allen Arnon medium (AA medium) for enriching the microalgae and incubated for a week at 25.5°C after shaking at 200 rpm for 24 h (**Figure 1**; Allen and Arnon, 1955). The supernatant was carefully transferred into sterilized flasks containing AA medium with imipenem at a final concentration of 100 μg mL^{-1}, which could inhibit prokaryotic cell growth. The culture was shaken at 150 rpm for 3 weeks at a light intensity of 100 μmol photons m^{-2} s^{-1} for 12 h per day at 25°C. Then, 1 mL of the mixed culture was serially diluted. Hundred microliters of each serial dilution was coated on 1.5% agar solid AA medium with imipenem for microalgae growth with a light intensity of 100 μmol photons m^{-2} s^{-1} at 25°C until green colonies were observed. Single colonies were repeatedly selected and streaked on a solid AA medium and incubated until the single colonies consistently had the same appearance and morphology as the microalgal cells under the microscope (BX3-URA, Olympus, Japan) (**Figure 1**).

The AA medium was prepared from a mixture of +pi and −pi stock solutions as follows. The +pi stock solution was prepared from autoclaved anhydrous K_2HPO_4 (42.8 g L^{-1}), and the −pi stock solution was prepared from four stock solutions that were mixed at a ratio of 1:1:1:1, including autoclaved macroelement stocks of 40 g L^{-1} $MgSO_4{\cdot}7H_2O$, 12 g L^{-1} $CaCl_2{\cdot}H_2O$, 40 g L^{-1} NaCl, and filter sterilized microelement stock (1,090 mL double-distilled water, 160 mL Fe-EDTA, 360 mg $MnCl_2{\cdot}4H_2O$, 36 mg MoO_3 85% purity, 44 mg $ZnSO_4{\cdot}7H_2O$, 15.8 mg $CuSO_4{\cdot}5H_2O$, 572 mg H_3BO_3, 4.6 mg NH_4VO_3 (NH_4^+ metavanadate), and 8 mg $CoCl_2{\cdot}6H_2O$). For microalgal growth, the +pi solution was used as 6.25 mL L^{-1} for solid plate culture and 3.1 mL L^{-1} for liquid culture, and the −pi solution was used as 25 mL L^{-1} for solid plate and 6.30 mL L^{-1} for liquid culture (Allen and Arnon, 1955).

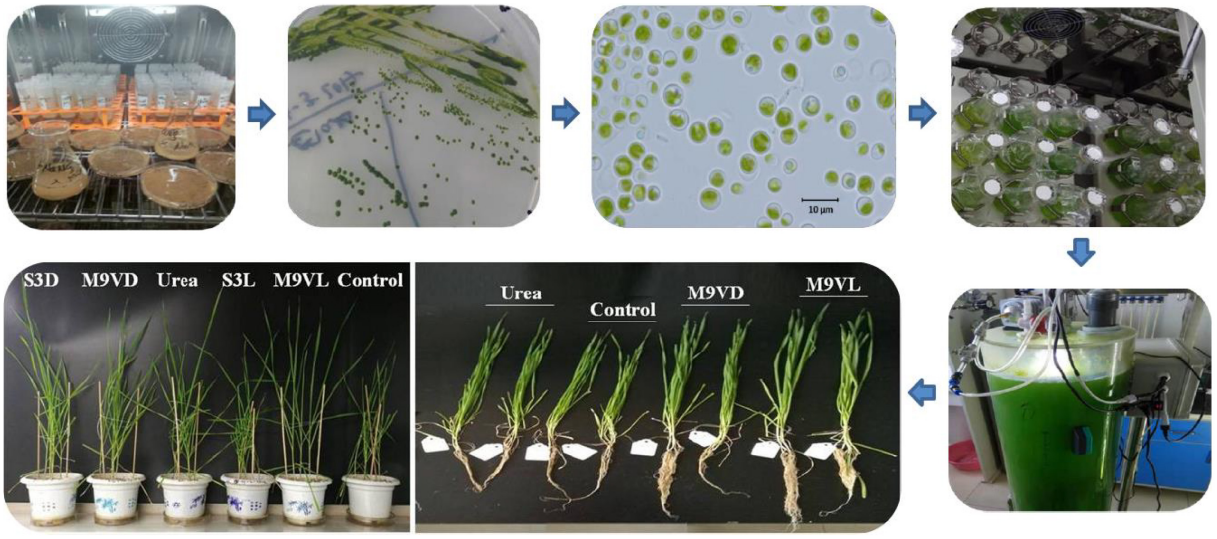

FIGURE 1
Isolation, purification, cultivation, and the application to pot experiment of microalgae *Chlamydomonas applanata* M9V and *Chlorella vulgaris* S3.

DNA extraction, PCR amplification, sequencing, and phylogenetic analysis

Genomic DNA was extracted from a 2 mL microalgal culture using an E.Z.N.A. @ HP fungal DNA kit (D3195-01, Omega, United States). The quantity and quality of the extracted DNA were tested using a NanoDrop One instrument and 1% (w/v) agarose gel electrophoresis. The small subunit ribosomal DNA (SSU rDNA) sequence was amplified using primer set 18F (5′-TGGTTGATCCTGCCAGT-3′) and 18R (5′-TGATCCTTCTGCAGGTTCACC-3′) (Song et al., 2016). The 50-μL PCR mixture contained 25 μL of Premix Ex Taq, 0.5 μL of each primer (10 μM), 3 μL of template DNA, and 21 μL of ddH_2O. The PCR was conducted with an initial denaturation at 94°C for 5 min, followed by 32 cycles of 50 s at 94°C, 50 s at 55°C, and 90 s at 72°C (Song et al., 2016). The PCR amplification products were checked by 1% (w/v) agarose gel electrophoresis at 180 V for 15 min with a 2,000 bp DNA ladder. Finally, the amplified PCR products were sequenced by Sangon Biotechnology Inc. (Shanghai, China). The closest relatives of SSU rDNA sequences of microalgal strains M9V and S3 were examined using the BLASTn search program on the National Center for Biotechnology Information (NCBI) website.[1] Reference sequences from microalgal organisms were retrieved from GenBank. After the sequences were aligned with CLUSTALX 1.81 (Thompson et al., 1997), neighbor-joining phylogenetic trees were constructed using the software MEGA7 with 1,000 bootstrap replicates (Kumar et al., 2016).

1 http://www.ncbi.nlm.nih.gov/BLAST

Pot experiment

The experiment was conducted in pots containing 3.0 kg fresh and 2-mm-diameter mesh-sieved soil. Four healthy wheat plants of a similar size of variety Kenong199 (KN199, provided by the State Key Laboratory of Plant Cell and Chromosome Engineering) were planted in each pot and cultured in a controlled environment for 56 days with a relative humidity of 65 ± 5% and light intensity of 248 μmol photons m^{-2} s^{-1} for a 16 h photoperiod per day at 25°C (**Figure 1**). In this pot experiment, the growth period of wheat plants growing for 56 days could be judged as seedling stage according to their characteristics of no-tillers and less than nine leaves. The main reason for this phenomenon was that the objective of our experiment was to investigate the relative difference in wheat growth between the treatment with microalgal fertilizer and the control and urea treatments without microalgal fertilizer, so the vernalization treatment of wheat seedlings was not carried out, and the growth environment was kept at 25°C without temperature difference. Six treatments (i.e., M9VL, M9VD, S3L, S3D, urea, and the control), were laid out in a completely randomized design (**Figure 1**). All treatments were repeated two times. To obtain a large amount of microalgae as biofertilizer, we used a 20 L volume light bioreactor to cultivate the microalgae *Chlamydomonas applanata* M9V and *Chlorella vulgaris* S3 with a light intensity of 100 μmol photons m^{-2} s^{-1} for a 12 h photoperiod per day at 25°C. M9VD and S3D were obtained from equal quantities of M9VL and S3L processed in an autoclave sterilizer at 121°C for 30 min. Urea treatment was employed by using 0.39 g urea fertilizer at 0.18 g N per pot (the recommended rate of N fertilizer, 120 kg N ha^{-1}, roughly

calculated according to 2.0×10^6 kg soil ha^{-1}) (Oad and Buriro, 2004; Maurya et al., 2016). Based on equal N nutrient content with urea treatment at 0.18 g N per pot, 3.28 g dry weight of microalgae M9V biomass was applied to each pot for the M9VL and M9VD treatments, and 3.75 g dry weight of microalgae S3 biomass was applied to each pot for the S3L and S3D treatments. All the treatments, including the control, were supplemented with the AA medium to normalize the effect of the AA medium on wheat growth. The recommended rate of phosphorus (60 kg P_2O_5 ha^{-1}) was supplied with triple super phosphate to all treatments (Maurya et al., 2016; Xiao et al., 2021).

Soil property analysis

The measurement of soil chemical characteristics, including pH, total carbon (TC), total nitrogen (TN), and soil organic matter (SOM) contents, was conducted according to the methods previously described by Lu (1999) and Chu and Grogan (2010). To determine the soil pH value, a soil to CO_2-free water ratio of 1:2.5 was used and the pH was measured using a pH meter (PHS-3C, Shanghai INESA, China). The soil samples were air-dried and sieved with a 2 mm mesh, and then ground using a mortar and pestle to determine soil TC, TN, and SOM contents. The TC and TN contents of the soil were measured using an elemental analyzer (Vario Pyro, Elementar, Germany). The SOM content was measured using the $K_2Cr_2O_7$ oxidation method.

Data collection on wheat growth parameters

After the potting soil was saturated with tap water to loosen the root attachment to the soil, we washed the wheat roots gently and thoroughly with tap water to keep them intact. The four wheat plants in each pot were separated into independent individuals, and finally, we obtained eight wheat plants for data collection for each treatment. Data on shoot fresh weight, leaf length, and root length of wheat plants were collected first, and data on root dry weight were collected after the wheat plant was dried in an oven. The leaf length was recorded from the longest leaf of each wheat plant. To determine the chlorophyll *a*/*b* and carotenoid contents, 0.5 g fresh leaf samples were homogenized using a mortar and pestle with 10 mL 80% acetone, and the extract was centrifuged at $650 \times g$ for 5 min at 4°C. Then, 1 mL supernatant was gently transferred into a new tube and diluted by adding 9 mL 80% acetone. The absorbance of the diluted extract was measured at 663.2 nm, 646.8 nm, and 470 nm, and then chlorophyll *a*/*b* and carotenoid contents were calculated using the method described by Wellburn (1994).

Microalgal element composition analysis

To remove medium nutrients, the biomass of the microalgae *C. applanata* M9V and *C. vulgaris* S3 was washed with sterilized double-distilled water and centrifuged at $2{,}600 \times g$ for 10 min. Then, the biomass was dried at 65°C and ground, and elemental composition, i.e., N, C, P, K, Fe, Zn, and Mn, was evaluated using an atomic absorption spectrophotometer (AAS, ZEEnit 700P, Analytik Jena, Germany) and elemental analyzer (Vario Pyro, Elementar, Germany).

Statistical analysis

The data are described based on the mean with standard deviation. Statistical analysis was performed using one-way analysis of variance (ANOVA) with the Statistical Analysis Software (SAS) version 9.2, and the significance level was defined as $p < 0.05$ level and labeled with letters based on Tukey's test. Origin 2021 software was used in plotting.

Results

Phylogeny of microalgal strains M9V and S3

The SSU rDNA sequences of microalgal strains M9V and S3 were deposited into the NCBI database with accession numbers MK793578 and MK652782, respectively. In the NCBI database, the SSU rDNA sequence of microalgae M9V has the highest 99.94% identity with 12 reference microalgal sequences, and the SSU rDNA sequence of microalgae S3 has the highest 99.88% identity with 34 reference microalgal sequences. These reference sequences are described as SSU rDNA, 18S rDNA, and genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2, 28S rRNA gene in the NCBI database. The SSU rDNA sequence of microalgae M9V was clustered with those 12 reference sequences from *C. applanata* organisms CCAP 11/2, SAG 11.36a, CCAP 11/9, CCAP 11/36B, CCAP 11/36F, CCAP 11/36C, CCAP 11/36E, UTEX 2399, ACSSI 068, ACSSI 126, ACSSI 003, and ACSSI 148, as well as two reference sequences from *C. applanata* organisms NIES-2204 and NIES-2202 of lower identity with 100% bootstrap support value in the phylogenetic tree (**Figure 2**). Thus, the microalgal strain M9V was named *C. applanata* M9V. The SSU rDNA sequence of microalgae S3 was clustered with those 34 reference sequences with 82% bootstrap support value in the phylogenetic tree (**Figure 3**), of which 29 sequences were from *C. vulgaris* organisms NJ-7, CCAP 211/19, CCAP 211/35, NIES-227, KNUA027, CCAP 211/75, CCAP 211/82, CCAP 211/74, CCAP 211/21B, CCAP 211/110, CCAP 211/11S,

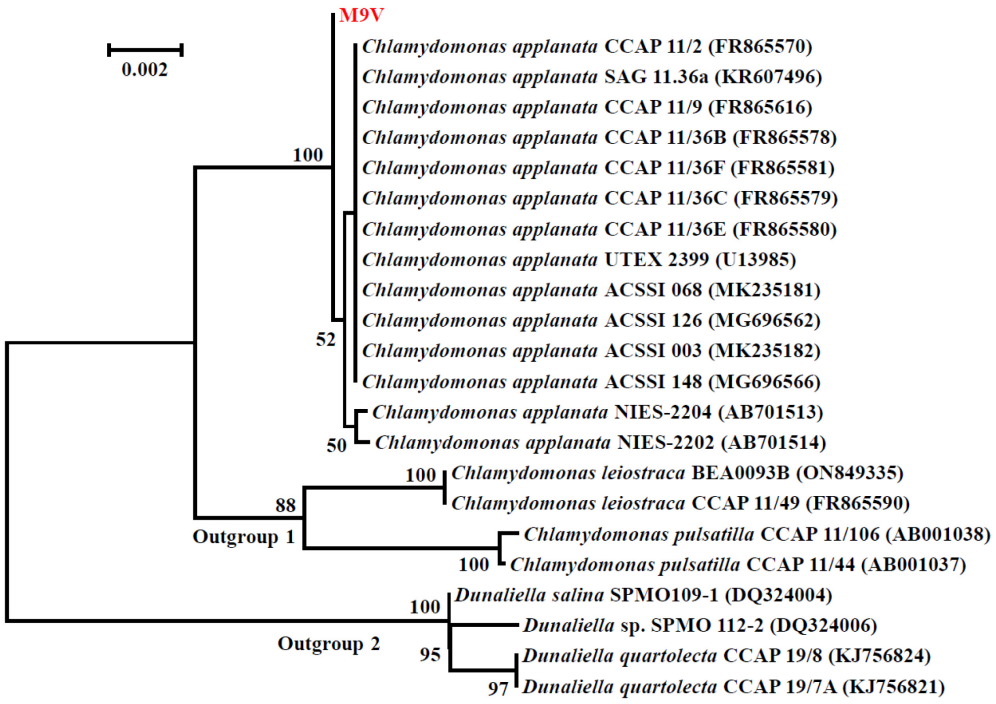

FIGURE 2
Neighbor-joining phylogenetic tree of the small subunit ribosomal DNA (SSU rDNA) sequence of microalgae M9V and the reference sequences retrieved from GenBank. Numbers in parentheses are the accession numbers of reference sequences in the National Center for Biotechnology Information (NCBI) database.

ACSSI 335, Ab5, ACSSI 249, ACSSI 374, ACSSI 378, S3, ChloN4, ZS1, ACSSI 361, CCAP 211/109, SAG 211-11b, CCAP 211/21A, CCAP 211/81, and CCAP 211/80, and five sequences were from *Neodesmus cf. pupukensis* CCAP 211-52, *Neochloris aquatica* CCAP 254/5, *Chlamydomonas chlamydogama* CCAP 11/48B, *Marvania coccoides* CCAP 251/1A, and *Chloroidium saccharophilum* CCAP 211/48 (dark gray background in Figure 3). In addition, two reference sequences with different accession numbers were from *C. vulgaris* organisms SAG 211-11b, CCAP 211/21A, CCAP 211/81, and CCAP 211/80, respectively (light gray background in Figure 3). Thus, the microalgal strain S3 was named *C. vulgaris* S3.

Influence of microalgal fertilizers on soil properties

All soils treated with M9VL, M9VD, S3D, and S3L had higher SOM, TC, and C:N ratio than the control and urea soils (Table 1). The M9VL soil had the highest TC and C:N ratio and significantly increased TC (13.10 and 14.46%) and C:N ratio (6.96 and 11.73%) compared to the control and urea soils, respectively ($p < 0.05$) (Table 2). The M9VD soil had the highest SOM and the second highest C:N ratio and significantly increased SOM (29.15 and 18.22%) and C:N ratio (6.06 and 10.79%) compared to control and urea soils, respectively ($p < 0.05$) (Table 2). In addition, the M9VD soil had significantly higher TC (8.43%) than the urea soil ($p < 0.05$), and the M9VL soil had significantly higher OM (23.10%) than the control soil (Table 2). The S3D soil had significantly higher TC (11.31 and 12.65%) and C:N ratio (5.24 and 9.93%) than the control soil and the urea soil ($p < 0.05$), respectively, and the S3L soil had significantly higher TC (10.24%) and C:N ratio (7.58%) than in the urea soil ($p < 0.05$) (Table 2). Although the soils treated with microalgal fertilizer did not exhibit a significant increase in TN, M9VL, S3D, and S3L soils did exhibit an increase in TN by approximately 2.31–5.56% compared to the control soil and the urea soil (Table 1). Soil pH decreased in all the soils treated with the microalgal fertilizers M9VL, M9VD, S3D, and S3L compared to that in the control and urea soils, and the M9VL, M9VD, and S3D soils had significantly lower pH than the control soil ($p < 0.05$), and the M9VD soil also had significantly lower pH than the urea soil ($p < 0.05$) (Table 1).

Influence of microalgal fertilizers on wheat growth parameters

Totally, M9VL was effective with respect to wheat growth promotion based on parameters, such as shoot fresh weight,

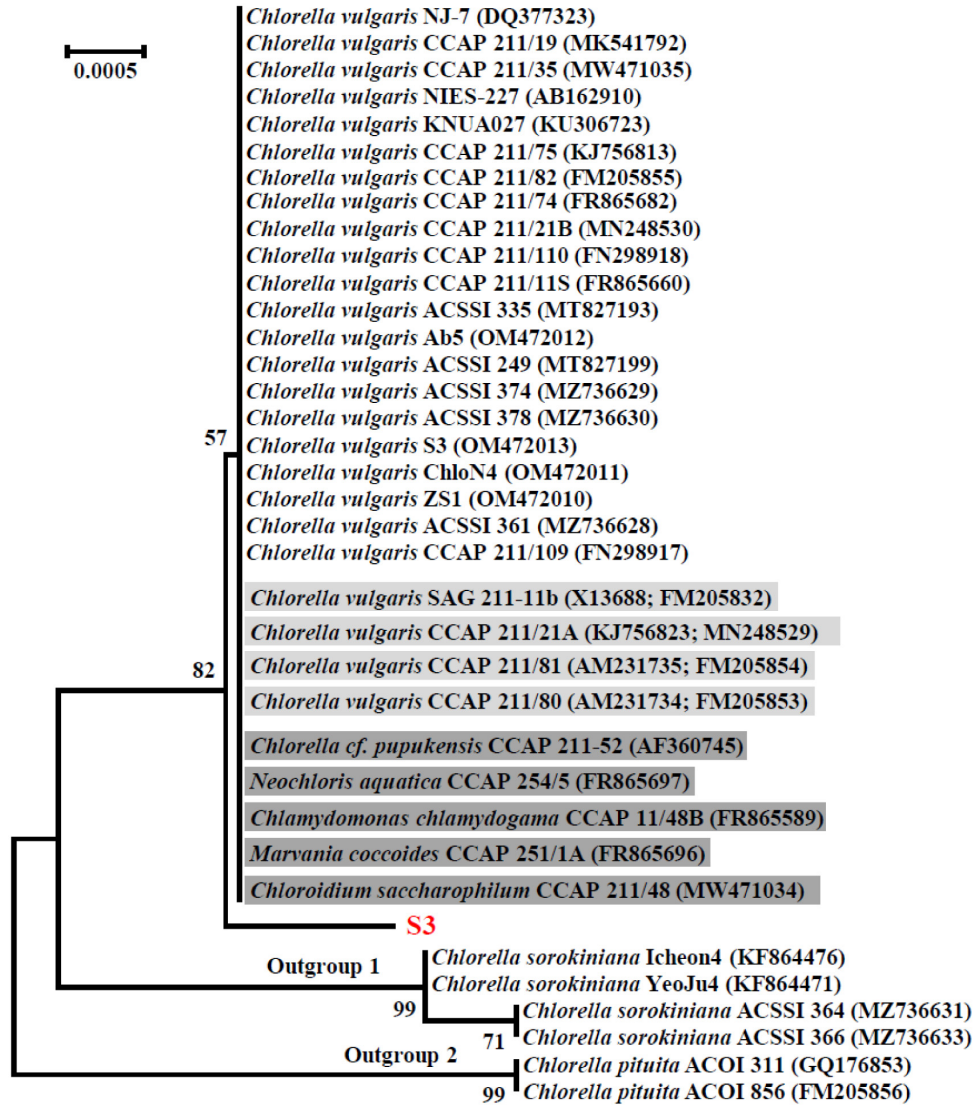

FIGURE 3
Neighbor-joining phylogenetic tree of the small subunit ribosomal DNA (SSU rDNA) sequence of microalgae S3 and the reference sequences retrieved from GenBank. Numbers in parentheses are the accession numbers of reference sequences in the NCBI database.

root dry weight, leaf length, and root length, followed by M9VD and S3D, while the lowest fresh weight was observed in the control (Figures 4–6 and Table 2). M9VL significantly increased the shoot fresh weight (166.77 and 125.68%), root dry weight (188.89 and 77.35%), and root length (46.04 and 43.93%), respectively, compared to the control and urea treatments ($p < 0.05$), and also significantly increased the leaf length (26.88%) compared to the control ($p < 0.05$) (Table 2). M9VD significantly increased shoot fresh weight (87.86 and 58.92%) and root dry weight (122.22 and 36.43%) compared to the control and urea treatments ($p < 0.05$), and S3D significantly increased shoot fresh weight (88.21 and 59.73%), respectively, compared to the control and urea treatments ($p < 0.05$) (Table 2). However, S3L showed a slight increase in shoot fresh weight (16.93%), root dry weight (11.11%), and root length (7.88%) compared to the control, and a slight reduction in leaf length (2.76%) compared to the control (Table 2).

Influence of microalgal fertilizers on chlorophyll *a/b* and carotenoid contents of wheat leaf

The highest chlorophyll *a* and *b* contents of wheat leaves were recorded from M9VL, followed by M9VD and S3D, while the lowest contents were obtained in the control (Figure 7). Compared with the control, M9VL, M9VD, and S3D significantly increased the chlorophyll *a* (by 257.81, 183.81, and

TABLE 1 Soil properties in different fertilization treatments (Mean ± standard deviation).

Treatment	Soil properties				
	SOM (g kg^{-1})	TN (g kg^{-1})	TC (g kg^{-1})	C:N	pH
M9VL	25.42 ± 1.06ab	1.33 ± 0.05a	19.00 ± 1.82a	14.29 ± 1.18a	7.89 ± 0.04bc
M9VD	26.67 ± 1.77a	1.27 ± 0.06a	18.00 ± 0.17ab	14.17 ± 0.61a	7.88 ± 0.02c
S3L	22.96 ± 1.53bc	1.33 ± 0.08a	18.30 ± 0.70ab	13.76 ± 0.99ab	7.95 ± 0.03abc
S3D	23.71 ± 1.12abc	1.33 ± 0.06a	18.70 ± 1.25a	14.06 ± 0.37a	7.89 ± 0.02bc
Urea	22.56 ± 0.26bc	1.30 ± 0.01a	16.60 ± 0.35c	12.79 ± 0.27c	7.98 ± 0.02ab
Control	20.65 ± 0.79c	1.26 ± 0.06a	16.80 ± 0.29bc	13.36 ± 0.85bc	8.00 ± 0.036a
BP	22.28 ± 1.01bc	1.28 ± 0.07a	17.70 ± 0.35abc	13.83 ± 0.96ab	7.97 ± 0.04ab

M9VL, living *Chlamydomonas applanata* M9V; M9VD, dead *Chlamydomonas applanata* M9V; S3L, living *Chlorella vulgaris* S3; S3D, dead *Chlorella vulgaris* S3; Urea, with urea fertilizer; Control, without fertilizer; BP, before planting; SOM, soil organic matter; TC, total carbon; TN, total nitrogen. Values followed by different letters in the same column are significantly different based on Tukey's test ($p < 0.05$).

TABLE 2 Influence of microalgal fertilizers on the wheat growth parameters and soil properties.

	Comparing to the control treatment				Comparing to the urea treatment			
	M9VL	M9VD	S3L	S3D	M9VL	M9VD	S3L	S3D
Shoot fresh weight	+ + + + 166.77%*	+ + 87.86%*	+ 16.93%	+ + + 88.21%*	+ + + + 125.68%*	+ + 58.92%*	− −1.08%	+ + + 59.73%*
Root dry weight	+ + + + 188.89%*	+ + + 122.22%*	+ 11.11%	+ + 111.11%	+ + + + 77.35%*	+ + + 36.43%*	− 31.79%	+ + 29.60%
Leaf length	+ + + + 26.88%*	+ + 13.48%	− 2.76%	+ + + 15.26%	+ + + + 14.56%	+ + 2.46%	− 12.20%	+ + + 4.07%
Root length	+ + + + 46.04%*	+ + 12.13%	+ 7.88%	+ + + 27.26%	+ + + + 43.93%*	+ + 10.52%	+ 6.33%	+ + + 25.43%
Chlorophyll *a*	+ + + + 257.81%*	+ + + 183.81%*	+ 16.78%	+ + 169.48%*	+ + + + 82.23%*	+ + + 44.55%*	− 40.52%	+ + 37.25%*
Chlorophyll *b*	+ + + + 269.00%*	+ + + 140.41%*	+ 26.86%	+ + 107.10%*	+ + + + 247.27%*	+ + + 126.25%*	+ 19.39%	+ + 94.90%*
OM	+ + + 23.10%*	+ + + + 29.15%*	+ 11.19%	+ + 14.82%	+ + + 12.68%	+ + + + 18.22%*	+ 1.77%	+ + 5.10%
TC	+ + + + 13.10%*	+ 7.14%	+ + 8.93%	+ + + 11.31%*	+ + + + 14.46%*	+ 8.43%*	+ + 10.24%*	+ + + 12.65%*
C:N	+ + + + 6.96%*	+ + + 6.06%*	+ 2.99%	+ + 5.24%*	+ + + + 11.73%*	+ + + 10.79%*	+ 7.58%*	+ + 9.93%*

M9VL, M9VD, S3L, and S3D indicate microalgal fertilizer treatments of living *Chlamydomonas applanata* M9V, dead *Chlamydomonas applanata* M9V, living *Chlorella vulgaris* S3, and dead *Chlorella vulgaris* S3, respectively. The "*" means a significant increase compared to the control or urea treatments. The "+" and "−," respectively, mean positive and negative influence on the wheat growth parameters and soil properties, and the counts of "+" indicate the degree of positive effects.

169.48%, respectively) and *b* contents in wheat leaves (by 269.00, 140.41, and 107.10%, respectively) (Table 2). Compared with the urea, M9VL, M9VD, and S3D significantly increased the chlorophyll *a* content (by 82.23, 44.55, and 37.25%, respectively) and *b* contents in wheat leaves (by 247.24, 126.25, and 94.90%, respectively) (Table 2). In addition, the chlorophyll *a* and *b* contents of M9VL were significantly higher than those of the other treatments ($p < 0.05$), and these contents were significantly higher in M9VD and S3D than those in S3L, urea, and control ($p < 0.05$) (Figure 7). For the carotenoid content in wheat leaves, the highest content was obtained from M9VD, followed by urea and S3L, while the lowest was obtained from M9VL (Figure 7). A significantly higher carotenoid content was observed in M9VD than in the case of the control, S3D, and M9VL, and a significantly higher content was observed in the urea treatment than in the case of M9VL ($p < 0.05$) (Figure 7).

Discussion

Application of microalgae for crop growth promotion

According to the results, *C. applanata* M9V and *C. vulgaris* S3 isolated in the present study, both in their living and

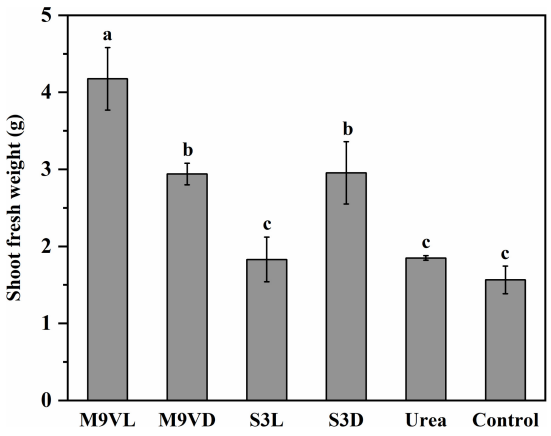

FIGURE 4
Effects of microalgal fertilizers and urea fertilizer on shoot fresh weight of wheat. The data are expressed as mean, and the error bars represent the standard deviation (SD). The significance level was defined as $p < 0.05$ using Tukey's test. The M9VL, M9VD, S3L, S3D, urea, and control indicated microalgal fertilizer treatments living *Chlamydomonas applanata* M9V, dead *Chlamydomonas applanata* M9V, living *Chiarella vulgaris* S3, dead *Chlorella vulgaris* S3, urea fertilizer, and control without fertilizer, respectively.

dead forms (M9VL, M9VD, S3L, and S3D), had different degrees of positive influence on wheat growth in the pot experiment (**Figure 1** and **Table 2**). M9VL was effective with respect to wheat growth promotion, followed by M9VD and S3D as measured based on shoot fresh weight, root dry weight, leaf length, root length, and photosynthetic pigment (chlorophyll *a* and *b*) content (**Figures 4–7** and **Table 2**). These growth-promoting characteristics resulted in thicker stalks, broader, longer, and darker green leaves, and a deeper developed root system in wheat (**Figure 1** and **Table 2**), which might herald higher yield productivity at the mature stage of wheat. The use of microalgae as fertilizers in agricultural crop production is mainly focused on the cyanobacterial members especially *Nostoc* and *Anabaena* that are capable of fixing atmospheric nitrogen (Garcia-Gonzalez and Sommerfeld, 2016; Renuka et al., 2016). Green algal members of microalgae, such as *Acutodesmus dimorphus*, *C. vulgaris*, *Scenedesmus quadricauda*, *Chlamydomonas reinhardtii*, *Chlorella sorokiniana*, *Asterarcys quadricellulare*, *Dunaliella salina*, and *Chlorella ellipsoidea*, have been gradually investigated their fertilizer properties on plants, i.e., wheat, maize, tomato, potato, and lettuce (Faheed and Abd-El Fattah, 2008; Garcia-Gonzalez and Sommerfeld, 2016; El Arroussi et al., 2018; Barone et al., 2019; Martini et al., 2021; Mutale-Joan et al., 2021; Cordeiro et al., 2022). These green algal biomass or extracts could positively affect the growth of plants through growth phytohormones, exopolysaccharides, and nutrient availability (Rana et al., 2012; Renuka et al., 2016; El Arroussi et al., 2018).

In this work, we speculate that the growth-promoting effect of microalgae M9V and S3 on wheat might mainly be attributed to the increased soil nutrient contents and microalgae-derived bioactivity. Microalgal biomass is a carbon-rich residue comprising carbohydrates, lipids, proteins, and diverse molecules, which has benefits, such as improved soil health, stability of soil aggregates, soil water retention, carbon sequestration, and prevention of nutrient losses (Metting and Rayburn, 1983; Anand et al., 2015; Maurya et al., 2016; Behera et al., 2021). SOM is responsible for storing nutrients and maintaining soil structure, which plays an important

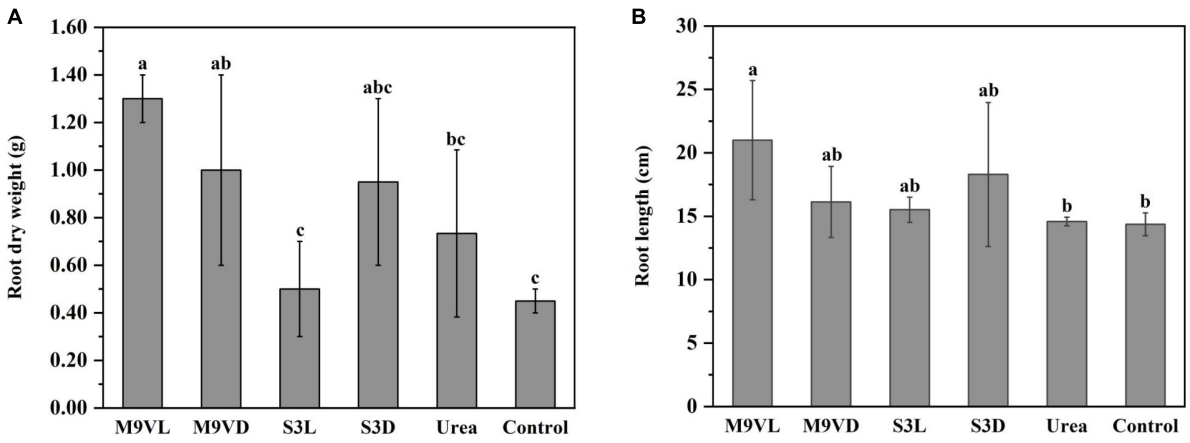

FIGURE 5
Effects of microalgal fertilizers and urea fertilizer on root dry weight **(A)** and root length **(B)** of wheat. The data are expressed as mean, and the error bars represent the standard deviation (SD). Significance was defined as $p < 0.05$ using Tukey's test. The M9VL, M9VD, S3L, S3D, urea, and control indicated microalgal fertilizer treatments living *Chlamydomonas applanata* M9V, dead *Chlamydomonas applanata* M9V, living *Chlorella vulgaris* S3, dead *Chlorella vulgaris* S3, urea fertilizer, and control without fertilizer, respectively.

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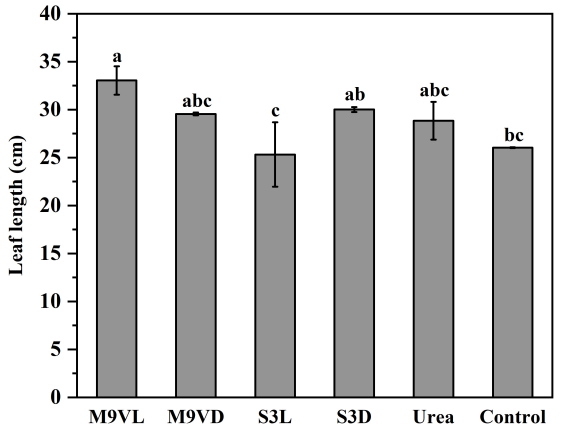

FIGURE 6
Effects of microalgal fertilizers and urea fertilizer on leaf length in wheat. The data are expressed as mean, and the error bars represent the standard deviation (SD). Significance was defined as $p < 0.05$ level using Tukey's test. The M9VL, M9VD, S3L, S3D, urea, and control indicated microalgal fertilizer treatments living *Chlamydomonas applanata* M9V, dead *Chlamydomonas applanata* M9V, living *Chlorella vulgaris* S3, dead *Chlorella vulgaris* S3, urea fertilizer, and control without fertilizer, respectively.

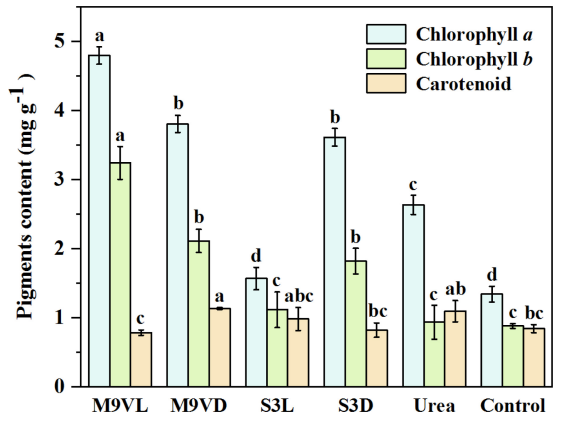

FIGURE 7
Effects of microalgal fertilizers and urea fertilizer on chlorophyll *alb,* and carotenoid contents in a wheat leaf. The data are expressed as mean, and the error bars represent the standard deviation (SD). Significance was defined as $p < 0.05$ using Tukey's test. The M9VL, M9VD, S3L, S3D, urea, and control indicated microalgal fertilizer treatments living *Chlamydomonas applanata* M9V, dead *Chlamydomonas applanata* M9V, living *Chlorella vulgaris* S3, dead *Chlorella vulgaris* S3, urea fertilizer, and control without fertilizer, respectively.

role in soil quality and crop nutrient supply in the agro-ecosystem (Menšík et al., 2018). In the current study, microalgae M9V and S3 contained 39.32 and 35.52% carbon, respectively (Table 3). Using microalgae M9V and S3 in their living and dead forms as biofertilizers increased the amount of SOM, TC, and the C:N ratio compared to that in the individual

TABLE 3 Elemental composition of microalgal biomass of *Chlamydomonas applanata* M9V and *Chlorella vulgaris* S3.

Elemental composition	*Chlamydomonas applanata* M9V	*Chlorella vulgaris* S3
N (%)	5.49	4.80
C (%)	39.32	35.52
P (%)	2.06	1.68
K (%)	0.76	0.57
Fe (mg kg^{-1})	811	966
Zn (mg kg^{-1})	68	53
Mn (mg kg^{-1})	435	285

urea and control treatments or a combination of the two (Tables 1, 2). The microalgae M9V and S3 in their dead forms had slightly higher SOM content than their living forms. Specifically, M9VD had higher SOM content (1.25 g kg^{-1}) than M9VL, S3D had higher SOM content (0.75 g kg^{-1}) than S3L. As for the nitrogen, not much significant increase in the soil TN content was observed in this study. The M9VL, S3D, and S3L treatments increased the TN compared to the control and urea treatments (0.07 g kg^{-1}, 5.56%; 0.03 g kg^{-1}, 2.31%, respectively). Furthermore, M9VD treatment increased the TN (0.01 g kg^{-1}, 0.79%) compared to the control treatment, suggesting that the continuous application of microalgae may have significant long term benefits with respect to improving the soil TN (Table 1). With respect to the vast majority of nitrogen-fixing organisms [prokaryotic bacteria or cyanobacteria (blue-green algae)] (Garcia-Gonzalez and Sommerfeld, 2016; Atnoorkar, 2021), we speculated that the increased nitrogen came from the chemical elements of microalgal cells or the recycling of elements directly or indirectly affected by microalgae. Finally, certain microalgal extracts that enhance the growth of crops have been found to contain high levels of macro- and micronutrients (Tarakhovskaya et al., 2007; Renuka et al., 2016). In this study, other nutrient elements including P, K, Fe, Zn, and Mn, released from the biomass of microalgae M9V and S3, in the background of autoclaving at 121°C, might also play an essential role in wheat growth promotion (Table 3).

Studies have shown that microalgae can promote crop growth by producing plant hormones (auxins, gibberellins, and cytokinins), amino acids, vitamins, and antifungal and antibacterial compounds (Maurya et al., 2016; Ronga et al., 2019; Behera et al., 2021). Considering that M9VL resulted in much higher values for shoot fresh weight, root dry weight, leaf length, root length, and photosynthetic pigment (chlorophyll *a* and *b*) content than M9VD, and did not result in any significant difference in soil nutrients compared to M9VD (Figures 4–7 and Table 2), we found that the living *C. applanata* M9V tended to possess some bioactivity with respect to positively affecting wheat growth. Consequently, the positive influence of

C. applanata M9V on crop growth might mainly be attributed to excellent biological activity followed by the improvement in the soil nutrient contents. On the contrary, the positive influence of *C. vulgaris* S3 on crops might mainly be attributed to the improvement of the soil nutrient contents because S3D resulted in much better results for most wheat growth parameters than S3L (**Figures 4–7** and **Table 2**). Although S3L did not result in any obvious growth promotion, it had an effect similar to that of urea and control. Reports indicate that plant growth is negatively influenced by much higher concentrations of microalgal extracts, with lower seed germination, fewer lateral roots, and shorter shoot length (Kumar and Sahoo, 2011; Hernández-Herrera et al., 2014).

Application of microalgae as urea alternatives

Nitrogen is essential for crop growth and significantly affects root and leaf growth and yield by affecting photosynthesis and the synthesis of proteins and nucleic acids during crop production. However, the low utilization rate of 30–50% of the nitrogen in the soil environment and emission of greenhouse gases (nitrous oxides), and leaching of nitrogen into the groundwater caused by excess usage of chemical fertilizers is very prominent (Fan et al., 2004; Quaggio et al., 2005; Chien et al., 2009; Shcherbak et al., 2014; Maurya et al., 2016). Urea is the most commonly used nitrogenous chemical fertilizer in crop production. The results of this study showed that the wheat plants treated with the microalgal biomass showed better results in terms of shoot fresh weight, root dry weight, leaf length, root length, and photosynthetic pigment (chlorophyll *a* and *b*) content than the wheat plants treated with urea fertilizer and no fertilizer (**Figures 4–7** and **Table 2**). Thus, it could be estimated that the microalgal biomass of M9V and S3 could be used as a substitute for a certain proportion of chemical urea fertilizer, which would help reduce the use of chemical fertilizers. With respect to the conventional fertilizer application rate of approximately 400–700 kg N ha^{-1} in the winter-wheat/summer-maize rotation system in the North China Plain (Zhao et al., 2009), microalgae might achieve approximately 17–30% substitution of chemical fertilizer application, which would have the potential to maintain crop yields, and alleviate a series of environmental pollution problems.

Additionally, all the microalgal fertilizer treatments, including M9VL, M9VD, S3L, and S3D, increased the root length of wheat compared to the urea and control treatments (**Figures 1**, **5B**). In addition, all the microalgal fertilizer treatments except the S3L treatment increased the root dry weight of wheat compared to the urea and control treatments, while the S3L treatment resulted in a higher root dry weight than the control treatment but not the urea treatment (**Figure 5A**). Furthermore, M9VL treatment significantly promoted the growth of wheat roots, including root dry weight and length compared to the urea and control treatments, and M9VD treatment significantly promoted root dry weight compared to the control treatment (**Figures 1**, **5**). The promotion of root growth using microalgal biomass would accelerate the uptake of nutrients from the soil, thereby increasing nitrogen utilization and alleviating the leaching of nitrogen into the groundwater, a phenomenon that is conducive to coordinated and sustainable environmental protection and agricultural production (Martini et al., 2021; Mutale-Joan et al., 2021).

Prospects of applying microalgae for crop production in a nature-friendly way

Excessive use of chemical fertilizers results in decreased crop yields and significant soil pollution (Rahman and Zhang, 2018). Thus, innovative technologies that would increase agricultural yields while minimizing inputs and environmental pollution are a crucial concern (Tilman et al., 2002; Foley et al., 2011; Garcia-Gonzalez and Sommerfeld, 2016; Singh et al., 2016). Biofertilizers are products that contain living microorganisms, natural compounds, or substances derived from organisms, such as bacteria, fungi, and algae, which can improve soil chemical and biological properties, stimulate plant growth, and restore soil fertility (Abdel-Raouf et al., 2012; Ronga et al., 2019). A few reports provide insights into the potential use of microalgae as biofertilizers, considering that microalgae are rich in biochemical components, bioactive metabolites, micronutrients, and macronutrients, such as proteins, carbohydrates and lipids, phytohormones, carotenoids, and vitamins, which would benefit plant growth with greater nutrient uptake, higher biomass accumulation, and greater crop yields (Shaaban, 2001; Faheed and Abd-El Fattah, 2008; Maurya et al., 2016; Behera et al., 2021). Microalgal biofertilizers provide a possible alternative to chemical fertilizers as they are considered environmentally friendly and economically feasible (Kawalekar, 2013; Garcia-Gonzalez and Sommerfeld, 2016). Not only do they increase agricultural productivity, but they have also been shown to decrease environmental pollution (Kawalekar, 2013; Garcia-Gonzalez and Sommerfeld, 2016). Meanwhile, the use of microalgal biofertilizers as a substitute for chemical fertilizers results in improved soil health, soil aggregate stabilization, enhanced soil water retention, nutrient loss prevention, and carbon sequestration (Metting and Rayburn, 1983; Anand et al., 2015; Maurya et al., 2016).

In recent years, although increasing worldwide interest in the use of microalgae in ecological crops production, poor soils remediation and adverse conditions of a changing climate has been observed, microalgal resources with biofertilizer properties

remain largely unexploited (Grzesik and Romanowska-Duda, 2014; Ronga et al., 2019). In this study, microalgal strains *C. applanata* M9V and *C. vulgaris* S3 promote crop growth and increase soil nutrient contents, favoring the application of microalgae as biofertilizers to reduce the usage of chemical fertilizers, thereby promoting sustainable crop production. Previous studies have shown that *C. vulgaris* seems to be a relatively common strain, the same specie as S3 in this study, which can enhance growth parameters and strengthen the metabolic aspects (Shaaban, 2001; Faheed and Abd-El Fattah, 2008; Feng et al., 2022). To the best of our knowledge, the *C. applanata* M9V might be the first *applanata* species of the *Chlamydomonas* genus that can promote crop growth. At present, the *reinhardtii* species of the *Chlamydomonas* genus are most commonly used as model organisms for basic research and biotechnological applications (Yang et al., 2019), and a recent study showed that *C. reinhardtii* strain 4a+ from the Culture Collection of Algae at Goettingen University (Germany) promotes the development of the maize root system (Martini et al., 2021). The *C. applanata* M9V exploited in this study has enriched the existing microalgal resources, and it might have huge development potential and broad application prospects for generating high-quality agriculture.

Conclusion

In the present study, *C. applanata* M9V and *C. vulgaris* S3, both in their living and dead forms (M9VL, M9VD, S3L, and S3D), were used as alternatives to chemical fertilizers for wheat growth. The results suggested that M9VL, M9VD, and S3D as microalgal fertilizers performed as well as and even better than a certain amount of chemical urea fertilizer with respect to wheat growth promotion, while S3L treatment exhibited an effect similar to that of the urea treatment and a slightly better effect than the control treatment, except in the case of leaf length. Moreover, all the microalgal fertilizer treatments improved soil properties, such as SOM, TC, and C:N ratio (compared to chemical urea fertilizer and the control without fertilizer). In particular, M9VL performed the best with respect to wheat growth promotion in the context of parameters, such as plant fresh weight, root dry weight, leaf length, root length, and plant photosynthetic pigment (chlorophyll *a* and *b*) content, which might mainly be attributed to its excellent biological activity and improved soil nutrient contents. The use of microalgae for crop growth promotion and as urea alternatives is a feasible strategy in the context of crop production. The *C. applanata* M9V obtained in this study has the potential for further development as a substitute for partial chemical fertilizers with a positive and eco-friendly influence on wheat growth and soil nutrient contents.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov, MK793578 and MK652782.

Author contributions

MYS designed and performed all experiments, analyzed the data, and wrote the first draft of the manuscript. YPT and XGW contributed to the project administration of the study and reviewed and edited the manuscript. XZW performed the pot experiments, analyzed the data, and revised the manuscript. All authors read and approved the submitted version.

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Conflict of interest

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

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Tengxiang Lian,
South China Agricultural University, China

REVIEWED BY
Yanzhao Zhang,
Luoyang Normal University, China
Xin Lou,
Dalian University, China
Shi Chuanqi,
Harbin University, China

*CORRESPONDENCE
Liqiang Mu
mlq0417@163.com

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Composition and diversity of soil bacterial communities under identical vegetation along an elevational gradient in Changbai Mountains, China

Mengsha Li[1,2], Guanhua Dai[3] and Liqiang Mu[1]*

[1]School of Forestry, Northeast Forestry University, Harbin, China, [2]Institute of Nature and Ecology, Heilongjiang Academy of Sciences, Harbin, China, [3]Research Station of Changbai Mountain Forest Ecosystems, Chinese Academy of Sciences, Erdaobaihe, China

Soil bacteria play important roles in biogeochemical cycling and biodiversity in mountain ecosystems. Past studies have investigated the bacterial community composition and diversity in elevation gradations covered by different vegetation types, but for a better assessment of elevation effects, here we studied bacterial communities in soil under identical vegetation cover. High-throughput amplicon sequencing of the V3-V4 region of bacterial 16S rDNA was used to investigate the diversity and composition bacterial communities in soil from 700 to 1,000 m above sea level collected on the north slope of Changbai Mountains, Northeast China. Obviously differences (p<0.05) in soil physicochemical parameters (i.e., total nitrogen, nitrate and ammonium nitrogen, soil moisture content, available potassium, microbial biomass carbon and nitrogen) were observed at different elevations. Soil bacterial abundance indices (Richness, Chao1, ACE) differed significantly along the elevation gradient, whereas the Shannon index remained unchanged. Principal Coordinates Analysis indicated separated soil bacterial communities of the different elevations. The dominant phyla in all soil samples were Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, and Bacteroidetes, which in combination reached 80%–85%. Soil pH to some extend related to soil bacterial community along altitude gradations. The relative abundance of a multiple phyla was negatively affected by the soil nutrients, such as ammonium and nitrate nitrogen, available potassium, soil moisture content, available phosphorus, microbial biomass nitrogen and soil organic C. The strongest effects were seen for Proteobacteria. The pH either positively or negatively correlated with specific genera. The soil bacterial function differed significantly among four elevations. The chemoheterotrophy, aerobic chemoheterotrophy and nitrification were the most dominant functions of soil bacteria among four elevations. Overall, the changes in soil physicochemical properties with elevation are important in shaping the bacterial diversity, composition and function in soil with the same above-ground vegetation of Changbai Mountains.

KEYWORDS

soil nutrients, bacterial diversity, temperate mountain forest, diversity index, soil bacteria

Introduction

Soil bacteria are an important component of the soil microbiota and play crucial roles in regulating ecological functions such as litter decomposition, biogeochemical cycling, and maintaining biodiversity (Frey et al., 2016). Since bacteria are sensitive to environmental changes, they can be considered as important indicators for assessing soil nutrient cycling and maintaining ecosystem balance (Shen et al., 2013; Deng et al., 2019). Elevation gradients provide geographic variation that can coincide with changes in environmental factors such as temperature, precipitation, vegetation type, and soil properties (Ren et al., 2021). These represent a natural experimental platform for assessing how soil bacterial communities respond to environmental change (Körner, 2007). There remains controversy regarding the patterns of changes in soil bacterial diversity and community structure along an altitudinal gradient, whose plot can produce declining (Nottingham et al., 2018), depressed (Shen et al., 2020), humped (Ren et al., 2018) or stepped curves of soil bacterial diversity along the altitudinal gradient. Soil bacterial community composition can differ significantly at different altitudes, but opposing results have been reported (Singh et al., 2014; Li et al., 2018). The functions of soil bacterial ecosystems are closely related to the structural characteristics of their communities (Bardgett and Van Der Putten, 2014). Shen et al. (2016) found that the abundance of soil bacterial functional genes in samples taken from the Changbai Mountains, China, tended to increase with elevation. An et al. (2021) also showed that the metabolic pathway genes of soil bacteria in the Sedi La Mountains, China, significantly changed along the altitude gradient and identified a strong correlation between the structure and function of the bacterial community. In general, the activity and structure of soil microbial communities and their diversity are influenced by a combination of biotic and abiotic factors that include plant species and the soil environment (Weng et al., 2021; Sui et al., 2022). At present, most available studies have focused on elevational gradients where there are large differences between vegetation and local ecosystem, which hampers interpretation of the results. The distribution patterns of bacterial community characteristics (diversity, structure and function) within ecosystems at different elevations but with the same vegetation type remain to be explored. Furthermore, there is uncertainty about how the structure and function of soil bacterial communities are related to each other.

Bacterial community composition significantly corresponds to vegetation and habitat selection (Chang et al., 2020), and therefore the composition and diversity of bacterial communities are strongly influenced by the composition of above-ground vegetation and soil environmental conditions (Sui et al., 2021). Previous studies have shown that changes in vegetation type and plant biomass on elevational gradients impact bacterial community diversity and structure by altering soil nutrient inputs (Xu et al., 2014). The vegetation type along an elevation can also affect the local input of organic matter into the soil and this potentially alters the soluble organic carbon content of soils, which in turn has a significant impact on the structure and function of soil bacterial communities (Shen et al., 2016). However, Carney and Matson (2006) showed that plant diversity only had a weak effect on soil microbial biomass. Consequently, the complexity of above-ground ecosystem species makes the interpretation of below-ground ecosystems more challenging (Bardgett et al., 1998). It can be expected that changes in vegetation type result in a more complex response of soil bacterial community characteristics to elevation.

The Changbai Mountains in the northeast of China represent an area with rich and well-preserved subtropical biodiversity gene pools at the same latitude. The area is sensitive to climate changes and is a current hotspot for research (Ping et al., 2017). Several studies have been conducted on the effects of soil microbial diversity and community composition at different altitude gradients in Changbai Mountains. For example, Han et al. (2018) compared the soil of broadleaf Korean pine forests at different altitudes and found that the composition and diversity of the bacterial communities were mutually influenced by soil physicochemical properties and the composition of above-ground vegetation. Zhang et al. (2022) reported that soil microbial community structure and enzyme activity in the vertical zone of the Changbai Mountains were closely related to vegetation community composition, soil environmental factors, and hydrothermal conditions. Yang et al. (2017) showed that different altitudinal gradients in Changbai Mountains affected above-ground vegetation composition, leading to changes in soil fungal communities and diversity through plant litter. Clearly, current research on soil microorganisms at different altitude gradients in the Changbai Mountains has been influenced by the composition of the above-ground vegetation, and the effect of above-ground vegetation composition on soil microorganisms could not be eliminated. The effect of different elevation gradients on soil microorganisms under the same conditions of above-ground vegetation remains to be determined.

The deciduous tree *Tilia amurensis* Rupr. is dominant at a wide altitude distribution on the northern slopes of Changbai Mountains (Kang et al., 2021). It is an important species in the Korean pine forests and plays important roles in biogeochemical

cycles, biodiversity, and other ecological functions and services of Korean pine forest ecosystem. The main distribution elevations are from 700 to 1,000 m in Changbai Mountain. Therefore, this supply an ideal field platform for us to investigate the distribution regulation of soil microbial diversity, composition and structure along with elevation gradations. So, this study investigated soil sampled between 700 m and 1,000 m altitude from the northern slopes of Changbai Mountains where the vegetation composition was identical (i.e., *T. amurensis*). The questions addressed were: (1) How do the soil bacterial community characteristics (diversity, structure and function) change along an altitudinal gradient under the same vegetation? (2) What are the driving factors for the elevational distribution patterns of soil bacterial community characteristics? In order to solve above questions, the high-throughput sequencing technology was applied to analyze the structural composition, diversity and function of soil bacterial communities from 700 m to 1,000 m under an identical trees (*Tilia amurensis*, which is a typical tree in these elevations). The results contribute to a better understanding of the bacterial diversity in soil and the ecological roles of bacterial communities, and assist in elucidating the driving factors shaping soil bacterial communities related to elevation in Changbai Mountains.

Materials and methods

Research site

This study location is within the Changbai Mountain Nature Reserve (126°55′–129°00′E; 41°23′–42°36′N) in Jilin Province, northeast of China, which belongs to a typical temperate climate with cold winters and warm summers. The average annual temperature is 4.3°C, and the average annual precipitation is 745 mm. The growing season lasts from May to September. The soil is dark brown soil developed from volcanic ash.

Four elevations were selected: 700, 800, 900, and 1,000 m above sea level, located on a north slope of Changbai Mountains. The GPS position of each elevation showed in Supplementary Table S1. The forest types between four elevations were Broad-leaved Korean Pine Forests. The dominant vegetations of 700 m were *Pinus koraiensis*, *Acer mono*, *Tilia amurensis*, *Fraxinus mandshurica*, *Quercus mongolica*, *Populus ussuriensis*. The dominant vegetations of 800 m were *P. koraiensis*, *Betula platyphylla*, *T. amurensis*, *F. mandshurica*, *Abies nephrolepis*, *Phellodendron amurense*, *Picea koraiensis*. The dominant vegetations of 800 m were *P. koraiensis*, *T. amurensis*, *Q. mongolica*, *A. nephrolepis*, *P. jezoensis*, *A. mandshuricum*, *A. mono*, *Ulmus laciniata*, *Larix gmelinii*, *B. costata*, *P. davidiana*. The dominant vegetations of 800 m were *P. koraiensis*, *P. jezoensis*, *A. nephrolepis*, *A. mono*, *T. amurensis*, *F. mandshurica*, *P. cathayana*, *L. gmelinii*, *A. tegmentosum*. For each elevation the above-ground vegetation was inspected and five plots were identified per elevation that all contained the same vegetation (*T. amurensis*). In October 2017, soil samples were collected at a depth of 0–20 cm with an 8 cm diameter soil auger from 10 to 15 locations along an S-shaped path. After that, the soils (10–15 soil samples) were mixed into one sample. Therefore, we finally had 5 mixed soil samples in each elevation. After removal of the surface litter and humus layer, the sampled soil sample was mixed per plot. Plant rests and coarse material was removed and the soil was homogenized and sieved through 2 mm meshes before transfer to the lab. There, the samples were divided into two parts; one was stored in the −80°C for soil microbial analysis and the other was air-dried for soil physicochemical properties analysis.

Measurements of soil chemical properties

Soil moisture content (SMC) was determined by weight loss following desiccation. The soil organic carbon (SOC) and total nitrogen (TN) contents were determined using an elemental analyzer (Flash 2000, Thermo Fisher, Austria). The total phosphorous (TP) content was determined by the digestion method of concentrated sulfuric acid and perchloric acid treatment (Adeloju et al., 1984), and NH_4^+-N and NO_3^--N were extracted with 2 mol·L^{-1} KCl (Miranda et al., 2001) and measured calorimetrically using an automated ion analyzer (Skalar San++, Holland, Netherlands). The contents of microbial biomass C (MBC) and N (MBN) in the soil were determined by a TOC analyzer (TOC-LCPH, Shimadzu, Japan). After mixing water and soil at a ratio of 2.5:1 (w/w), the soil pH was measured with a pH meter. Total potassium (TK) and the available potassium (AK) and the available phosphorus (AP) fraction were following a NaHCO3 extraction (Olsen et al., 1954). TK, AK and AP were determined using inductively coupled plasma atomic emission spectrometry (ICP-AES-7500, Shimadzu, Japan).

Soil DNA extraction and Illumina MiSeq sequencing

Total DNA was extracted from the soil with an E.Z.N.A.™ Soil DNA kit (Omega Biotek, Norcross, GA, United States). The V3-V4 region of the bacterial 16S rDNA gene was amplified by PCR using bacterial universal primers 338F (5′-ACT CCT ACG GGA GGC AGC A-3′) and 806R (5′-GGA CTA CHV GGG TWT CTA AT-3′; Liu et al., 2016). Each PCR had three amplification replicates and then mixed these three replicates into one PCR mixed for sequencing. So finally there were five PCR products ready to sequence. The PCR amplification reaction was carried out using TransGen AP221-02 with TransStart Fastpfu DNA Polymerase. The total volume was 20 µl contained 4 µl 5 × FastPfu buffer, 2 µl 2.5 mmol·L^{-1} dNTPs, 1.0 µl each of 5 µmol·L^{-1} upstream primer 338F and downstream primer 806R, 0.4 µl FastPfu polymerase, 0.2 µl BSA, and 20 ng DNA template. The amplification protocol was 95°C for 5 min, followed by 33 cycles with 30s at 95°C, 20s at 55°C, and 45 s at 72°C and final extension

of 10 min at 72°C. Triplicate amplification products of the same sample were mixed and checked by electrophoresis on a 2% agarose gel, excised and were recovered with a TAKARA DNA Gel Extraction kit (TAKARA Biosciences, Japan). The purified amplicons were subjected to high-throughput sequencing using the Illumina MiSeq platform. The high-throughput sequencing was performed by the Biomarker Technologies Company (Beijing China).

Analysis of the sequencing data and statistical analysis

Sequences were analyzed using QIIME (version 1.17, http://qiime.org) software on the Biomaker bioinformation cloud platform.[1] Forward and reverse reads were merged using the PEAR software (version 0.9.8). The merged reads were removed if the mean quality score < 20 or the length < 200 bp and the ambiguities were also removed. Chimeras were removed using Usearch software (version 7.1, https://www.drive5.com/usearch/). Exact barcode matching was implemented, which allowed for a two-nucleotide mismatch during primer matching. Operational taxonomic units (OTU) were generated at a similarity level of 97% for which the RDP (ribosomal database project) classifier Bayesian algorithm was used (Wang et al., 2007). Taxonomic analysis was performed on the representative sequences of OTU, with a confidence threshold of 0.7, and the Silva 132/16 s bacteria database was used for comparison.[2] Before calculate the alpha diversity, we first normalized the reads according to the lowest number of reads. The alpha diversity indices Ace, Chao1, Shannon and Simpson were calculated. R software (R Development Core Team, 2016) was used to produce graphs and Principal Coordinates Analysis was used to calculate the beta diversity distance matrix based on Bray–Curtis dissimilarity (Frey et al., 2021). One-way ANOVA and Duncan's tests were performed to detect significantly different phyla and genera and physicochemical properties for the different elevations using SPSS 22.0 (IBM SPSS Statistics for Windows). Linear discriminant analysis (LDA > 4.0) effect size (LEfSe) analysis, based on the OTU table, was performed by R software using the microeco package and this was also used for a Mantel test on the relationship of soil physicochemical parameters and soil bacterial community. A correlation heatmap for soil physicochemical properties with the most abundant 30 genera was likewise produced. The functional prediction of soil bacteria was based on the FAPROTAX code and database that were downloaded at http://www.loucalab.com/archive/FAPROTAX/lib/php/index.php?section=Download. The heatmap of functions were calculated *via* R software using "microeco" package.

1 https://international.biocloud.net

2 https://www.arb-silva.de/

Results

Soil physicochemical properties

Table 1 shows that no significantly differences were observed ($p > 0.05$) at different altitudes for soil pH (range: 5.48–6.15), and contents of TK (2.74–4.21 g·kg^{-1}), TP (0.05–0.8 g·kg^{-1}), AP (9.74–16.12 mg·kg^{-1}), and SOC (10.77–17.31 g·kg^{-1}). Other physical and chemical properties were significantly different ($p < 0.05$). A relatively wide range was observed for NH_4^+ (18.48–59.96 mg·kg^{-1}) and SMC (19.17%–49.08%), while NO_3^- (2.37–11.32 mg·kg^{-1}), TN (11.69–33.27 g·kg^{-1}) and AK (12.28–21.70 mg·kg^{-1}) also significantly varied. The microbial parameters MBC also strongly varied, with a range of 55.52–510.54 mg·kg^{-1} for MBC and a range of 7.30–54.87 mg·kg^{-1} for MBN (Table 1).

Bacterial alpha diversity

The variable region of bacterial 16S rDNA was amplified from DNA isolated from the soil samples and sequenced to compare the bacterial communities that were present (Table 2). From the obtained sequences, diversity indices were calculated (Table 2). The Shannon index did not vary significantly between the four elevation gradients ($p > 0.05$), but the abundance indices (Richness, Chao1, and ACE index) all varied significantly ($p < 0.05$). The Chao1 index showed significant differences at 900 m and 1,000 m, whereas no differences were observed at other altitudes; the ACE index was significant different at 900 m and 1,000 m, and the Richness index differed between 700 and 900 m (Table 2).

Correlation analysis between these indices and the soil physicochemical properties revealed a strongly significant positive correlation between the Shannon index and soil pH (Supplementary Table S2, $p < 0.01$), while the Richness, ACE and Chao1 indices were strongly negatively correlated with soil NH_4^+, and SMC ($p < 0.01$). Significant negative correlations were also observed between these three indices and MBC ($p < 0.05$, Supplementary Table S2).

Bacterial beta diversity

The bacterial community structure along the altitudinal gradient was graphically compared by Principal Coordinates Analysis (Figure 1). The five soil samples per altitude were separately analyzed. The ordination showed that the bacterial communities were clearly separated by altitude, and indeed the PERMANOVA analysis of the bacterial community among 700 m, 800 m, 900 m and 1,000 m was significantly different ($F = 2.847$, $p < 0.01$).

TABLE 1 Soil physicochemical characteristics along an altitudinal gradient in the Changbai Mountains, Northeastern China.

Elevation (m)	pH	NH_4^+ (mg·kg^{-1})	NO_3^- (mg·kg^{-1})	SMC (%)	TN (g·kg^{-1})	TK (g·kg^{-1})	TP (g·kg^{-1})	AK (mg·kg^{-1})	AP (mg·kg^{-1})	MBC (mg·kg^{-1})	MBN (mg·kg^{-1})	SOC (g·kg^{-1})
700	5.48 ± 0.28a	53.06 ± 7.05a	5.99 ± 1.48ab	48.19 ± 1.49a	26.64 ± 7.07ab	4.21 ± 0.47a	0.06 ± 0.01a	21.09 ± 3.29a	10.28 ± 1.93a	510.54 ± 76.55a	54.87 ± 10.44a	13.99 ± 0.97a
800	6.05 ± 0.23a	59.96 ± 5.69a	6.61 ± 2.09ab	38.52 ± 2.25b	33.27 ± 4.63a	3.44 ± 0.71a	0.08 ± 0.02a	21.70 ± 2.60a	16.12 ± 1.95a	420.52 ± 25.92a	49.12 ± 3.11b	17.31 ± 1.74a
900	5.88 ± 0.17a	18.48 ± 1.55b	2.37 ± 0.28b	19.17 ± 1.09c	11.69 ± 2.26b	2.74 ± 0.33a	0.05 ± 0.00a	12.28 ± 0.96b	9.74 ± 2.98a	55.52 ± 11.85c	7.30 ± 0.90c	10.77 ± 1.39a
1,000	6.15 ± 0.11a	54.97 ± 9.80a	11.32 ± 3.18a	44.59 ± 3.71ab	18.02 ± 8.12ab	2.74 ± 0.84a	0.07 ± 0.01a	18.21 ± 2.87ab	14.48 ± 2.20a	249.32 ± 25.54b	26.41 ± 3.81b	15.85 ± 1.22a

Values represent means ± standard deviations ($n = 3$). Different letters indicate significant ($p < 0.05$) differences between individual means assessed by one-way ANOVA followed by Tukey's HSD *post-hoc* testing. NH_4^+, ammonium nitrogen; NO_3^-, nitrate nitrogen; SMC, soil moisture content; TN, total nitrogen; TK, Total potassium; AK, available potassium; AP, available phosphorus; MBC, Microbial biomass carbon; MBN, Microbial biomass nitrogen; SOC, soil organic carbon.

TABLE 2 Bacterial α diversity in the soil collected at different altitudes.

Elevation (m)	Richness	Chao1	ACE	Shannon
700	1,666 ± 51.3b	1739.9 ± 18.9ab	1726.5 ± 16.90b	6.4 ± 0.06a
800	1707 ± 28.6ab	1758.1 ± 13.6ab	1746.7 ± 13.0ab	6.4 ± 0.02a
900	1738 ± 18.3a	1787.4 ± 8.6a	1776.4 ± 8.3a	6.45 ± 0.02a
1,000	1,680 ± 44.8bab	1746.37 ± 13.2b	1728.4 ± 15.1b	6.41 ± 0.03a

Values represent means ± standard deviations ($n = 5$). Different letters indicate significant ($p < 0.05$) differences between individual means assessed by one-way ANOVA followed by Tukey's HSD *post-hoc* testing.

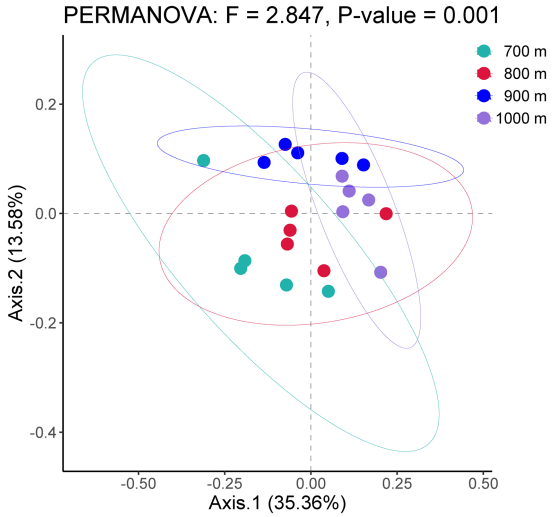

FIGURE 1
Principal coordinates analysis of bacterial communities in the soil at four altitudes with similar vegetation cover in Changbai Mountains, Northeastern China.

Composition of the soil bacterial community

The obtained sequences were attributed to OTUs to investigate the bacterial community composition. A total of 52 bacterial phyla were identified; sequences that could not be classified to a known phylum were collectively reported as 'others' (Figure 2A). The highest relative abundance (r.a.) was observed for Proteobacteria. Other phyla reaching r.a. > 1% were, in descending order: Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Nitrospirae, Gemmatimonadetes, and Planctomycetes, and this order did not vary with altitude, although variation in r.a. between the elevations was observed (Figure 2A). The r.a. of the 8 phyla (relative abundance > 0.1%) at each altitude is summarized in Table 3. Significant differences in r.a. between the four elevations was found for the phyla Gemmatimonadetes and Latescibacteria (highest r.a. at 900 m and lowest at 700 m for both), Firmicutes (highest at 900 m and lowest at 1,000 m), Parcubacteria, Actinobacteria and WS2 (highest at 1,000 m and lowest at 700 m for all three; Supplementary Figure S1, $p < 0.05$).

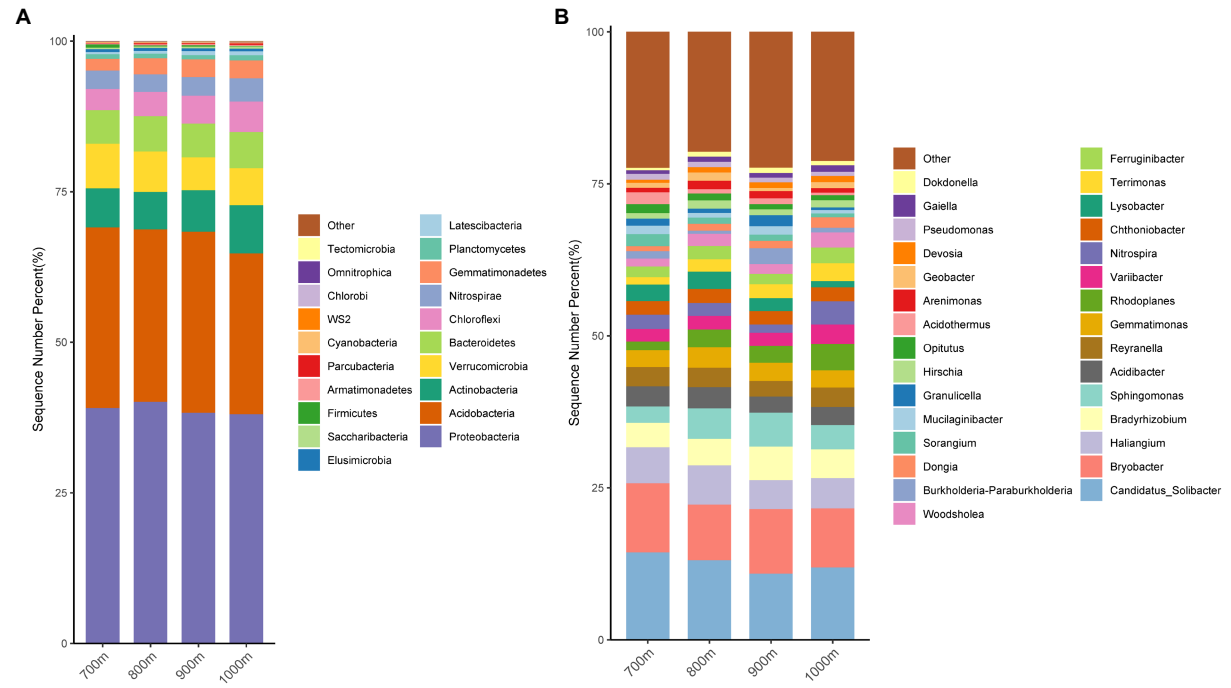

FIGURE 2
Relative abundance of the dominant bacterial phyla **(A)**, and genera **(B)**, in soils collected at the four indicated altitudes in the Changbai Mountains, Northeastern China.

TABLE 3 Relative abundance of the dominant bacterial phyla (top) and genera (bottom) in the soil along an altitudinal gradient in the Changbai Mountains, northeastern China.

Phylum	700 m	800 m	900 m	1,000 m	*p*
Proteobacteria	39.1%	40.0%	38.3%	38.0%	
Acidobacteria	30.0%	28.7%	30.0%	26.8%	
Verrucomicrobia	7.4%	6.8%	5.4%	6.2%	
Actinobacteria	6.5%	6.2%	6.9%	8.0%	<0.05
Bacteroidetes	5.6%	5.8%	5.6%	6.0%	
Chloroflexi	3.5%	4.0%	4.6%	5.1%	
Nitrospirae	3.1%	2.9%	3.1%	3.9%	
Gemmatimonadetes	1.9%	2.7%	2.9%	3.0%	<0.05
Genus (>1%)					
Variibacter	2.0%	2.2%	2.2%	3.2%	<0.05
Rhodoplanes	1.4%	2.9%	2.7%	4.4%	<0.05
Lysobacter	2.7%	2.9%	2.2%	1.0%	<0.05
Woodsholea	1.2%	2.0%	1.6%	2.5%	<0.05
Dongia	0.8%	1.1%	1.2%	1.7%	<0.05
Terrimonas	1.1%	2.0%	2.2%	2.9%	<0.05
Sorangium	2.0%	1.0%	1.0%	0.6%	<0.05
Nitrospira	2.1%	2.1%	1.3%	3.7%	<0.05

The r.a. of the other phyla listed in Table 3 did not differ significantly with altitude ($p > 0.05$).

The sequences were also analyzed at the genus level (Figure 2B). For all four altitudes, the most abundant genera were (in descending order) *Candidatus Solibacter*, *Bryobacter*, *Haliangium*, *Bradyrhizobium*, *Sphingomonas*, *Acidibacter*, *Reyranella*, *Gemmatimonas*, *Rhodoplanes*, and *Variibacter*, which in combination accounted for 50% or more of the identified genera (Figure 2B). Ten more genera reached a relative abundance >1% (Figure 2B). Statistically significant differences ($p < 0.05$) in r.a. between the altitudes was observed for the genera *Variibacter*, *Rhodoplanes*, *Lysobacter*, *Woodsholea*, *Dongia*, *Terrimonas*, *Sorangium*, and *Nitrospira* (Table 3, $p < 0.05$).

A total of 52 genera were identified in the four elevations (Figure 3), as indicated by LDA effect size scores of >4.0. Indicator genera were identified: *Acidothermus* for 700 m, *Lysobacter* for 800 m, *Bauldia* and *Granulicella* for 900 m and *Rhodoplanes*, *Variibacter*, *Dongia*, and *Woodsholea* were indicator genera for 1,000 m.

Relationship between bacterial community structure and soil physicochemical properties

A Mantel analysis of soil bacterial OTU levels and environmental factors showed that pH was the main soil factor affecting the bacterial communities (Table 3, $p < 0.05$), with differences noted at the different altitudes: strong correlations were seen at 800 m and 900 m for pH ($p < 0.01$) but the correlation was weaker at 1,000 m ($p < 0.05$) and not significant at 200 m.

The correlations between the relative abundance of the top 20 bacterial phyla and the soil physicochemical properties for all altitudes combined is graphically summarized in a heat map

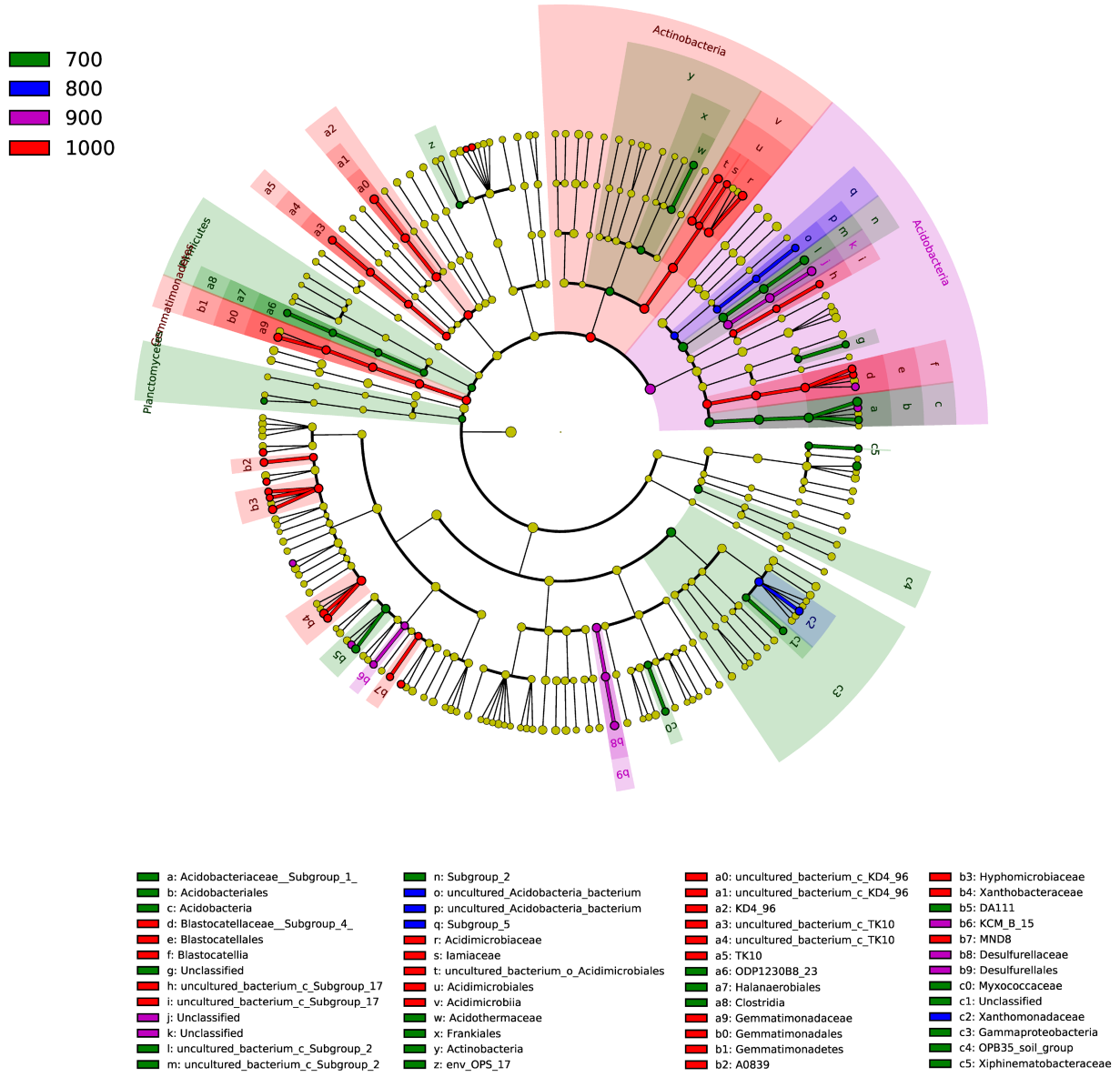

FIGURE 3
Cladogram of the soil bacterial communities with LDA>4.0 in the four elevations. The circular plot shows bacterial taxa from phyla to genera starting from the center.

(Figure 4). A strong positive correlation was observed between Verrucomicrobia and pH, Fircumicutes and TN, as well as Omnitrophica and AP (Figure 4). Strong negative correlations were observed for Proteobacteria and NH_4^+, SMC and AK together with weaker correlation for AP and NO_3^-. Strong negative correlations were also observed for Bacteroidetes with AP. Acidobacteria correlated strongly negatively with NH_4^+ and AP. Weaker negative correlations were observed for Chloroflexi with soil MBN, and SMC for Gemmatimonadetes with SOC and NH_4^+; for Latescibacteria with MBN, and NH_4^+. Weaker positively correlations were observed for Fimicutes with soil TN, and Verrucomicrobia with pH.

The correlations between genera and the soil characteristics are summarized in Supplementary Figure S1. The soil factors affecting relative abundance of multiple genera were pH, MBC, MBN, NO_3^-, AK, and SMC, and these produced positive or negative correlations, depending on the genus (Supplementary Figure S1). TK was the only factor correlating with a single genus (*Pedobacter*). The correlation patterns for the various genera of NO_3^-, NH_4^+, AK, and SMC clustered together, as did the patterns for MBC and MBN (Supplementary Figure S1).

Inferred functionality of soil bacteria and their differences between different altitudes

FAPROTAX analysis was used to infer a total of 39 functional groups, and their relative abundance at the different

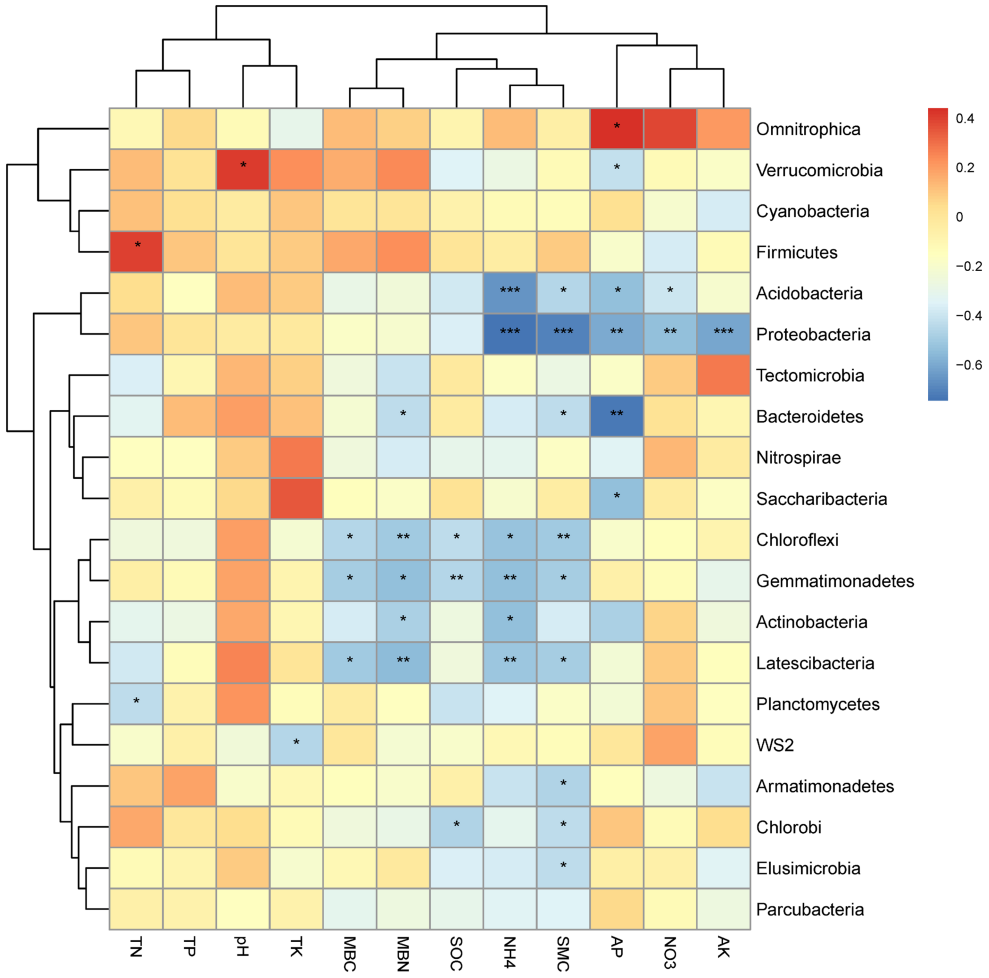

FIGURE 4
Heat map of the correlations between soil physicochemical properties and the top 20 bacterial phyla, with significance indicated as * at the 0.05 level (one-tailed) and ** at the 0.01 level (two-tailed).

altitudes were compared in a heatmap (Figure 5). Chemoheterotrophy was the most dominant functional category, followed by aerobic chemoheterotrophy and nitrification, but they did not correlate with altitude as they were found across the elevation gradient ($p > 0.05$, Figure 5). Many functional categories identified in the microbial communities of the soil collected at 800 m and 900 m resembled each other, while those at 700 m was quite distinct. As expected, trends along the elevation gradient were similar for the functions of nitrate denitrification, nitrite denitrification, nitrous oxide denitrification, denitrification, nitrate_respiration, and nitrogen respiration, as these are all involved in related N metabolic processes. Those trends resembled the trends for anoxygenic photoautotrophy S oxidizing, anoxygenic photoautotrophy, photoautotrophy, photoheterotrophy, and phototrophy. Other functions that varied along the gradient were sulfur_respiration/respiration of sulfur compounds, chitinolysis, nitrite respiration, cellulolysis, animal parasite or symbionts, and nitrate reduction ($p < 0.05$, Figure 5).

Discussion

Effects of elevation on bacterial alpha diversity

A number of studies have suggested that specific soil microbial communities exist at specific altitudes (Liu et al., 2013; Shen et al., 2013; Wang et al., 2015). The present study showed that soil bacterial abundance changed under different altitude gradients, but the diversity of the community did not change (Figure 1). In our study, the above-ground vegetation did not vary between altitudes, which might explain why the soil bacterial Shannon index did not change significantly. Further, at the investigated elevation between 700 and 1,000 m, elevation may not be the main driver of Shannon diversity in soil bacteria. The observed changes in ACE and Chao richness indices may be attributed to the effect of soil physicochemical factors. Our results show that SMC, NH_4^+ and MBC significantly and negatively correlated with soil ACE and Chao1 indices. This

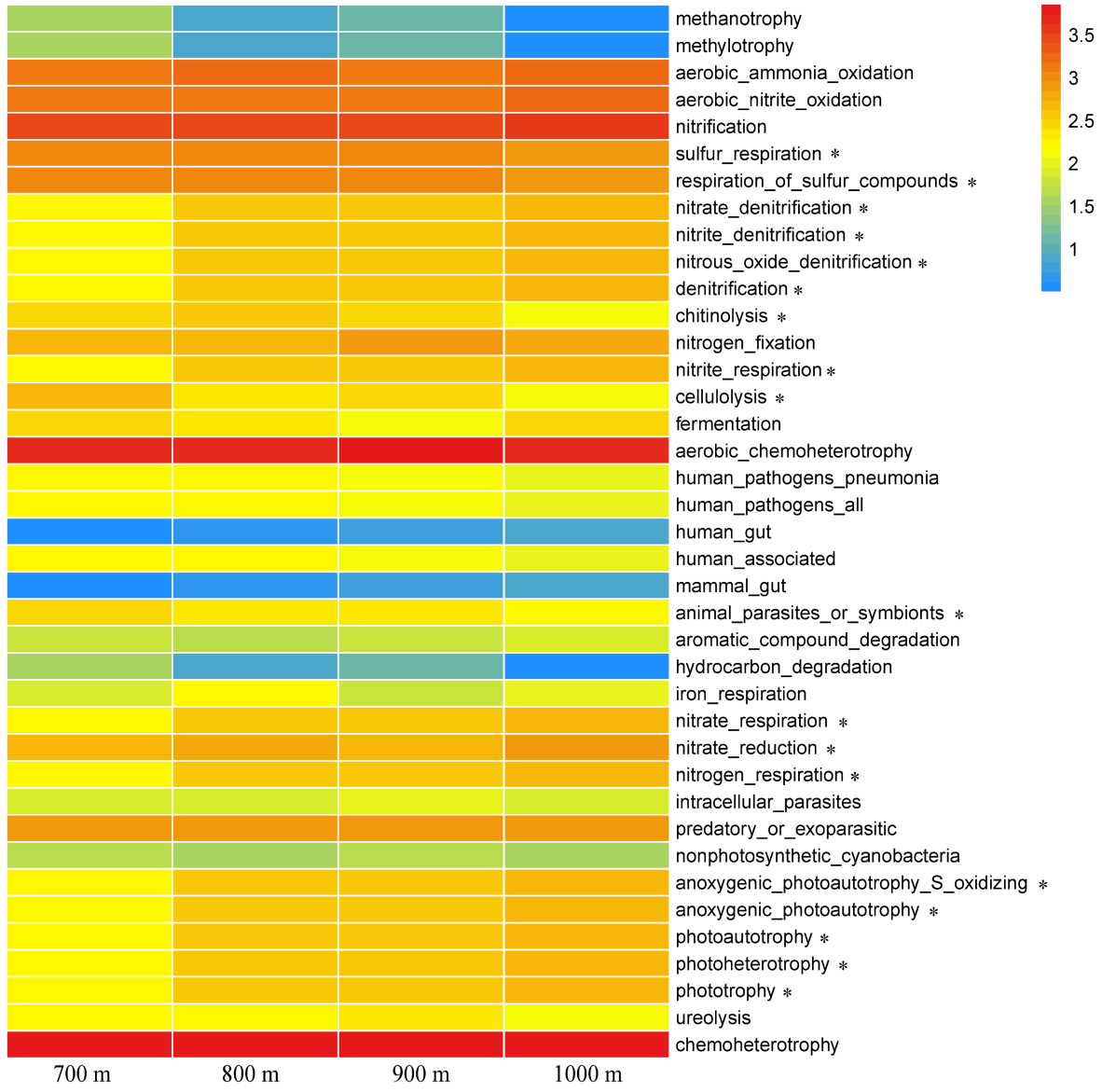

FIGURE 5
Heatmap of functional processes inferred from the relative abundance of the soil bacteria at the five different elevations. The color gradient (red, yellow, and blue) represents the relative abundance of the inferred bacterial functions from high to low. * indicated difference at the 0.05 level based on one-way ANOVA.

indicates elevation can affect changes in soil physicochemical factors that influence the abundance of bacteria. Han et al. (2018) studied the diversity of soil bacteria between 699 and 1,177 m in broadleaf Korean pine forests in Changbai Mountains and reported no change in the Shannon diversity index, which is consistent with the results of this study: we found that altitude did not lead to changes in Shannon diversity of soil bacteria from 700 to 1,000 m altitude. However, whereas we detected variation in ACE and Chao indices, Han et al. (2018) did not observe such an effect. The discrepancy may be explained by soil physicochemical factors that remained unaltered in their comparison, as Ping et al. (2017) showed that soil TOC, TN, SMC, and pH were not significantly altered under broadleaf red pine forests at 699–1,044 m. Shen et al. (2013) found that soil bacterial PD diversity indices and OTUs did not change at 530–760 m in Changbai Mountains, probably because the low altitude scale did not significantly affect bacterial diversity. Our study only covered a difference of 400 m, which may also account for the fact that soil bacterial diversity was not significantly altered. Moreover, it is worth noting that Han et al. (2015) found a significant change in the functional Shannon diversity of soil bacteria at 699–1,177 m using the Biolog-Eco technique. This suggests that different technical tools may also have some influence on the results. Above studies showed that the above-vegetation and the analysis method would have the different results of soil microorganism. Collectively, the distribution of soil bacterial diversity and

composition along the altitudes is complex and bacterial communities respond to different driving processes at the altitudinal scale (Shen et al., 2019). The trend of the soil ACE abundance index along the altitude was also not consistent with Shannon, suggesting that a uniform pattern of bacterial diversity may be difficult to achieve along the altitude gradient.

Bacterial compositions in different altitudes in Changbai Mountains

Our study identified a number of dominant bacterial phyla whose relative abundance differed at different elevations, although they were not necessarily present in high abundance (Figure 2A, Table 3). The already mentioned study by Han et al. (2018) described as bacterial phyla Acidobacteria, Proteobacteria, Actinobacteria Verrucomicrobia, and Chloroflexi, which was mostly consistent with our results, but they identified Acidobacteria to differ at different altitudes, which is not what we found. Instead, among the dominant bacterial phyla we detected (>3% relative abundance), only Actinobacteria significantly varied (Table 3). Although the elevation gradients of both studies were similar, Han et al. (2018) did not consider the influence of aboveground vegetation. Soil bacteria are not only affected by changes in soil microhabitats caused by the elevation gradient, but also by aboveground plant diversity. The soil phylum acidobacteria, a relatively high abundant phylum in soil, may change due to the diversification of the composition of above-ground plant diversity. In the present study, the main soil bacterial phyla, such as Acidobacteria and Proteobacteria, did not change significantly because the above-ground vegetation was the same and therefore the effects caused by above-ground vegetation and litter could be ignored. As changes in soil bacterial species under the altitude gradient are also influenced by above-ground vegetation, studies in which above-ground vegetation is not uniform are difficult to interpret and compare.

A number of dominant genera were also detected at significantly different abundances along the altitude gradient, including *Variibacter*, *Rhodoplanes*, *Lysobacter*, *Woodsholea*, *Dongia*, *Terrimonas*, and *Sorangium*, while the most abundant genera were *Candidatus_Solibacter*, *Bryobacter*, *Haliangium*, *Bradyrhizobium*, *Sphingomonas*, *Acidibacter* (Figure 2B; Table 3). Surprisingly, in the comparable study by Han et al. (2018) the dominant bacterial genera were *DA101_soil_group_norank*, *Xanthobacteraceae_uncultured*, *Subgroup_6_norank* and *Bradyrhizobium* while Li et al. (2017) reported *Devosia*, *Dokdonella*, *Phaselicystis*, *Rhodobacter*, and *Conexibacter* as the main genera in the inter-rhizosphere soil community at 2,000 m of Changbai Mountain. This indicates a strong variation in composition of soil bacteria at the genus level between different locations and studies, even within the same area. Differences in temperature, soil nutrients and plant composition related to altitude all contribute to the distribution of soil bacteria, and differences become more apparent at lower taxonomic levels.

The relationships of bacterial structure and soil physicochemical properties in different altitudes

The soil bacterial community structure composition is regulated by soil physicochemical factors. The results of the mantel analysis in this study showed that soil physicochemical properties were significantly correlated with soil bacterial community structure (Table 3). The soil bacterial community structure at different altitude gradients was influenced by different soil physicochemical properties. At 700 m and 800 m, the soil bacterial community structure was correlated with MBC, MBN, TN, AK, TK, TP, AP, NO_3^-, MC, and NH_4^+; pH showed a positive correlation with the soil bacterial community structure at 1,000 m (Table 3). This indicated that the structural composition of the soil bacterial community is influenced by soil physicochemical factors and that the driving factors for soil bacteria are not the same at different altitudes. In mountain forest ecosystems, altitude can lead to dramatic changes in climatic factors such as temperature and precipitation (Chen et al., 2011), and microorganisms are extremely sensitive to environmental changes. Xia et al. (2022) found that soil microbial community structure was positively correlated with soil SOC and TN. This is inconsistent to the results of our study. Tan et al. (2013) found that changes in soil TP and AP levels affected the composition of soil bacterial communities. Shen et al. (2013) showed that soil microorganisms were most significantly influenced by pH. This indicated that the structure and diversity of soil microbial communities in a given area were influenced by a combination of factors (Liu et al., 2010).

In addition, according to Pearson correlation analyses, the 20 most abundant bacterial phyla were mainly influenced by NH_4^+-N, NO_3^--N, TK, MBC, MBN, TC, MC (Figure 4), and Proteobacteria, Acidobacteria, Bacteroidetes, Chlroflexi, Gemmatimonadetes, Latescibacteria, Actinobacteria, Planctomycetes, Armatimonadetes, Elusimicrobia, and Chlorobi all showed significantly negative correlations with soil nutrients. This is inconsistent with previous studies, for example Proteobacteria are eutrophic microorganisms, which have been shown a positive correlation with soil nutrients (He et al., 2021). This may be because at the bacterial phylum level, not all Proteobacteria may be constrained by nutrient content when nutrient availability is adequate. The Changbai Mountains is a volcanic mountain ecosystem (Zhang et al., 2022) with sufficient nutrient content to provide for the growth of Proteobacteria. It is also worth noting that our study was carried out on the composition of the soil bacterial community under *T. amurensis*, which may have affected the bacterial distribution. The mechanisms regulating the abundance of soil microorganisms under the trees need to be further investigated.

Changes in soil bacterial function under purple linden trees at different altitudes

FAPROTAX is an effective tool to predict the soil bacterial function (Louca et al., 2016), and this tool revealed bacterial functional groups that changed significantly with different elevations (Figure 5). It can be expected that changes in bacterial composition influence soil carbon and nutrient functions (Frey et al., 2016). Moreover, phototrophy, nitrification, nitrate respiration, and nitrite respiration play crucial functions in soil carbon and nitrogen cycling in 800–1,000 m altitudes, while functions related to parasites and cellulolysis and sulfur respiration may be more important in 700 m altitudes. Altitudes changes usually alter soil physicochemical properties, which sets broadleaf forests apart from conifer forests. Therefore, because of variation in soil nutrient contents, the soil bacterial functions may differ significantly between the four altitudes.

Conclusion

Soil bacterial richness differed significantly, but Shannon diversity did not change under same above-ground vegetation in Changbai Mountains, which supports our 1st hypothesis. Soil dominant phyla relative abundance were affected by soil nutrient (i.e., SMC, SOC, MBN, MBC, AP, NH_4, and NO_3), but the dominant genera relative abundance were affected by pH, MBC, MBN, NO_3, AK, and SMC. This showed that the composition of soil bacterial has different correlations with soil physicochemical parameters, which supports our 2nd hypothesis. This comprehensive study to characterize bacterial communities and their diversities at different elevations under same above-ground vegetation in Changbai Mountains revealed that the soil physiochemical characteristics have a significant effect on the overall diversity of bacterial communities along the elevation gradient, but from the available data it is difficult to predict overall bacterial diversity along elevation. Nevertheless, this study contributes to the understanding of bacterial community composition and diversity patterns at local altitudinal scales, and provides a theoretical basis for predicting the response, adaptation and feedback of microbial communities to changes in environmental conditions.

Data availability statement

The data presented in the study are deposited in the Sequence Read Archive repository, accession number PRJNA888966.

Author contributions

ML designed and performed the experiment and prepared this manuscript. GD helped to collect the soil samples and revised this manuscript. LM designed this experiment. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2022.1065412/full#supplementary-material

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EDITED BY
Puneet Singh Chauhan,
National Botanical Research Institute (CSIR), India

REVIEWED BY
Qibiao Sun,
Jiujiang University, China
Yanzhao Zhang,
Luoyang Normal University, China
Dalong Ma,
Harbin Normal University, China
Qin Yao,
Heilongjiang Bayi Agricultural University, China

*CORRESPONDENCE
Rongtao Zhang
✉ zhangrongtao14@163.com
Hongwei Ni
✉ nihongwei2000@163.com
Mai-He Li
✉ maihe.li@wsl.ch.

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Soil Acidobacterial community composition changes sensitively with wetland degradation in northeastern of China

Xin Sui[1,2,3,4], Beat Frey[3], Libin Yang[4], Yingnan Liu[4], Rongtao Zhang[4]*, Hongwei Ni[5]* and Mai-He Li[3,6,7]*

[1]Engineering Research Center of Agricultural Microbiology Technology, Ministry of Education, Heilongjiang University, Harbin, China, [2]Heilongjiang Provincial Key Laboratory of Ecological Restoration and Resource Utilization for Cold Region, School of Life Sciences, Heilongjiang University, Harbin, China, [3]Snow and Landscape Research WSLSwiss Federal Institute for Forest, , Birmensdorf, Switzerland, [4]Institute of Nature and Ecology, Heilongjiang Academy of Sciences, Harbin, China, [5]Heilongjiang Academy of Forestry, Harbin, China, [6]Key Laboratory of Geographical Processes and Ecological Security in Changbai Mountains, Ministry of Education, School of Geographical Sciences, Northeast Normal University, Changchun, China, [7]School of Life Science, Hebei University, Baoding, China

Acidobacteria are a major component of the soil bacteria and are conducted for many soil functions, and the soil Acidobacterial structure and diversity are affected by climate changes and human activities. However, soil Acidobacterial structure and diversity in wetland ecosystems are still limited recognized. The current study aimed to study the Acidobacterial community and diversity in relation to soil environmental factors along a typical degradation series from primitive wetland to forest in a representative fresh wetland in northeastern China. In this research, we assessed the soil Acidobacterial community composition, using Illumina MiSeq sequencing along a typical degradation series from primitive wetland to forest in a representative fresh wetland in northeastern China. The soil physico chemical properties changed significantly among the eight degrade stages ($p < 0.05$). The α diversity index (Shannon and Chao1 index) of soil Acidobacteria changed significantly between different degradation stages ($p < 0.05$). Principal Coordinates Analysis (PCoA) revealed that the soil acidobacteiral communities obviously separated into wetland group and forest group. The most abundant subgroups of Acidobacteria accounted for 31% (Gp1), 5% (Gp2), 12% (Gp3), 2% (Gp4), 5% (Gp6), and 2% (Gp7) in soils within eight successional series. The compositions of soil Acidobacteria in wetland stages were significantly affected by soil moisture content, soil total nitrogen and available nitrogen contents, while those in forest stages were significantly driven by soil pH, soil organic carbon, total nitrogen, available phosphorus and soil moisture content. Our results indicated that the soil Acidobacterial community was mainly structured by soil physico chemical parameters, and wetland degradation towards forests will greatly influence the soil Acidobacterial structure and thus the wetland functions.

KEYWORDS

Sanjiang plain, soil bacterial diversity, β diversity, high-throughput sequencing, forest, community structure

Introduction

Acidobacteria are a major bacterial phylum recently classified based on research of molecular ecology and widely distributes in the natural soil and the proportion of Acidobacteria is almost same with Proteobacteria (Janssen, 2006; Wang et al., 2010, 2016). Acidobacteria plays a crucial role in soil element cycle and ecological function (Ward et al., 2009; Liu et al., 2014), such as cellulose, methylcellulose, xylan and pectin (Pankratov et al., 2008; Eichorst et al., 2011). Blöthe et al. (2008) found that Acidobacteria can participate in iron circulating. Radajewski et al. (2002) also indicated Acidobacteria may be participated in the carbon metabolism. However, due to its difficult cultivation, the ecological functions of Acidobacteria are still very limited.

Acidobacteria can be divided into 8 different subgroups from Gp1 to Gp8, most of which are acidophiles (Wang et al., 2010). The composition of vegetation has an important influence on the composition of Acidobacteria. For example, Naether et al. (2012) used primers 31F/1492R to amplify and construct a clone library of Acidobacteria in soils collected from different grasslands and forests in Germany. They found that Acidobacteria in forest soils were mainly Gp1 (relative abundance: 26–85%), Gp6 (1–41%), Gp3 (7–11%), Gp4 (6%) and Gp5 (12–13%), while those in grasslands were mainly Gp1 (59–62%), Gp4 (8–20%), Gp5 (3–17%), and Gp17 (6–7%) (Naether et al., 2012). Fierer et al. (2011) used high-throughput sequencing and cloning methods to investigate the distribution of Acidobacteria in 87 forest soils collected from North and South America and found Acidobacteria accounted for 30.9% of the total bacteria identified, and there were 8,600 Acidobacterial genotypes, and the relative abundance of different Acidobacterial subgroups ranged from 2.4 to 78.5% following a decreasing order of Gp4 > Gp1 > Gp3 > Gp2 > Gp6. Zhang et al. (2014) used high-throughput sequencing technology and found that Acidobacteria in forest soils had at least 4,480 OTUs, of which Gp1, Gp2, Gp3, Gp4, and Gp6 were the dominant subgroups accounting for 85% of the total Acidobacteria. Wang et al. (2010) used high-throughput sequencing technology and discovered Gp1, Gp2, Gp3, and Gp5 were the principal Acidobacteria in forest soils. Liu et al. (2014) and Yao et al. (2021) found that Gp1, Gp3, Gp4 and Gp6 were the vital Acidobacteria in mollisol of northeastern China. For wetland ecosystems, Pankratov et al. (2008) studied the Acidobacteria of Sphagnum-dominated wetlands of West Siberia and European North in Russia and found that Gp1 and Gp3 are the dominant Acidobacteria in wetland soils. Sui et al. (2019) found that Gp1, Gp2, Gp3, Gp6, and Gp7 were the dominant Acidobacteria in the wetland soil.

The composition of Acidobacteria correlates with soil physicochemical properties. Some previous studies reported soil pH was a vital environmental factor affecting the Acidobacterial composition. For example, Fierer et al. (2011) indicated that the composition of Acidobacteria was affected significantly by soil pH, and the same results were also reported in other studies (Männistö et al., 2007; Jones et al., 2009; Griffiths et al., 2011; Navarrete et al., 2013). However, Liu et al. (2014) found the composition of Acidobacteria did not correlate with soil pH. Fierer et al. (2011) also proved soil organic carbon and soil C/N ratio were the key soil environmental factors affecting the soil Acidobacterial composition. The occurrence of these results perhaps related to the variations in the response of different subgroups of Acidobacteria, or even different Acidobacterial species in same subgroup, to soil environmental factors. The distribution of phylum of Acidobacteria, different subgroups of Acidobacteria, and even different Acidobacterial genotypes (OTUs) in the same subgroup varies greatly in soil, and the distribution of Acidobacteria is regulated by a variety of environmental factors. Therefore, we speculate that the compositions of soil Acidobacteria are varied among different vegetations and soil properties according to the studying ecosystems, and more work is needed to discover the compositions and diversity of Acidobacterial communities in soils.

Through an Illumina MiSeq Sequence technique, we examined the composition and diversity of bacterial and fungal communities across the wetland gradient types in Sanjiang plain (Sui et al., 2021), which is one of the most important wetland ecosystem for northeast of China (Wang et al., 2022). Our work revealed that Acidobacteria was the most abundant bacterial phylum in the wetland soils of Sanjiang plain (Sui et al., 2019, 2021). Moreover, we also found that wetland degradation resulted in the composition of Acidobacteria in forest type was higher than that of wetland type and the driving physicochemical parameters were also significant different (Sui et al., 2021). Given the knowledge of soil Acidobacteria in most studies were retrieved from high-throughput sequencing data using universal bacterial primers (Lauber et al., 2008; Jones et al., 2009; Liu et al., 2014; Zhang et al., 2014), we worried that some of the valuable information about Acidobacteria in soils had not been well described.

Due to the serious agricultural activities and global climate changes, the water level of the wetlands in the Sanjiang Plain continues to decline, leading to significant degradation of the original wetlands. Sui et al. (2021) reported that wetland degradation resulted in the composition of Acidobacteria in forest type was higher than that of wetland type. Although the research on niche and lifestyle for the phylum Acidobacteria has been studied (Hartmann et al., 2017), but we still lack deep understand on the differential response at subgroup level to changes in soil physicochemical parameters, and how changes of Acidobacterial subgroups with vegetation change. In Sanjiang Plain Field Experiment Platform, there are original natural wetland (NW), shrub-invaded wetland (IW), shrub-dominant wetland (DW), wetland edge (EW), young-*Betula* forest (YB), mature-*Betula* forest (MB), *Populus* and *Betula* mixed forest (PB), and conifer forest (*CF*) within a small distance, and the soil physicochemical properties in different degradation stages also changed significantly. This provides a unique base for us to study the impact of wetland degradation on the composition and diversity of soil Acidobacteria. Therefore, we investigated the composition of Acidobacteria at eight stages of wetland degradation in

particular of the transition from wetland types to forest types. We hypothesized that: (1) the subgroups of Acidobacteria in soils change with wetland degradation from wetland (wetter) to forests (dry); and (2) soil physicochemical properties differently affect Acidobacterial structure between wetland and forests due to changes in soil water content. This study will provide basic data for further explaining the ecological functions of Acidobacteria in soil and also for in-depth revealing of the function of Acidobacteria in the element cycle in the process of wetland degradation and wetland ecosystems.

Materials and methods

Experimental site and soil sampling

This study was performed on in the Honghe National Nature Reserve (47°35'N, 133°31'E) of Sanjiang plain, China (Supplementary Figure S1). The mean annual temperature and precipitation is approximately and 1.9°C and 560 mm, respectively. Eight vegetation types were selected along slopes from the real wetland to the degraded wetlands for this study: i.e. NW, IW, DW, EW, YB, MB, PB, and *CF* (Supplementary Table S1).

In 2016, soils were collected from the eight vegetation types. Each vegetation type set three plots (20 m × 20 m). Ten to fifteen soil samples along an S-shaped path (0–20 cm) were sampled, using a sterile soil drill (5 cm in diameter, 20 cm deep) after removing the litter layer. The soil samples in each plot were pooled and mixed into one soil. The soil samples were then immediately transferred in refrigerator to keep at 4°C. The soil samples were sieved (2 mm mesh) to remove stones, roots and other debris, and divided into two parts-one was stored at −60°C for sequencing, and the other one was air-dried to conduct physicochemical analyses. For the above ground plant's diversity, the details and Shannon diversity were shown in the Supplementary Table S1 and the methods were in our previous study (Sui et al., 2021).

The soil physicochemical properties were described in our previous study (Sui et al., 2021). Briefly, the soil pH was measured using a pH meter and soil to water ratio of 1:2.5 w/v. Soil organic carbon and total nitrogen were measured using an elemental analyzer (Elementar, Langenselbold, Germany). Available nitrogen was examined with a continuous flow analysis (SAN++, Skalar Analytical, Netherlands). Soil moisture content (MC) measured gravimetrically. Total phosphorus was measured with a spectrophotometer. Available phosphorus was measured using a colorimetric method upon extraction with 0.5 M $NaHCO_3$.

Soil DNA extraction and PCR amplification

Soil total DNA was extracted using a MOBIO-12888 soil DNA Isolation Kit (Mo Bio Laboratories, Carlsbad, CA, United States). DNA quantity and quality were first detected by a garose gel electrophoresis (1%) and then detected by using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific Waltham, MA, United States). The special primers ACIDO (5′GCTCAGAATSAACGCTGG3′)/342r(5′CTGCTGCSYCCCGTAG3′) (~336 bp) were selected to amplify the Acidobacterial region (Lee and Cho, 2011). The PCR amplification system was conducted in a 25 μl reaction systems, consisted of 12.5 μl of PCR mix (Invitrogen, Shanghai, China), 1.5 μl of forward and reverse primers (10 μmol·L^{-1}), 1 μl of DNA template (100 ng·L^{-1}), and enough ultrapure water (ddH_2O) to reach a 25 μl reaction volume. The amplification program were the following: pre-denaturation at 95°C for 8 min, 30 cycles of denaturation at 95°C for 60 s, annealing at 55°C for 60 s, and extension at 72°C for 60 s, and finally an extension step at 72°C for 20 min. The PCR products were inspected by 2% agarose electrophoresis, and the PCR products were purified with the AxyPrep DNA purification kit (Axygen Biosciences, Union City, CA, United States). Three independent PCR replicates per sample and then three PCR samples were pooled at equal amounts and paired-end sequenced on the Illumina MiSeq v3 platform (2 × 300 bp).

Bioinformatics

QIIME Pipeline (version 1.8.0) was used to conduct the raw fasta sequences. Forward and reverse sequences were merged using the PEAR software (version 0.9.8). The sequences were removed if the mean quality score < 20 or the length < 200 bp and the ambiguities sequence were also removed by using the Trimmomatic (V0.33) software[1] (DeSantis et al., 2006). The chimeras were removed by Usearch (version7.1,[2]) (Edgar et al., 2011). Exact barcode matching was implemented, which allowed for a two-nucleotide mismatch during primer matching by using the QIIME Pipeline (version 1.8.0). The obtained operational taxonomic units (OTUs) were clustered by Uprase at similarity threshold of 97% (Li, 2015) and the taxonomy were annotated to the SILVA database (v138.1) (Quast et al., 2013). If the OTU did not belong to plylum Acidobacteria, the OTU will be removed before next analysis. Before further analysis of α diversity, the reads were normalized according to the lowest number of reads for a single soil sample.

Statistical analyses

The α diversity index were performed on QIIME1 platform at OTU level. The difference of Soil physicochemical properties, PD index, OTU, Chao1 and Shannon index in eight vegetation types

1 http://www.usadellab.org/cms/?page=trimmomatic

2 http://drive5.com/aparse/

were calculated by one-way ANOVA and Duncan test at a 0.05 significance level by using the software SPSS 17.0 software (SPSS Inc., Chicago, IL, United States). Principal Coordinates Analysis (PCoA) was also performed by using OTU table in the package of "vegan" R software (version 3.3.0; R Development Core Team, 2006) based on the Bray–Curtis dissimilarity (Hartmann et al., 2017; Frey et al., 2021). Permutational Multivariate Analysis of Variance (PERMANOVA) was performed in the "microeco" package of R software (version 3.3.0; R Development Core Team, 2006). Heatmap was generated using the relative abundance of Acidobacterial subgroups in R according to Frey's method (Frey et al., 2016). The correlation heatmap between the soil physicochemical properties and soil Acidobacterial subgroups were performed in the "microeco" package of R software (version 3.3.0; R Development Core Team, 2006). Mantel test was also used to analyze at the wetland group and forest group and all soil Acidobacteria with soil physicochemical properties by using the "vegan" package of R software (version 3.3.0; R Development Core Team, 2006).

Results

Soil physicochemical properties in eight vegetation types

All the soil physiochemical properties changed significantly ($p < 0.05$) between the eight vegetation types (Table 1). MC was higher for the wetland group than for the forest group (Table 1). Soil pH was between 5.47 (PB) and 5.75 (NW) (Table 1). SOC was between 27.82 (*CF*) and 57.54 g/kg (DW) (Table 1). The TN was between 2.58 (*CF*) and 7.62 g/kg (NW). AN was between 165.86 (PB) and 455.25 mg/kg (NW). TP was between 0.32 (YB) and 6.36 mg/kg (NW). AP was between 25.18 (DW) and 51.99 mg/kg (YB).

Acidobacterial α- and β-diversities

The Shannon diversity index, Chao1 index and OTU and PD index of soil Acidobacteria differed significantly (one-way ANOVA, $p < 0.05$) among the eight vegetation types (Table 2). The Shannon diversity index was between 4.8 (IW) and 5.7 (EW), and the Chao1 index was between 823 (MB) and 1032 (EW) (Table 2), and the OTUs was between 732 (MB) and 981 (EW) (Table 2). The PD index was between 112.4 (*CF*) and 153.1 (NW).

The PCoA separated the Acidobacterial community found in the eight vegetation types into three significantly different groups depending on their similarity, i.e., an wetland group (NW, IW, DW), a forest group (YB, MB, PB, and *CF*) and EW group (Figure 1; Table 3).

Composition of soil Acidobacterial communities

A total of 1,545 Acidobacterial OTUs belonging to 22 subgroups were detected, following a decreasing order of relative abundance Gp1 > Gp3 > Gp7 > Gp6 > Gp2 > Gp4 > Gp13 > Gp5 > Gp17 > Gp15 > Other>Gp18 > Gp16 > Holophagae>Gp11 > Gp25 > Gp10 > Gp22 > Gp19 > Gp12 > Gp20 > Gp23 (Figure 2). Moreover, all the Acidobacterial subgroups, except Gp23, varied significantly among the eight vegetation types (Figure 3, $p < 0.05$).

The relative abundance of Gp7, Gp12, Gp20, Gp23, Gp13, Holophagae, Gp16, Gp25, Gp18, Gp19 in the wetland group (NW, IW, and DW) was higher than those in the forest group (YB, MB, PB and *CF*) (Figure 3). The relative abundance of Gp2, Gp3, Gp1, Gp15 in the wetland group (NW, IW and DW) was smaller than those in the forest group (YB, MB, PB and *CF*) (Figure 3). The relative abundance of Gp4, Gp11, Gp17, Gp22, Gp6, Gp10, Gp5 in EW was higher than those in the wetland group (NW, IW and DW) and forest group (YB, MB, PB, and *CF*) (Figure 3).

TABLE 1 Soil physicochemical properties and soil enzymes in the eight vegetation types along a successional gradient in a degraded wetland.

Type	Soil moisture	pH	Total organic carbon (g/kg)	Total nitrogen (g/kg)	Available nitrogen (mg/kg)	Total phosphorus (mg/kg)	Available phosphorus (mg/kg)
NW	**52.79 ± 2.00a**	*5.47 ± 0.04d*	56.43 ± 3.13a	**7.62 ± 0.12a**	**455.25 ± 17.06a**	**6.36 ± 1.17a**	26.34 ± 1.05c
EW	23.52 ± 1.63d	5.58 ± 0.08bcd	56.09 ± 2.17a	3.00 ± 0.10e	214.47 ± 28.711d	0.52 ± .05c	26.45 ± 2.15c
IW	44.70 ± 2.07b	5.52 ± 0.03 cd	38.82 ± 3.33b	5.56 ± 0.11b	381.53 ± 23.97c	0.60 ± .11c	26.09 ± 2.50c
DW	35.62 ± 1.46c	5.69 ± 0.02ab	**57.54 ± 2.37a**	4.75 ± 0.02c	418.34 ± 10.88 ac	5.18 ± .19a	*25.18 ± 1.97c*
YB	20.07 ± .80de	*5.47 ± 0.03d*	37.88 ± 1.65b	4.28 ± 0.32d	329.55 ± 11.09b	*0.32 ± .06c*	**51.99 ± 2.13a**
MB	21.92 ± 1.83de	5.66 ± 0.02abc	36.49 ± 1.07b	7.25 ± 0.07a	437.61 ± 12.02 ac	0.62 ± .01c	43.15 ± 1.38b
PB	*16.09 ± .54e*	**5.75 ± 0.01a**	57.10 ± 2.34a	4.96 ± 0.16c	*165.86 ± 13.47d*	0.48 ± .13c	49.23 ± 4.35b
CF	17.18 ± 1.26e	5.58 ± 0.02bcd	*27.82 ± 2.14c*	*2.58 ± 0.17e*	189.12 ± 5.07d	2.43 ± .04b	29.60 ± 1.00c

Statistical significance (One-way ANOVA, $p < 0.05$) is indicated by different superscript letters in the same column. In each column, the largest value is shown in bold and the smallest value is shown in italics. Vegetation types: original natural wetland (NW), wetland edge (EW), shrub-invaded wetland (IW), shrub-dominated wetland (DW), young-*Betula* forest (YB), mature-*Betula* forest (MB), *Populus* and *Betula* mixed forest (PB), and conifer forest (CF).

TABLE 2 Soil Acidobacterial α-diversity along a degraded wetland in Sanjiang plain.

Types	OTU	Shannon	Chao1	PD
NW	924.0 ± 10b	5.0 ± 0.01d	1032.6 ± 23a	153.1 ± 0.66a
IW	801.3 ± 13d	4.8 ± 0.01f	905.7 ± 28d	137.1 ± 1.98c
DW	822.0 ± 23d	5.0 ± 0.01 cd	959.6 ± 20bc	142.6 ± 0.39b
EW	981.7 ± 24a	5.7 ± 0.01a	1079.7 ± 40a	152.8 ± 2.14a
YB	742.3 ± 18e	5.1 ± 0.04bcd	867.6 ± 30de	117.2 ± 2.22d
MB	732.3 ± 15e	4.9 ± 0.08e	823.3 ± 21e	117.6 ± 2.06d
PB	877.0 ± 20c	5.1 ± 0.01b	977.5 ± 37b	120.7 ± 3.47d
CF	799.3 ± 8d	5.1 ± 0.04bc	918.3 ± 30 cd	112.4 ± 0.97e

Different lowercases indicate statistical significance identified by the Duncan test at $p < 0.05$.

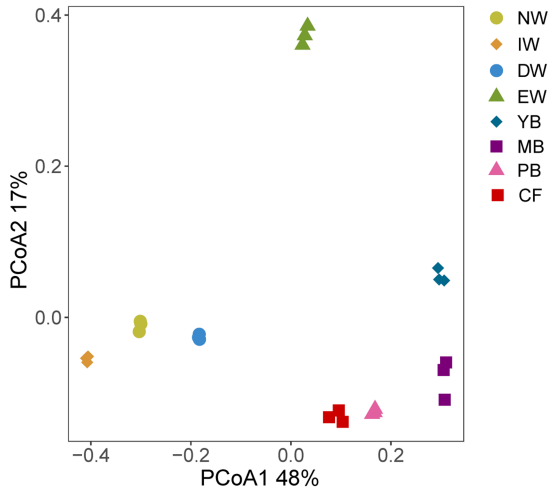

FIGURE 1
PCoA analysis of eight degradation vegetation types in Sanjiang plain. Vegetation types: original natural wetland (NW), shrub-invaded wetland (IW), w shrub-dominated wetland (DW), etland edge (EW), young-*Betula* forest (YB), mature-*Betula* forest (MB), *Populus* and *Betula* mixed forest (PB), and conifer forest (*CF*).

TABLE 3 Adonis analysis of the difference or similarity in Acidobacterial community between the wetland group and forest group in a degraded wetland of Sanjiang plain.

Vegetation	Sum of Sqs	Mean Sqs	*F*	R^2	*p*
All habitat	3.14	0.44	134.24	**0.98**	0.001
Wetland group versus forest group	1.25	1.25	14.16	**0.39**	0.001

The bold vales indicated that the significant difference at 0.05 level.

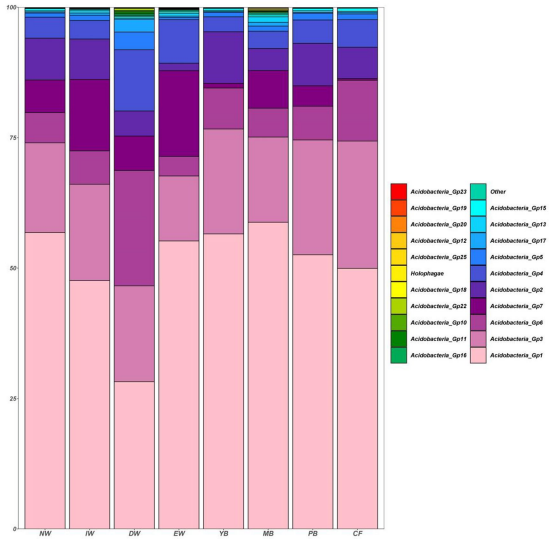

FIGURE 2
Histogram of the relative abundance of soil Acidobacterial communities in a degraded wetland.

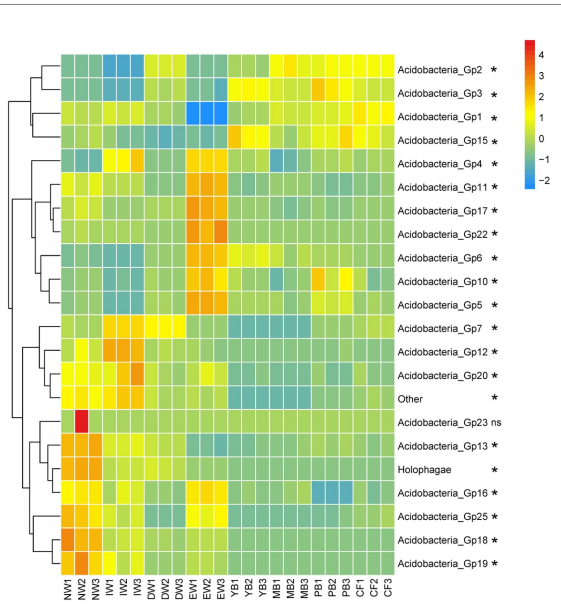

FIGURE 3
Heatmaps of Acidobacterial composition differences in different vegetation types in Sanjiang plain. *means one-way ANOVA significant at $p < 0.05$. The color gradient (red, yellow, blue) represents the relative abundance of the soil Acidobacteria from high to low in the different vegetation types.

The relationships between soil physicochemical properties and soil Acidobacterial communities

Mantel tests (Table 4) indicated that soil AP and MC were the key soil physicochemical properties affecting the soil Acidobacterial communities in all vegetation types (Table 4). But for wetland types, the soil TN, AN, and MC were the key soil physicochemical properties affecting the soil Acidobacterial communities, and for forest types, soil pH, SOC, AN, AP, MC, and TP significantly affected the soil Acidobacterial community (Table 4).

TABLE 4 Mantel test to determine the correlations between the environmental variables and acidobacterial community compositions at two habitats of wetland and forest.

Variables	Wetland group		Forest group		All samples	
	r	*p*	*r*	*p*	*r*	*p*
pH	0.086	0.217	**0.393**	0.001**	0.068	0.120
SOC	0.058	0.235	**0.372**	0.004**	0.080	0.104
TN	**0.605**	0.004**	0.102	0.173	0.067	0.143
AN	**0.667**	0.009**	**0.607**	0.006**	0.080	0.081
TP	0.111	0.117	**0.348**	0.009**	0128	0.051
AP	−0.057	0.632	**0.252**	0.048*	**0.385**	0.003**
MC	**0.733**	0.001**	**0.232**	0.04*	**0.666**	0.001**

**$p < 0.01$, *$p < 0.05$. The bold value indicated the significant difference. SOC, soil organic carbon; TN, total nitrogen; AN, available nitrogen; TP, total phosphorus; AP, available phosphorus; MC, moisture content; Wetland group included NW, IW, DW, and EW; Forest group included YB, MB, PB, and CF.

The heamap analysis showed that Gp2 and Gp3 correlated significantly positively with soil AP ($p < 0.05$) and significantly negatively with MC ($p < 0.05$) and pH ($p < 0.05$), but the abundant Gp6 and Gp7 had the opposite correlation with these properties (Figure 4). Gp1 was significantly negative correlated with soil pH ($p < 0.05$). Moreover, Gp5 and Gp 17 were significantly positively correlated with SOC ($p < 0.05$), but the Gp5 significantly negatively correlated with TN ($p < 0.05$) and AN ($p < 0.05$), the Gp 17 significantly negatively correlated with AP ($p < 0.05$). Gp4 was significantly negatively positively with TP ($p < 0.05$) and TN ($p < 0.05$). Gp13 significantly positively correlated with MC ($p < 0.05$), AN ($p < 0.05$) and TN ($p < 0.05$), but negatively correlated with AP ($p < 0.05$) (Figure 4).

Discussion

In line with our hypothesis I, α diversity of Acidobacteria significantly changed with wetland degradation (Table 2; Figure 1), and the OTU, Shannon and Chao1 indices all showed the highest values in EW type and the lowest ones in MB. Since soil Acidobacteria in wetlands have been rarely studied, it is still unclear how the wetland vegetation affects the diversity of the soil Acidobacteria. However, from the results of this study, the EW type belongs to the transitional type with both wetland plants and forest plants, and its special habitat may be one of the reasons for the high diversity of its soil Acidobacteria. Another reason may be related to the overall bacterial diversity in soil, our previous study found that soil bacterial diversity was highest among EW types (Sui et al., 2021). Therefore, due to its high overall diversity, it may affect α diversity of Acidobacteria. However, it is worth noting that the Chao1 index in MB was the lowest but the Chao1 index of total bacteria was the highest (Sui et al., 2021). Naether et al. (2012) found that there were significant differences in the diversity of Acidobacteria between grasslands and forests, and even between different locations in the same ecological environment. Therefore, further in-depth research is needed in the future.

Our results indicated the Shannon index was significantly negatively correlated with soil TN and AN (Supplementary Table S2). However, we did not find that α diversity was significantly correlated with pH (Supplementary Table S2). Liu et al. (2014) reported that Acidobacteria in northeastern black soil farmland was significantly correlated with soil pH, and Fierer et al. (2007), Männistö et al. (2007), and Lauber et al. (2008) also pointed out that α diversity was significantly correlated with pH. However, the results of our research are not consistent with theirs. This is possible that the soil pH did not change significantly from wetland to forest (range from 5.5 to 5.8). In addition, we further found that the diversity of five Acidobacterial subgroups also highly correlated with soil pH (Figure 4). Our results reveal that soil pH acts as an effective habitat filter to restrict the Acidobacterial community in the Sanjiang wetland soils. Similar results were also reported by Jones et al. (2009), who indicated that Acidobacterial communities in soils were more phylogenetically clustered as pH departed from neutrality. Another reason is possible that Liu et al. (2014) study was based on a regional scale in northeast China, so the results may be in line with the distribution rule of microbial biogeography, that is, the distribution of soil microorganisms at a large scale is driven by pH. And because of a small scale of our study, the acidobacteriral diversity may be more significantly affected by soil nutrients. However, our previous study found that the soil Chao1 index under different vegetation types was the lowest in EW and the highest in MB (Sui et al., 2019), which was contrary to the result of Chao1 index of soil Acidobacteria. Different primers will lead to different results (Lee et al., 2008; Liu et al., 2014). Lee et al. (2008) found that the amplification efficiency of different primers for subgroup of Acidobacteria was different. The amplification efficiency of universal bacterial primers (515F/907R and 515F/806R) and Acidobacterial special primers (ACIDO/1492R, 31F/1492R, 341F/805R, and ACIDO/342R) for Acidobacteria is also different (Supplementary Table S3). Therefore, the diversity and distribution of the main bacterial phyla are not consistent, so further research is needed on the distribution of the diversity of different bacterial phyla in the wetlands of the Sanjiang Plain.

We hypothesized that changes in vegetation type resulted by wetland degradation would lead to changes in the composition of Acidobacteria. The proportion of Gp1, Gp2, Gp3 and Gp4 were higher in forest group than wetland group (Figure 3). Some studies showed that Acidobacterial composition was related to plant community types (Navarrete et al., 2013). Wang et al. (2010) found that Gp1, Gp2, Gp3, and Gp4 were dominant subgroups in forest soil. Naether et al. (2012) found that Gp4 and Gp6 were dominant subgroups in grassland soil. Yao et al. (2021) found that Gp4 and Gp6 were dominant subgroups in farmland soil. Therefore, the composition of Acidobacteria in the forest group in this study is consistent with the above studies. After the transition from wetland type to forest type, the wetland vegetation mainly composed of *Deyeuxia angustifolia* is transformed into broad-leaved forest and coniferous forest (e.g., *Betula platyphylla*,

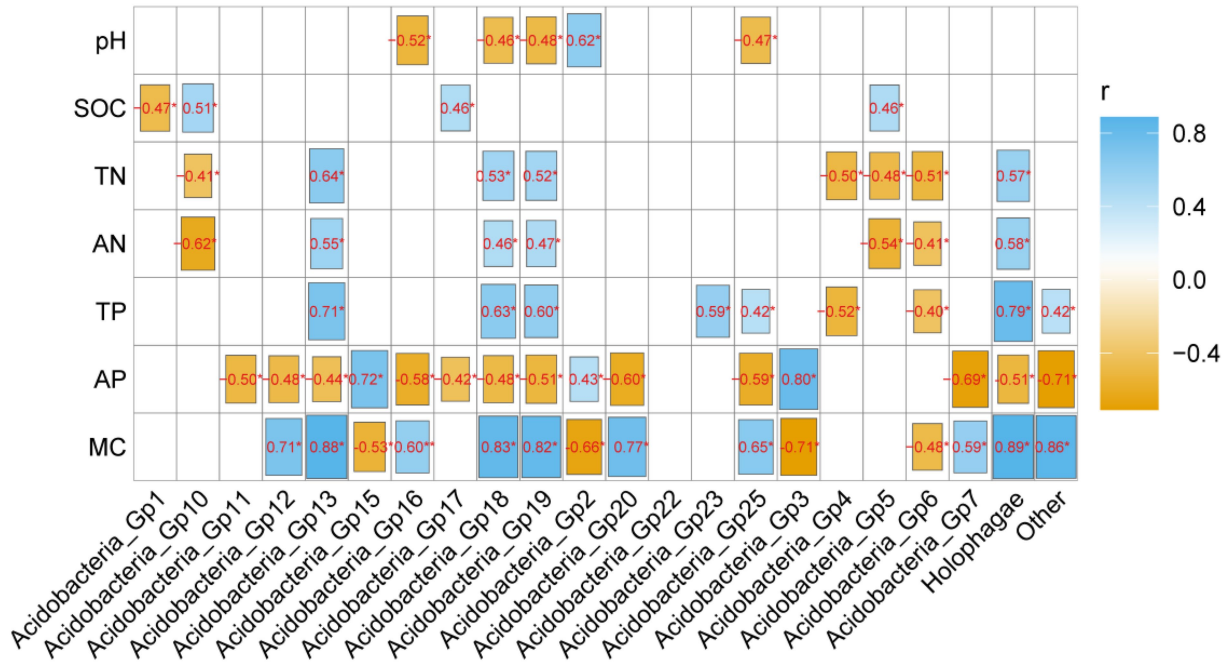

FIGURE 4
The correlation analysis of soil physicochemical properties and Acidobacterial subgroups. Blue colors indicate positive and yellow colors negative correlations. *, **, and*** mean 0.05, 0.01, and 0.001 levels, respectively.

Populus davidiana, and *Larix gmelinii*), resulting the composition, litter, soil physicochemical properties were all changed, which may be the reason for the change in the abundance of the subgroup of Acidobacteria. Addition, Lee et al. (2008) proved that different primers lead to different amplification efficiencies for phylum Acidobacteria. Moreover, Supplementary Table S3 indicated that the use of different primers could affect the subgroup abundance of Acidobacteria. Even the different DNA extraction methods (e.g., CTAB and soil extraction kit) and sequencing methods (e.g., 454, Miseq and Hiseq) also influence the results of composition of Acidobacteria. Hence these may also be important reasons for the inconsistency between this study and other studies.

Through the correlation analysis between soil physicochemical properties and the community structure of soil Acidobacteria (Table 4), it can be found that the abundance of soil Acidobacterial community structure in the Sanjiang Plain is generally affected by soil available phosphorus and soil water content, but the forest group is mainly affected by soil pH, SOC, TN, AN, TP, AP, MC, while wetland types were mainly affected by TN, AN, and MC (Table 4). These are in accordance with our 2nd and 3rd hypothesis. Overall, our study is consistent with previous studies (Fierer et al., 2007; Jones et al., 2009), but in terms of forest type and wetland type, it seems to contradict to previous studies. Acidobacteria is an oligotrophic type of bacteria, mainly distributed in soils with low nutrient content (Fierer et al., 2007; Jones et al., 2009). However, a number of recent studies have shown that Acidobacteria can grow well under high nutrient conditions (Eichorst et al., 2007; de Castro et al., 2013). Navarrete et al. (2013) and Liu et al. (2014) proved the composition of Acidobacteria significantly positively related to soil organic carbon. Therefore, we speculate in the soil of the Sanjiang wetland type with high soil nutrient content, the relationship between the content of soil aciddobacteria and soil nutrients may have the following reasons. First, Acidobacteria is not correlated with soil organic carbon (Table 4), only Acidobacteria in forest types showed a significant positive correlation with soil organic carbon (Table 4). We found in a previous study that the total bacterial content in the Sanjiang wetland forest type were positively correlated with soil organic carbon (Sui et al., 2019), so this may be the reason of the positive correlation between Acidobacteria and soil organic carbon content. Second, it may be because there is a significant correlation between Acidobacterial subgroups in forest soil and soil nutrients. We found that Gp5 and Gp10 were significantly correlated with SOC (Figure 4). Naether et al. (2012) also found SOC had a positively relationship with soil Acidobacteria in grassland and forest soils.

At present, there were 28 subgroups in the Acidobacteria phylum (Barns et al., 2007). In this study, we found that there were 22 Acidobacterial subgroups in all soil samples, meaning that Acidobacteria is widely spread in the wetland soil of Sanjiang plain. Addition, Gp1，Gp2，Gp3，Gp4, Gp7, and Gp6 were the most abundance subgroups (Figures 2, 3; Supplementary Table S3). This result was consistent with other reports (Kielak et al., 2009; Wang et al., 2010; Liu et al., 2014; Yao et al., 2021). Liu et al. (2014) reported that the proportion of Gp1, Gp3, Gp4, Gp6 was higher in black soils in northeastern of China. Yao et al. (2021) also proved that the proportion of Gp4 and Gp6 was higher in

farmland in northeastern of China. This indicated that Acidobacteria in black soil in northeastern China was dominated by Gp1, Gp3, Gp4, and Gp6, and our research is consistent with it. However, our study also found that the wetlands of the Sanjiang Plain also contain higher abundance of Gp2 and Gp7. Some studies showed that Gp7 was highly abundant in forests (Shen et al., 2013) and grasslands (Naether et al., 2012). However, we found that Gp7 was also high abundant in wetlands, so we speculate that Gp7 is can be widely distributed in different habitats. Navarrete et al. (2015) stated that the relative abundances of Gp7 were positively correlated with soil nutrient content, while the soil organic carbon of the Sanjiang Plain wetland was high, which may be the reason why the Sanjiang Plain wetland contains higher Gp7, but the reason of Gp7 could be adapted to different habitats is still unclear and further research is needed. Liu et al. (2014) found that Gp2 was very low in black soil in northeastern farmland, which seems to contradict this study. However, a large number of studies have shown that Gp2 is the main subgroup in forests (Jones et al., 2009; Wang et al., 2010; Wei et al., 2018), which is consistent with the results of this study because the abundance of Gp2 was highest in the forest group (Figure 3).

Through the correlation analysis between soil physiochemical properties and different subgroups of Acidobacteria (Figure 4), it can be found that the abundance of Acidobacteria in the Sanjiang Plain is closely related to soil organic carbon, total nitrogen, total phosphorus, available phosphorus, soil moisture content. Our hypothesis guess soil moisture is one of the key factors affecting Acidobacteria in wetlands. The soil moisture content of the wetlands in the Sanjiang Plain is high, and the soil moisture content fluctuates significantly with the change of rainfall, which is one of the main characteristics of wetland habitats. Moreover, Hartmann et al. (2017) found that soil moisture is the main environmental factor affecting Acidobacteria, which is consistent with this study. However, only Gp2, Gp16, Gp18, Gp19, and Gp25 showed significant correlation with soil pH (Figure 4). These findings suggest that the growth characteristics of different Acidobacterial subgroups are not identical. Some subgroups were more sensitive to soil pH, while others were sensitive to soil nutrient content (e.g., SOC, TN, TP and AP), probably because subgroups within Acidobacteria select different ecological niches (Hartmann et al., 2017). Acidobacteriaceae can be differentiated into copiotrophic or oligotrophic categories (Naether et al., 2012; Navarrete et al., 2015), so we speculate that this may be the reason that soil Acidobacteria was highly correlated with SOC, TN, TP, AP. It needs to be emphasized that since large-scale surveys on Acidobacterial abundances are rare, further studies are needed to reveal the relationships of abundances of different subgroups of Acidobacteria and soil physicochemical properties.

Conclusion

To our knowledge, this is the first comprehensive study, using high-throughput sequencing, on Acidobacterial communities across wetland degradation stages. We found that Acidobacterial diversity changed significantly after vegetation conversion (from wetland to forests). Soil physicochemical properties were the key environmental factors regulating Acidobacterial communities, while the relative abundances of many Acidobacterial subgroups were dominantly controlled by soil water content, suggesting that the physiological characteristics of Acidobacteria are different at the subgroup level in wetland environment. We revealed that Gp1, Gp2, Gp3, Gp4, Gp6 and Gp7 were dominant in all vegetation types, while Gp5 was minor in all vegetation types in this study. Changes in soil water content and vegetation type following wetland degradation were confirmed as the primary factors in determining the diversity and distribution of Acidobacterial communities, which will further affect biogeochemical cycling and lead to changes in ecosystem functioning and service at the regional level.

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

XS: designed and performed the experiment and prepared this manuscript. BF and M-HL: revised this manuscript and language editing. YL and RZ: helped to do the experiment and finish the bioinformatic analysis. HN and M-HL: designed this experiment. All coauthors contributed to manuscript editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

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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.2022.1052161/full#supplementary-material

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EDITED BY
Xin Sui,
Heilongjiang University, China

REVIEWED BY
Dongxing Zhou,
Northeast Agricultural University, China
Zhijie Chen,
Fujian Normal University, China
Xiang-Min Fang,
Jiangxi Agricultural University, China

*CORRESPONDENCE
Rong-Tao Zhang,
zhangrongtao14@163.com
Hong-Wei Ni,
469909761@qq.com

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Latitude variations of soil bacterial community diversity and composition in three typical forests of temperate, northeastern of China

Xiao-Yu Fu[1,2], Zhi-Chao Cheng[2], Hong-Wei Ni[1,3]* and Rong-Tao Zhang[2]*

[1]College of Geographical Science, Harbin Normal University, Harbin, China, [2]Institute of Nature and Ecology, Heilongjiang Academy of Sciences, Harbin, China, [3]Heilongjiang Academy of Forestry, Harbin, China

Soil bacteria are a crucial component of forest soil biodiversity and play important functions in numerous ecosystem processes. Hence, studying the variation of diversity and composition of soil bacteria between latitude gradients and the driving factors responsible for these differences is important for understanding the changes of soil bacteria. We used Illumina MiSeq sequencing of bacterial 16S rRNA to investigate the distribution pattern and driving factors of bacterial diversity and composition in temperate forest soils at three different latitudes in northeast China, with samples taken at low, middle and high latitude. Each sample area was located at a distance of 1,200 km. Our results indicate that the soil bacterial diversity decreased with increasing latitude. Members of the phyla *Acidobacteria* and *Proteobacteria* were the dominant in all investigated soils, the highest relative abundances of these phyla were: *Acidobacteria* and *Proteobacteria* in high-latitude forest, *Rokubacteria* and *Actinobacteria* in low-latitude forest. The dominant bacterial genera in the three different latitude forests were *Candidatus_Solibacter*, *Bryobacter*, *Roseiarcus* and *Granulicella*. Mean average temperature, soil pH and total nitrogen content were the key environmental factors shaping the soil bacterial diversity and composition in different latitudes of these temperate forests. The results of this study contribute to a deeper understanding and better predictions the latitudinal pattern of soil biodiversity.

KEYWORDS

temperate forest, latitude pattern, bacterial diversity, effect factors, soil nutrient

1 Introduction

Soil microorganisms represent some of the most abundant and diverse biological populations on Earth, and play an important role in material conversion, energy flow, and biogeochemical cycling (Cardinale et al., 2015; Bahram et al., 2018; Deng et al., 2020). Understanding the distribution patterns of soil microorganism on a spatial scale is a central goal of ecology (Wang et al., 2015). However, compared with macrobiology, the distribution and driving mechanisms of soil microorganisms is still limitedly investigated (Fuhrman, et al., 2008; Zhou et al., 2016). It has been reported that soil microorganisms produce a random distribution pattern on a global scale (Finlay, 2002), although on a local or regional scale, regular, spatial distribution patterns of soil microbial community structures have been observed (Shen et al., 2014; Sun et al., 2021; Ji et al., 2022). However, the conclusions of previous studies,

performed at different scales, are inconsistent (Tedersoo et al., 2014; Neu et al., 2021). Even under the same environmental conditions can the distribution of soil microorganisms be inconsistent (Han et al., 2015; Li et al., 2019; Tian et al., 2022). Therefore, further studies are needed to reveal the driving mechanism of changes in the spatial distribution of soil microorganisms.

Forest ecosystems represent highly important terrestrial ecosystems with a rich species diversity, which plays an important role in the material cycle and energy flow (Andrew, 2020; Hu et al., 2021; Chen et al., 2022). Soil microorganisms are highly abundant and diverse in forest soils. Understanding their community composition and diversity, predicting dynamic changes, and exploring the ecosystem's response mechanism to habitat changes, all have great significance on the protection of forest ecosystems and their stability (Chen et al., 2020; Chen et al., 2022; Zhou et al., 2022). Tripathi et al. (2014) showed that environmental factors played a decisive role in the variation of soil microorganisms community structure and diversity, among the environmental factors, pH had the most significant effect followed by total nitrogen and altitude (Tripathi et al., 2014). Temperature also regulates the diversity of microorganisms in forest soil on a continental scale, as was demonstrated by Zhou et al. (2016) who investigated a temperature gradient showed it affected the diversity of different microbiota variably. Tian et al. (2018) studied microorganisms in forest soil at different latitudes (including tropical, subtropical, and temperate forest), and found that soil bacterial diversity was significantly higher in temperate forests than the others. In that study, climate factors were the main driving factors of forest soil bacterial community structure, together with pH and organic matter, but vegetation diversity had relatively little impact on forest soil bacterial community structure (Tian et al., 2018). Although studies have focused on the spatial and temporal distribution patterns of soil microorganisms and their main driving factors, a unified conclusion has not been reached, especially due to the lack of large-scale data and the lack of universal rules, that hinder an accurate prediction of soil bacterial diversity changes and the development of ecological theories (Zhang et al., 2020; Sun et al., 2021). Therefore, there is an urgent need to study the distribution and driving mechanism of soil microorganisms in a more systematic way on a large scale.

Along latitude gradients, the geographic variation can be associated with changes in environmental factors such as temperature, precipitation, vegetation type, and soil properties (Yang et al., 2019; Zheng et al., 2021). These provide a natural experimental platform for assessing how soil bacterial communities respond to environmental change (Tian et al., 2018). There remains controversy regarding the patterns of changes in soil bacterial diversity and community structure with increasing latitude, with available works describing patterns with declining (Zhou et al., 2016), humped (Tian et al., 2018) or stepped (Lee et al., 2018) trends of soil bacterial diversity. Furthermore, Zhang et al. (2020) studied the variation of soil bacterial community diversity in 11 latitudinal gradients in eastern China and considered the variation of bacterial diversity patterns could be explained by a local community aggregation mechanism. The inconsistent conclusions of these studies may be caused by the different response patterns of soil bacteria to latitudinal changes in different vegetation types and ecosystems. Bacterial community composition significantly corresponds to vegetation and habitat selection (Srivastava et al., 2021), and therefore the composition and diversity of bacterial communities are strongly influenced by the composition of above-ground vegetation and soil environmental conditions (Sui et al., 2021). As the most representative forest type in a certain area, typical forests are often used as the research objects to explain the spatial distribution characteristics of soil (Lee et al., 2018), plants (Liu et al., 2022) and microorganisms communities (Gaytán et al., 2022) in different latitude forests. There are various temperate forest ecosystems in northeast China, which create an important barrier to the agricultural development of the Northeast Great Plain and play an important ecological role in the maintenance and regulation of the regional climate (Zheng et al., 2018; Sang et al., 2021). The typical forests in this area are cold temperate *Larix gmelinii* forest, temperate Coniferous-broad Leaved *Korean pine* Mixed Forest and warm temperate broadleaf *Quercus* forest (Jia et al., 2019; Chen et al., 2022). Patterns of plants and animals decreasing with increasing latitude have been well documented (Berdugo et al., 2018; Yu et al., 2021), but it is unknown whether microorganisms exhibit similar latitudinal diversity gradients (Zhou et al., 2016). This study was performed to fill in this knowledge gap.

Three typical temperate forest areas were selected from the China Forest Biodiversity Monitoring Network to investigate the spatial distribution pattern of soil bacterial community composition and diversity by high-throughput sequencing. We aim to elaborate: (1) the distribution pattern of soil bacterial community in the three different latitudes forest ecosystems. (2) the key environmental factors driving the changes of soil bacterial community and diversity in three different latitudes forest ecosystems. The obtained results can not only promote the development of biogeography, but also provide a theoretical basis for the improvement of forest latitudinal diversity patterns. At the same time, it is conducive to the functional regulation and management of terrestrial ecosystems, so as to better cope with important environmental problems such as global warming.

2 Materials and Method

2.1 Site description

The study areas covered a longitude range of 115°–129°E and latitude range 39°–54°N, and included a warm, a moderate, and a cold temperate zone, with altitudes ranging from 786 m to 932 m. The average annual precipitation ranges from 383.53 mm to 836.23 mm. Three sampling areas were identified in each zone of low, middle, and high latitude. The survey was mainly conducted in national nature reserves or scientific research bases, away from habitation, and mature forests with good preservation and strong regional representation were selected. The specific areas of low latitude to high latitude included: Long-term Biodiversity Monitoring Plot of Dongling Mountain in Beijing (LL): The experimental samples were located in Beijing Forest Ecosystem 20 hm^2 Research Station, Chinese Academy of Sciences, five 20 m × 20 m standard quadrates were selected, and each quadrate was 100 m apart, the vegetation type of the sample plot is *Quercus liaotungensis* forest; Changbai Mountain National Nature Reserve of Jilin Province (ML): The experimental samples were located in the forest monitoring plots of 25 hm^2 in Changbai Mountain, five 20 m × 20 m standard quadrates were selected, and each quadrate was 100 m apart, the vegetation type of the sample plot are *Quercus mongolica* and *Pinus koraiensis* mixed forest, and Huzhong National Nature

TABLE 1 Basic information of the selected forests in China temperate zones.

Location[1]	Latitude (N)	Longitude (E)	Altitude (m)	Climate type	Dominant species
LL	39°57′27″	115°25′24″	932	Warm temperate continental monsoon climate	*Quercus liaotungensis*
					Betula dahurica
ML	42°24′16″	128°05′27″	786	Temperate continental monsoon climate	*Pinus koraiensis*
					Querus mongolica
HL	51°46′48″	123°01′12″	855	Cold temperate continental monsoon climate	*Larix gmelinii*

LL, low-latitude; ML, mid-latitude; HL, high-latitude.

Reserve in Daxing'anling, Heilongjiang Province (HL): the experimental samples were located in the forest monitoring plots of 25 hm^2 in Daxing'anling, five 20 m × 20 m standard quadrates were selected, and each quadrate was 100 m apart, the vegetation type of the sample plot is *Larix gmelinii* forest. A summary of these three research areas is shown in Table 1.

2.2 Soil sampling

From each of the three areas, forest soil samples were acquired in July 2020. For this, standard experimental plots were set up in the core areas of the national nature reserves (see Table 1). Within each forest type, five sampling plots were established, and within each plot soil samples (0–20 cm) were collected using an 8 cm diameter soil auger from 15–20 locations along an S-shaped path. Before sampling, superficial debris (e.g., leaves and dry vegetation) was removed. The collected soil samples were transferred to sterile seal bags and stored in an icebox. Following transport to the laboratory, the soil was immediately sieved (2 mm mesh) to remove stones and plant material. One portion of the samples was air-dried to conduct physical and chemical analyses, whereas the remainder was kept at −80°C until required for microbial analysis.

2.3 Characterization of soil chemical parameters

A soil-water (1:2.5 w/v) suspension was shaken for 30 min prior to measuring the pH with a pH meter. The soil total N (TN) and organic C (SOC) were quantified using an Elemental Analyzer (Elementar, Langenselbold, Germany). Available nitrogen (AN) was sequentially digested in H_2SO_4–$HClO_4$, 0.5 M $NaHCO_3$, and 2.0 M KCl, and then examined with a continuous flow analysis system (SKALAR SAN++, the Netherlands). After wet digestion with $HClO_4$–H_2SO_4, total phosphorus (TP) was determined spectrophotometrically using standard procedures.

2.4 DNA extraction, PCR amplification, and MiSeq sequencing

Total DNA of soil microorganisms was extracted using the QIAGEN DNeasy PowerSoil Pro Kit according to the manufacturer's instructions. The V3, V4 region of the bacterial 16S rRNA region was amplified using universal primers 338 F and 806 R. Each sample was tagged with a different six bp barcode present at the 5′end of the primer. The PCR amplification protocol consisted of an initial denaturation stage at 94°C for 10 min, followed by 30 cycles of 90°C for 60 s, 55°C for 60 s, and 72°C for 60 s, and final extension at 72°C for 10 min. The product was purified using the QIAquick PCR Purification Kit (QIAGEN). The PCR products of different samples were sequenced by BMC Biotechnology Co., Ltd., using an Illumina MiSeq sequencer.

The raw sequences were processed using QIIME1. Forward and reverse reads were merged using FLASH software. 'Trim' was used to remove low-quality sequences shorter than 150 bp, reads with a mean quality score below 20, and sequences containing ambiguous nucleotides. Chimeric sequences were identified and removed with the uchime algorithm. A similarity of 97% was applied to divide the reads into operational taxonomic units (OTU) through UPARSE (V.7.0.1090). Operational taxonomic identities were determined using QIIME by executing the BLAST algorithm against sequences in the SILVA database (https://www.arb-silva). Diversity indices Chao1, ACE, Shannon and Simpson were calculated using 'alpha_diversity.py' to reflect the *a* diversity of the bacterial community, and 'beta_diversity.py' was used to calculate the Bray-Curtis distance matrix to reflect the *ß* diversity.

2.5 Statistical analyses

Differences in soil physicochemical properties between the three treatments were analyzed using one-way ANOVA at a 0.05 significance level coupled with Duncan's tests, using SPSS 23.0 (IBM SPSS Statistics for Windows). OTU- level *a* diversity indices, such as Chao1 index, ACE index, Shannon index and Simpson index, were calculated using the OTU table in QIIME1. Venn diagrams and heatmap representations of the relative abundances of bacterial OTUs among soil samples were generated using the "vegan" package in R. Non-metric multidimensional scaling (NMDS) was conducted based on the Bray—Curtis dissimilarity at the OTU level, also using "vegan" (Frey et al., 2021). Redundancy analysis (RDA), implemented in "vegan", was used to generate compositional profiles, agglomerating the OTUs to the phylum level and genus level. Indicator species analysis based on the OTU level was performed as outlined in Rime et al. (2015) using "vegan" and the "labdsv" (version 1.2–2) R package.

TABLE 2 Chemical properties of forest soil at three different latitudes.

Latitude (low to high)	MAT (°C)	pH	SOC (g·kg^{-1})	TN (g·kg^{-1})	An (mg·kg^{-1})	TP (g·kg^{-1})
LL	6.55 ± 0.09 a	6.69 ± 0.22 a	38.49 ± 1.98 b	4.49 ± 0.66 a	35.78 ± 1.48 a	0.88 ± 0.03 a
ML	2.79 ± 0.08 b	4.69 ± 0.14 b	46.13 ± 3.54 a	4.06 ± 0.57 a	32.17 ± 2.01 a	0.78 ± 0.02 a
HL	−3.67 ± 0.08 c	4.58 ± 0.15 b	43.85 ± 1.96 a	2.21 ± 0.22 b	20.79 ± 1.24 b	0.49 ± 0.04 b

Different letters in the same column indicate significant differences among different areas ($p < 0.05$). LL, low-latitude; ML, mid-latitude; HL, high-latitude.

TABLE 3 α Diversity of the soil bacterial community at different latitudes.

	Latitude gradients	ACE	Chao1	Shannon	Simpson
Bacterial community	LL	1347.73 ± 31.06 a	1366.77 ± 29.59 a	8.85 ± 0.23 a	0.9948 ± 0.04 a
	ML	1388.44 ± 28.54 a	1404.93 ± 31.21 a	8.22 ± 0.28 ab	0.9885 ± 0.03 ab
	HL	1211.98 ± 26.28 b	1218.12 ± 25.06 b	7.56 ± 0.21 b	0.9813 ± 0.03 b

Different letters in the same column indicate significant differences among different areas ($p < 0.05$). LL, low-latitude; ML, mid-latitude; HL, high-latitude.

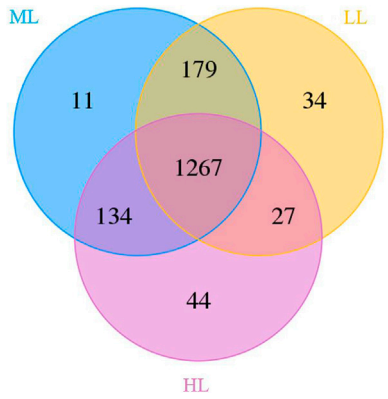

FIGURE 1
Venn diagram of soil bacterial OTUs. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

3 Results

3.1 Forest soil chemical properties at different latitudes

The determined chemical properties of the soil collected at three different latitudes is summarized in Table 2. The mean annual temperature (MAT) decreased with increasing latitude in the three locations. The pH and content of SOC, TN, AN and AP differed as well. The SOC content was lower and the soil pH was higher at the lowest latitude but they did not differ for the other two locations. Contents of TN, AN and AP values were lower for the highest latitude than for the other two locations.

3.2 Latitude variation pattern of forest soil bacterial *a*- and *ß*-diversities

Analysis of 16S rDNA gene sequences in the 15 samples revealed information on the structures of the bacterial communities. The vast majority of OTUs were detected at all three latitudes (n=1,267), but unique and partially shared OTUs were recognized as well, as visualized in a Venn diagram (Figure 1). There were 1,472 identified bacterial OTUs recognized in HL, 1,591 in ML and 1,507 in LL, and these soils contained 44, 11 and 34 unique OTUs, respectively (Figure 1). The fewest number of OTUs were exclusively shared by LL and HL (n=27), whose locations were most distant, and the most OTUs were exclusively shared by ML and LL (n=179).

As shown in Table 3, the bacterial *a* diversity of the forest soils at different latitudes varied significantly. HL produced the lowest values for the ACE, Chao 1, Shannon and Simpson index and these were all significantly lower compared to LL ($p<0.05$). The highest values for Shannon and Simpson index were observed in LL with 8.85 and 0.9948, respectively, followed by ML and HL. The higher ACE and Chao one index of ML was not significant different compared to HL or LL ($p>0.05$) (Table 3).

NMDS plots based on confidence intervals ($p<0.05$) were produced (Figure 2A) that showed an absence of overlap between the different sites, which illustrated that the soil bacterial communities (stress = 0.0225) were clearly different in the three areas. This was confirmed by PERMANOVA analysis, whose findings are shown in a box plot (Figure 2B) (significant difference between groups, $R^2 = 0.743$, $p<0.01$).

3.3 Variation in bacterial composition at different latitudes

At the phylum level (Figure 3), the soil bacterial communities of the LL, ML, and HL were dominated by *Acidobacteria* (35%, 43%, and 43%, respectively) and *Proteobacteria* (37%, 35%, 39%). Much lower levels were reported for *Rokubacteria* (8.6%, 7%, 0.5%), *Actinobacteria* (7%, 4%, 6%), and *Gemmatimonadetes* (4.9%, 2.6%, 1.8%), and even lower abundance was noted for other phyla (Figure 3). There were differences between the samples taken at the different latitudes, notably for lower levels of *Acidobacteria* in LL ($p<0.001$), but also for the other phyla shown in the figure. *Proteobacteria* members were lowest in soil collected in ML($p<0.01$). The relative abundances of Gemmatimonadetes, *Rokubacteria*, *Chloroflexi* and *Nitrospirae*

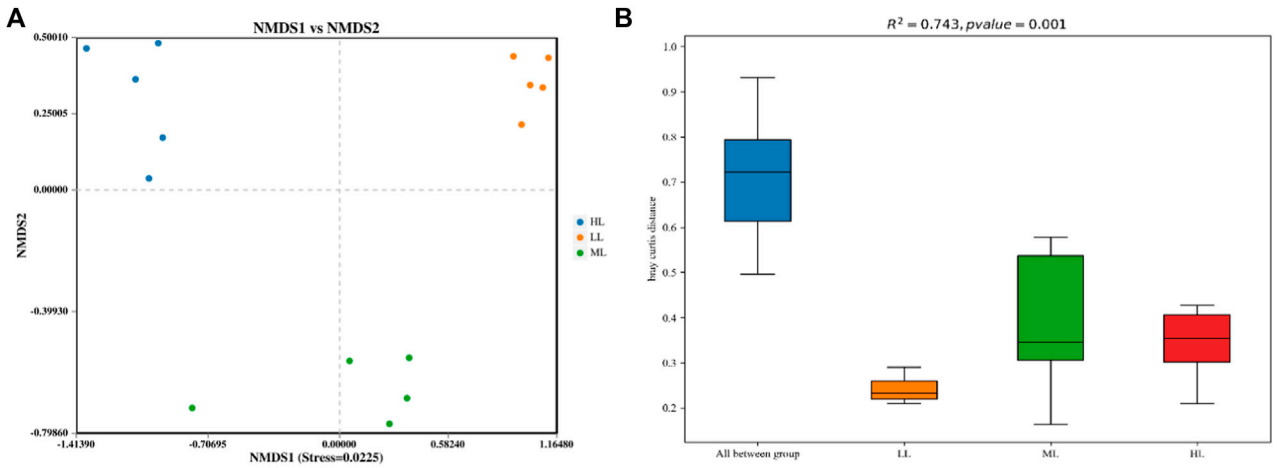

FIGURE 2
NMDS plot **(A)** and PERMANOVA analysis **(B)** of the soil bacterial OTUs based on Bray Curtis metrics among all samples. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

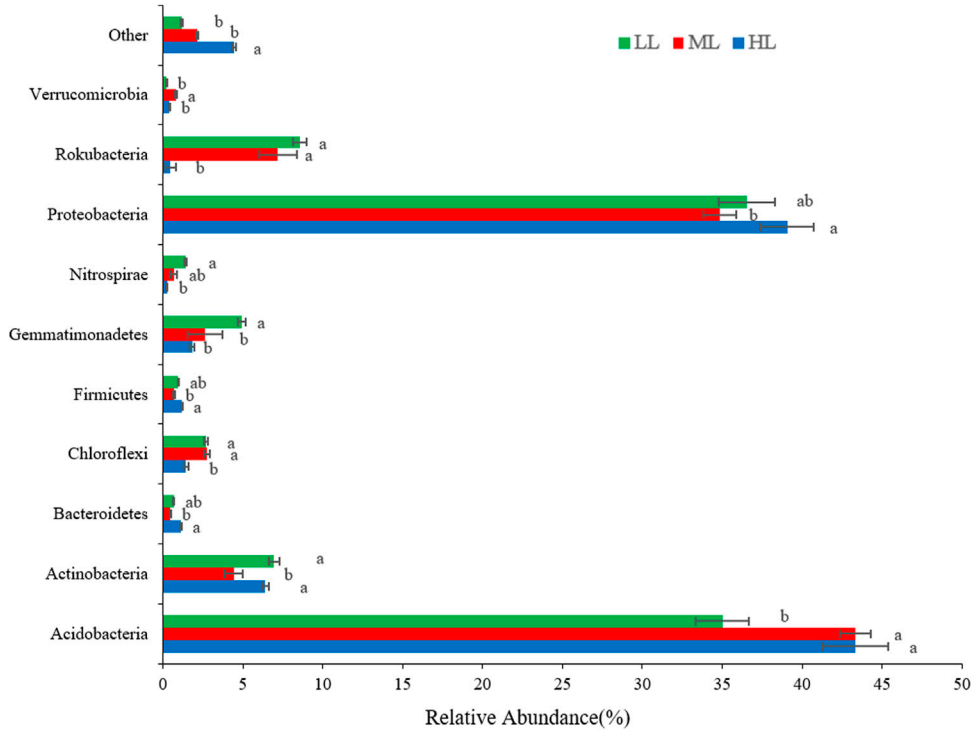

FIGURE 3
Histogram of the relative abundance of bacterial phyla detected in the forest soil at different latitudes. Different lowercase letters indicate significant differences at the $p < 0.05$ level among the treatments. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

produced decreasing trends (of variable strengths) with an increase of latitude (Figure 3).

At the genus level, the soil of HL and LL was dominated by *Candidatus_Solibacter* (4.1%, and 5.0%, respectively) and *Bryobacter* (4.5% and 3.8%), while these genera were present at significantly lower (p<0.001) levels in ML (Figure 4). *Roseiarcus* (4.5%) and *Granulicella* (4.3%) were particularly abundant in HL but undetected in ML. Conversely, *Sphingomonas* and *MND1* were dominant in ML, while *Lactococcus* was particularly abundant in LL (p<0.001). Thus, *Bryobacter* and *Roseiarcus* were the most abundant genera in the HL where *Nitrospira* and *MND1* were the least abundant. *Sphingomonas* was the most abundant genus in ML, where *Roseiarcus* and *Granulicella* were least abundant. Lastly, *Candidatus_Solibacter* was the most abundant genus in LL while *Granulicella* was present at lowest abundance here (Figure 4).

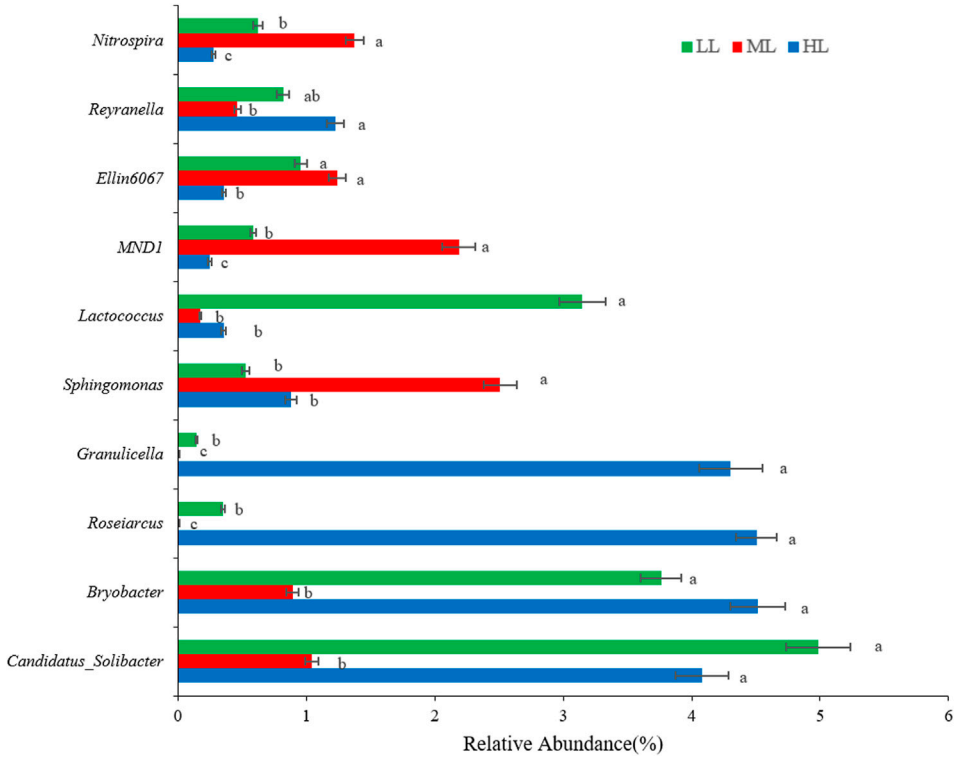

FIGURE 4
Genus-level composition of bacteria detected in the forest soil at different latitudes. Different lowercase letters indicate significant differences at the $p< 0.05$ level among the treatments. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

A heatmap analysis was performed to compare over- and under-abundance of the 24 most abundant bacterial genera in the five replicates for each latitude (Figure 5). This revealed co-enrichment patterns, as indicated that the genera *Elin6067*, *MND2*, *Sphingomonas* and *Dongia* in LL, *Candidatus_Udaeobacter*, *GAS113*, *Reyranella* and *Candidatus-Koribacter* in ML, *Burkhoderia*, *Rosearcus*, *Granulicella* and *Occallatibacter* in HL, (Figure 5).

3.4 Indicator species

A LEfSe analysis was conducted to determine the classified bacterial taxa with significant relative abundance differences among the different soil samples. Significant differences were observed among 16 taxa in the three latitudes, as indicated by LDA effect size scores of >4.5 (Figure 6). These are shown to the right of the cladogram in the figure.

3.5 Relationship between soil chemical properties and bacterial communities

Pearson correlation analysis was used to detect correlations between soil bacteria *a*-diversity and soil chemical factors. The results (Table 4) indicated that the ACE and Chao1 diversity indices correlated with TN, AN and TP ($p<0.05$), while the Shannon diversity was correlated with MAT and pH ($p<0.05$). The Simpson diversity did not correlate with any of the determined soil chemical properties (Table 4).

An RDA analysis indicated that soil chemical properties were important factors driving the structural and compositional changes of the soil bacterial communities (Figure 7A). The cumulative variations in the first and second RDA axes were 42.95% and 13.40%, respectively. Soil pH, MAT, SOC and TN were the major factors explaining the composition differences of the soil bacterial community. Further, through the correlation heat map analysis found the soil pH produced significant positive correlations with *Rokubacteria*, *Nitrospirae* and *Chloroflexi*, and negative correlations with *Acidobacteria*. Members of *Rokubacteria* correlated positively with all soil parameters except for SOC, while that soil parameter produced significant negative correlations with *Bacteroides*, *Proteobacteria* and *Actinobacteria*. Lastly, Cloroflexi correlated positively with AN and *Nitrospirae* with MAT (Figure 7B).

4 Discussion

4.1 Effects of latitude changes on bacterial alpha diversity

Most available studies have suggested presence of specific soil microbial communities at specific latitudes (Zhou et al., 2016; Tian et al., 2018; Zheng et al., 2021). The present study shows that the bacterial Shannon index of forest soil decreased gradually with increasing latitude, and the difference between highest and lowest latitude was significant (Table 3). There are two possible reasons for this. On the one hand, higher temperatures at lower latitudes promote

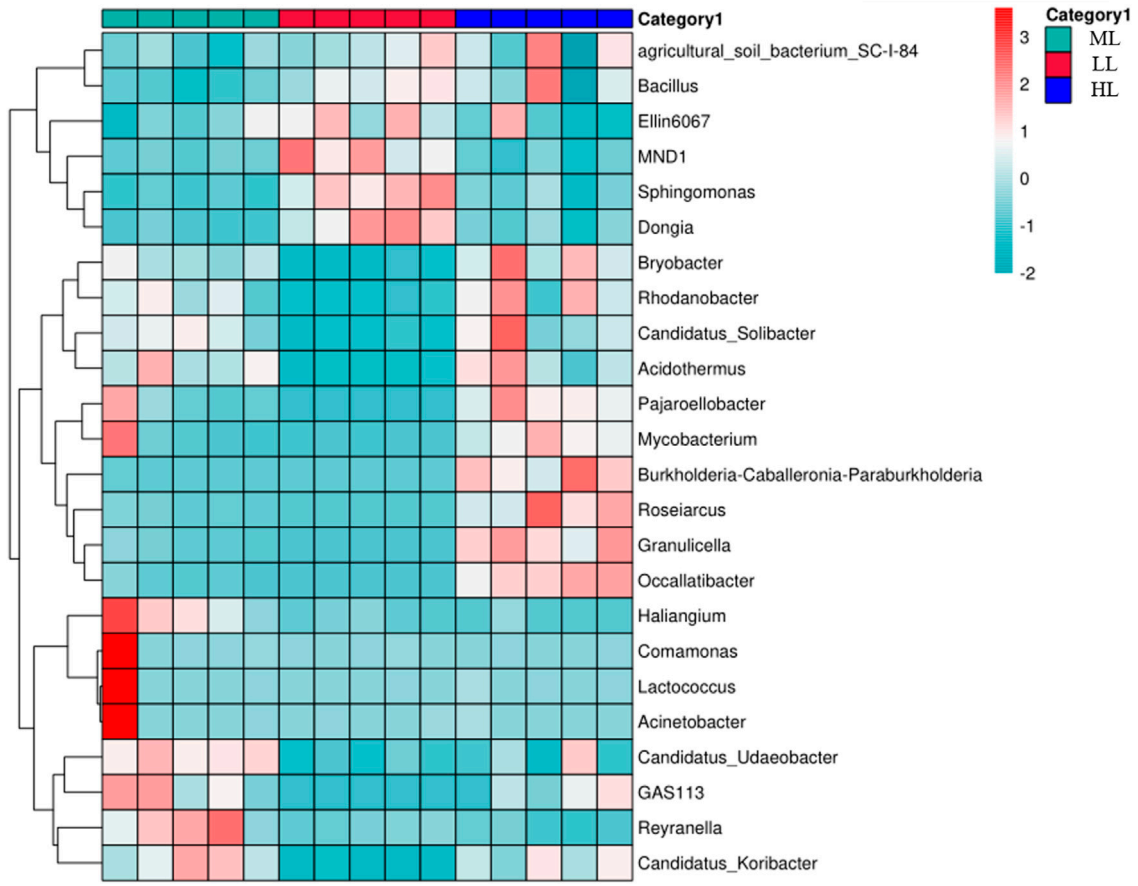

FIGURE 5
Heatmap and hierarchical clustering of the relative abundances of the top 24 genera detected in the individual soil samples. Co-enriched genera are ioutlined in red. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

the metabolism and population growth of soil bacteria, and accelerating litter decomposition and improving nutrient availability (Anderson et al., 2009; Zhou et al., 2020). On the other hand, lower pH at high latitudes acidifies forest soils, leading to an increase in acidophilus, which may reduce the number of other species and thus reduce soil bacterial diversity. The mid-latitude soil exhibited the highest bacterial ACE and Chao1 index, though this was not significantly different from low-latitude. Variations in ACE and Chao1 indices may be attributed to the effect of soil physicochemical factors, as levels of TN, AN and TP correlated significantly with soil ACE and Chao1 indices. This indicates that changes in latitudes can affect soil physicochemical factors and therefore affect the abundance of bacteria.

Explaining how biodiversity changes with spatial, temporal, and environmental gradients and its main drivers remains a central focus on biodiversity science. In recent years, research was performed to identify the factors that influence and drive the spatial distribution of microorganisms. Most studies have identified soil pH as the main factor driving soil microorganisms diversity and spatial distribution (Fiere and Jackson, 2006; Griffiths et al., 2011; Tripathi et al., 2018; Chen et al., 2022). In this study, we found that pH directly affected the diversity of soil bacterial communities in different latitudes and produced significant correlations with four phyla, suggesting that pH is a major factor driving alpha diversity in forest soil bacterial communities. This is consistent with results from the literature. Likewise, Bahram et al. (2018) found that pH caused niche differentiation of soil bacteria and fungi on a global scale and identfied certain trends of composition and diversity of soil bacterial communities with pH. However, according to a recent study, on a continental scale temperature regulates the diversity of microorganisms in forest soils (Zhou et al., 2016). Our study found that the soil bacterial alpha-diversity decreased with increasing latitude, with a significant correlation between temperature and soil bacterial Shannon diversity (Table 3), indicating that soil bacteria had strong latitudinal diversity patterns related to temperature in the temperate forests of China. Temperature can affect soil bacterial diversity through various mechanisms. First of all, the most important direct mechanism is that a higher temperature will increase the soil bacteria's metabolic rates and growth (Wang et al., 2009; Segura et al., 2015). Secondly, higher temperatures promotes plant diversity and productivity, which in turn provides substrates and nutrients for bacterial growth, thus improving soil bacterial diversity (Lange et al., 2015; Deng et al., 2020). Thirdly, higher temperatures support more effective litter decomposition and promote nutrient release, increasing nutrient availability that allows higher bacterial diversity (Zhou et al., 2012; Gao et al., 2022). Finally, the temperature may interact with soil

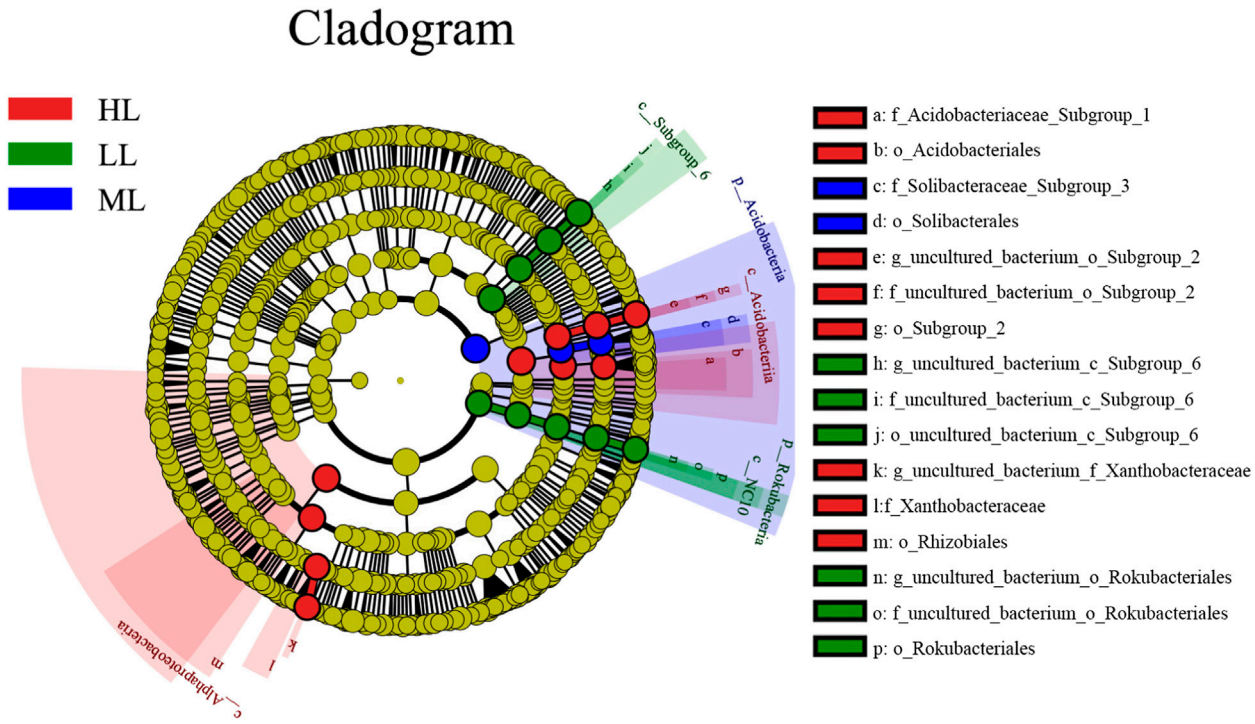

FIGURE 6
Cladogram of the bacterial communities at the three latitude soils with LDA >4.5 (A). LL, low-latitude; ML, mid-latitude; HL, high-latitude.

TABLE 4 Correlation coefficients of bacterial alpha diversity indices and soil chemical factors.

	MAT	pH	SOC	TN	An	TP
ACE	0.629	0.205	0.536	0.762*	0.752*	0.723*
Chao1	0.665	0.216	0.579	0.792*	0.793*	0.765*
Shannon	0.726*	0.760*	0.046	0.681*	0.643	0.616
Simpson	0.537	0.605	0.115	0.557	0.471	0.434

Different letters in the same column indicate significant differences among different areas ($p < 0.05$).

pH, soil nutrients and other environmental factors to regulate the change of soil bacterial diversity (Shen et al., 2013; Delgado-Baquerizo and Eldridge, 2019).

4.2 Effects of latitude changes on bacterial compositions

Our results identifiud the main bacterial phyla at different latitudes as *Acidobacteria* and *Proteobacteria*, followed by *Rokubacteria*, *Actinobacteria* and *Gemmatimonadetes*. Furthermore, we found that increasing latitude significantly decreased the abundance of *Acidobacteria*. Members of that phylum have a strong sensibility to the environment, such as vegetation type and soil pH, and these can affect the diversity and community composition of soil *Acidobacteria*. In both HL and ML, the soil pH was under 5.0, *Acidobacteria* in these soils can degrade cellulose under *microaerobic* and anoxic conditions, and the refractory needles in coniferous forests increased, which promoted the expansion of *Acidobacteria* populations. Previous studies have shown that *Proteobacteria* and *Actinobacteria* are mainly involved in the decomposition of organic matter, and the relative abundance of *Proteobacteria* is positively correlated with soil carbon content (Li et al., 2018; Li et al., 2022; Zhang et al., 2022). Indeed, both the SOC content and the relative abundance of *Proteobacteria* was highest in HL, which was consistent with previous results. Most *Actinomycetes* are aerobic, grow at an optimum temperature of 28°C–30 °C, and are abundant in neutral or slightly alkaline soils. The relative abundance of *Actinobacteria* was highest in LL, this is mainly because the low latitudes provides suitable temperature and soil pH for the growth of *Actinobacteria*. Increasing latitude brought about a reduction in the relative abundances of *Gemmatimonadetes*, *Rokubacteria*, *Chloroflexi* and *Nitrospirae*, which was somewhat inconsistent with published observations that described revegetation resulted in initial increases and then decreases

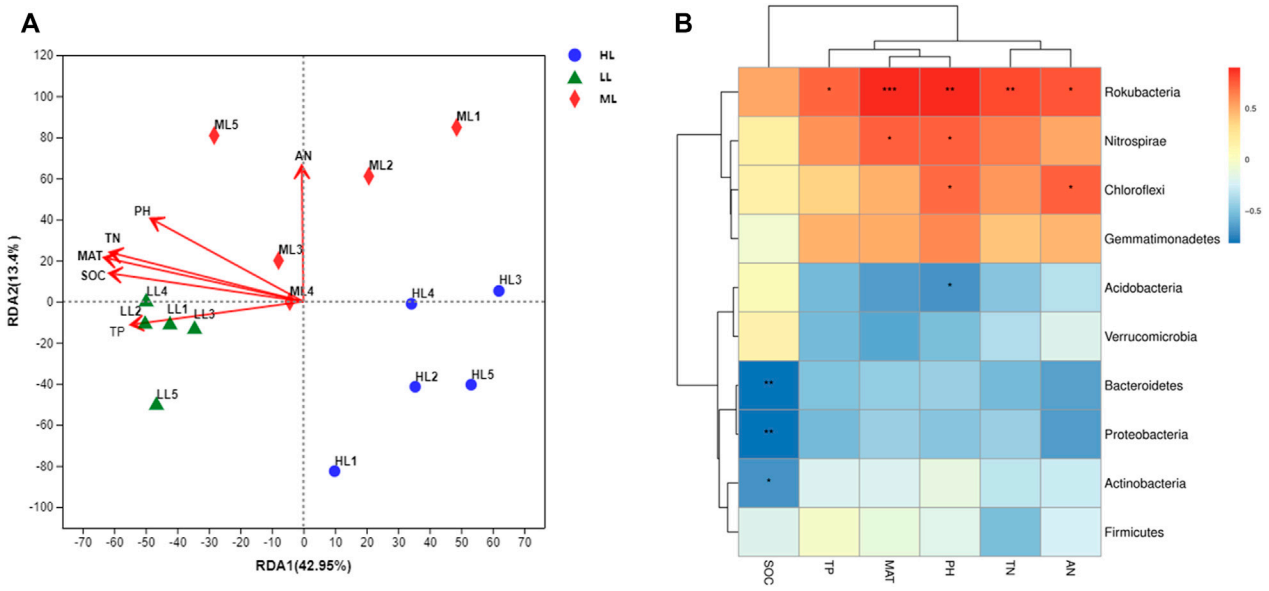

FIGURE 7
Correlations between soil chemical properties and bacterial phyla. RDA **(A)**, Heatmap **(B)**. LL, low-latitude; ML, mid-latitude; HL, high-latitude.

of *Gemmatimonadetes*, *Chloroflexi*, and *Nitrospirae* (Zhou et al., 2016; Tian et al., 2018). Further research is needed to address the reasons for this discrepancy, which may be attributed to the different vegetation types and different latitudinal gradients.

At the genus level, *Candidatus_Solibacter* and *Bryobacter* were highly abundant in two or three of the investigated soils, while *Roseiarcus*, *Granulicella* and *Lactococcus* were abundant in one soil type (Figure 4), which was consistent with other studies (Challacombe et al., 2017; Zhang et al., 2021; Kim et al., 2021). Members of the most abundant *Candidatus_Solibacter* and *Bryobacter* genera are involved in decomposing organic matter and use this as a carbon source. Interestingly, their relative abundance was significantly lower in ML. This may be due to the acidity of soil in high latitude coniferous forest, which provides a suitable environment for their growth, while low latitude broad-leaved forest provides sufficient carbon sources. The high relative abundance of *Roseiarcus* in the high-latitude coniferous forest soil of HL was consistent with previous results (Zhang et al., 2014; Rime et al., 2015; Du et al., 2016). The effects of latitude on the diversity and abundance of bacteria have been linked to the quantity and characteristics of the organic matter derived from plant litter, as it constitutes an important nutrient source for soil microorganism growth (Wagai et al., 2011).

The main drivers of soil bacterial composition can result in inconsistent responses at different study scales. Many studies showed that difference of soil factors at local and regional scales had a major impact on microorganisms communities, while climate factors and geography plays a more important role at larger scales (Tripathi et al., 2012; Zhang et al., 2020). In the present study, the soil bacterial community structure was affected by multiple environmental factors and was not determined by a single factor, which was similar to other results (Griffithset al., 2011; Delgado-Baquerizo and Eldridge, 2019). According to Pearson correlation analyses, the 10 most abundant bacterial phyla were mainly influenced by pH, MAT, SOC and TN: *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Rokubacteria* and *Nitrospirae* all produced significant, though variable correlations with one or more of these soil factors (Figure 7B). Although temperature is considered a major factor regulating soil bacterial composition at large scales, it only signficiantly affected abundance of *Rokubacteria* and Nitrospirae in our study, wherea pH played an important role in the distribution of soil bacterial core species in the different latitudes. This may be because pH can affect specific communities, such as increasing the relative abundance of *Acidobacteria*, while decreasing the relative abundance of actinomycetes, thus affecting the spatial distribution of the communities (Xia et al., 2016; Tian et al., 2021). In addition, soil nutrient factors such as SOC and TN are also closely related to bacterial composition, suggesting these too are main drivers of soil bacteria at different latitudes. Thus, there are no single climatic factors or soil physical and chemical properties, but more likely multiple factors interact with each other to regulate changes in soil bacterial composition. Therefore in the future, environmental factors shoud more comprehensively be considered in combination to explore their interactions and respective contributions, it can better reveal the driving mechanism of soil microbial distribution and stability on a large scale.

5 Conclusion

Our results indicate that the bacterial diversity of temperate forests in China decreases with increasing latitude. MAT, pH and TN were significantly correlated with bacterial diversity. At the same time, *Acidobacteria*, *Proteobacteria* and *Actinobacteria* were the dominant phyla in soil bacteria at different latitudes. MAT, pH and TN were significantly correlated with the relative abundance of most dominant phyla. These results indicated that they were the main driving factors affecting the latitudinal patterns of soil bacteria in temperate forests. From a microbiological point of view, this study revealed that the distribution of bacterial communities in temperate forests followed the

latitudinal diversity pattern, which has important reference value for enrich the latitudinal pattern of biodiversity.

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

X-YF performed this experiment, analyzed the data, and wrote this MS. Z-CC performed this experiment and help to write this MS. H-WN, and R-TZ designed this experiment and revised this manuscript. The author(s) read and approved the final MS.

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Conflict of interest

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

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/feart.2022.1096931/full#supplementary-material

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EDITED BY
Tengxiang Lian,
South China Agricultural University,
China

REVIEWED BY
Kou Yongping,
Chengdu Institute of Biology (CAS),
China
Qiwu Sun,
Chinese Academy of Forestry,
China

*CORRESPONDENCE
Mai-He Li
✉ maihe.li@wsl.ch
Lianghua Qi
✉ qlh@icbr.ac.cn

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Soil fungal community characteristics vary with bamboo varieties and soil compartments

Wen Guo[1,2], Jian Zhang[1], Mai-He Li[2,3,4]* and Lianghua Qi[1,5]*

[1]Key Laboratory of National Forestry and Grassland Administration/Beijing Bamboo and Rattan Science and Technology, International Centre for Bamboo and Rattan, Beijing, China, [2]Forest Dynamics, Swiss Federal Institute for Forest, Snow and Landscape Research WSL, Birmensdorf, Switzerland, [3]Key Laboratory of Geographical Processes and Ecological Security in Changbai Mountains, Ministry of Education, School of Geographical Sciences, Northeast Normal University, Changchun, China, [4]School of Life Science, Hebei University, Baoding, China, [5]Sanya Research Base, International Centre for Bamboo and Rattan, Sanya, China

Soil fungi play an important role in nutrient cycling, mycorrhizal symbiosis, antagonism against pathogens, and organic matter decomposition. However, our knowledge about the community characteristics of soil fungi in relation to bamboo varieties is still limited. Here, we compared the fungal communities in different soil compartments (rhizosphere vs. bulk soil) of moso bamboo (*Phyllostachys edulis*) and its four varieties using ITS high-throughput sequencing technology. The fungal α diversity (Shannon index) in bulk soil was significantly higher than that in rhizosphere soil, but it was not affected by bamboo variety or interactions between the soil compartment and bamboo variety. Soil compartment and bamboo variety together explained 31.74% of the variation in fungal community diversity. Soil compartment and bamboo variety were the key factors affecting the relative abundance of the major fungal taxa at the phylum and genus levels. Soil compartment mainly affected the relative abundance of the dominant fungal phylum, while bamboo variety primarily influenced the dominant fungal genus. Network analysis showed that the fungal network in rhizosphere soil was more complex, stable, and connected than that in bulk soil. A FUNGuild database analysis indicated that both soil compartment and bamboo variety affect fungal functions. Our findings provide new insights into the roles of both soil compartments and plant species (including variety) in shaping soil fungal communities.

KEYWORDS

fungal diversity, network analysis, FUNGuild, *Phyllostachys edulis*, soil compartment, soil fungi, bamboo variety

1. Introduction

Changes in rhizosphere fungal communities can affect plant health and development, while bulk soil, as a resource pool for the rhizosphere soil, has a long-term effect on the rhizosphere fungal assemblage (Bledsoe et al., 2020; Fiore-Donno et al., 2022). Hence, soil compartments (rhizosphere and bulk soil), as defined by Steer and Harris (2000) and Schmidt et al. (2019), may help shape the variation of microbial communities. For instance, fungal diversity and functional guild relative abundance (arbuscular mycorrhizae, soil saprotroph) were reported to be higher in the bulk soil of moso bamboo than in the rhizosphere soil, while the relative abundance of the dominant fungal taxa was lower (Li S. et al., 2022). However, other studies indicated that the diversity of fungal communities in rhizosphere soil was higher than in bulk soil while the complexity of the network was lower (Schmidt et al., 2019; Ye et al., 2021). This difference may be caused by differences in the

quality and quantity of organic matter substrate, nutrient availability, and the amount of root secretions (Waldrop et al., 2006; Zhang et al., 2017).

The species and even the variety of plants can influence the formation of soil fungal communities, and different plants can exude different qualities and quantities of compounds through the root system, thus causing differences in the diversity and composition of rhizosphere fungal communities (Gomes et al., 2003; Mouhamadou et al., 2013). Moreover, some fungi can indirectly influence the composition of microbial communities by altering the physiology of the host plant or the pattern of root exudation (Söderberg et al., 2002; Gomes et al., 2003). Different plant varieties therefore may lead to variation in fungal community characteristics. For example, differences were observed in the diversity, composition and symbiotic network of soil fungal communities among different varieties of *Zea mays* in China (Kong et al., 2020; Gil-Martínez et al., 2021). However, Li et al. (2018) found that rice variety did not significantly affect fungal abundance or community composition. It is crucial to explore the effects of plant host specificity on fungal communities in order to distinguish between the effects of plants and of soil characteristics.

As the most important economic bamboo varieties, moso bamboo (*Phyllostachys edulis*) accounts for 73% of China's total bamboo forest area. It is characterized by rapid growth, a thick litter layer, and a well-developed whip-root system (Dixon and Gibson, 2014; Ramakrishnan et al., 2020). Its unique biological properties can influence the characteristics of the soil fungal community, thereby affecting soil nutrient cycling (Fang et al., 2022). To our knowledge, few studies have been conducted to simultaneously investigate fungal community characteristics in relation to both bamboo variety and soil compartment. Differences in root exudates and soil microenvironment due to the large phenotypic variation may affect soil fungal communities and host selection. Here, we sequenced the fungal ITS regions in the bulk soil and rhizosphere soil compartments under moso bamboo and its four varieties to test our hypotheses that (I) the characteristics of soil fungal communities in rhizosphere soil are significantly different from those in bulk soil, and (II) the characteristics of soil fungal communities differ among bamboo varieties.

2. Materials and methods

2.1. Study site and experimental design

In August 2018, rhizosphere and bulk soils of five moso bamboo varieties were collected from a bamboo germplasm garden in Taiping, Anhui, China, which was planted 10 years earlier. The study area is located at the northern edge of the subtropics, at an elevation of 250 m, and the average annual temperature and precipitation are 15.8°C and 1,560 mm, respectively. The soil type is yellow-red, with a pH of 4.55. The following moso bamboo varieties were selected: PE (*P. edulis*), FT (*P. edulis* f. *tao kiang*), FL (*P. edulis* f. *luteosulcata*), FP (*P. edulis* f. *pachyloen*), and FG (*P. edulis* f. *gracilis*). The size of the sample plots was 20 m × 20 m, and the plots were separated by concrete walls. The soil physical and chemical properties and plant morphological characteristics of the sample plots were provided by Guo et al. (2022). The rhizosphere and bulk soil samples were collected according to the method of Semenov et al. (2020). In this study, there were 30 samples (5 bamboo varieties × 3 replicates × 2 soil compartments), and each soil sample was a composite of five randomly collected subsamples. Visible debris was removed from the samples, which were then sieved, packed in sterile bags, transported to the laboratory at low temperature, and stored in a refrigerator at −80°C for fungal extraction and sequencing analysis.

2.2. Fungal community analysis

Genomic DNA extraction was performed using the Power Soil DNA Isolation Kit (MOBIO Laboratories, Carlsbad, CA, United States). The fungal gene region was amplified using the fungal primers ITS3 (5′-GCATCGATGAAGAACGCAGC-3′) and ITS4 (5′-TCCTCCGCTT ATTGATATGC-3′) with barcodes (Zhang et al., 2017; Engelhardt et al., 2018; Perez-Mon et al., 2022). The polymerase chain reaction was as follows: 2× Premix Taq (25 μL) + primer-F (1 μL) and primer-R (1 μL) + DNA (3 μL, 20 ng/μL) + nuclease-free water (20 μL). The polymerase chain reaction and purification operations were performed according to the method of Zheng et al. (2021). The PCR reactions contained 25 μL of 2× Premix Taq, 1 μL of each primer, 3 μL DNA (20 ng/μL), and 20 μL ddH_2O. The thermal cycling conditions of fungi were as follows: 5 min at 94°C, 30 cycles of 30 s at 94°C, 30 s at 52°C, 30 s at 72°C, and 10 min at 72°C.

2.3. Bioinformatics analysis

The raw sequences were quality filtered using the QIIME2 pipeline. The forward and reverse reads were merged using PEAR software (version 0.9.8; Gdanetz et al., 2017). Sequences were removed if their mean quality score was <20 or if their length was <200 bp, and ambiguous sequences were also removed. Illumina amplified sequence data were detected and corrected using the DADA2 denoising algorithm, and random resampling was performed at a constant depth of 8,000 sequences per sample. Fungal OTUs were taxonomically identified using the UNITE 8.0 database (Tedersoo et al., 2018). Before alpha (α) analysis, the sequences were normalized according to the lowest number of sequences for a single sample. The sequence files were submitted to the NCBI Sequence Read Archive (SRA) under BioSample accession number SUB12473231.

2.4. Statistics analysis

The α diversity (Shannon index) of the fungal communities was calculated using the vegan package (v2.5-7; Oksanen et al., 2020) in the R environment (v4.1.2; R Core Team, 2020). Differences between bamboo varieties and soil compartments were assessed using two-way analysis of variance (ANOVA) using the R package rcompanion (v2.4.15; Fox and Weisberg, 2018). Based on a Bray–Curtis distance matrix, a principal coordinate analysis (PCoA) of the fungal communities was conducted using the vegan package. Permutation multivariate analysis of variance (PERMANOVA) was used to test whether the fungal communities were affected by soil compartment and bamboo variety ($p < 0.05$) at the operational taxonomic unit (OTU) level. The statistical visualization packages ggplot2 (v3.2.1) and ggpubr (v0.4.0) in R were used as part of the above analyses. The relative abundances of major fungal taxa (phyla and genera) were visualized using the R packages statnet (v2019.6) and circlize (v0.4.15), and the effects of soil compartment and bamboo variety on major fungal taxa (phyla and genera) were assessed using two-way ANOVA. Moreover, the FUNGuild database was used to predict fungal

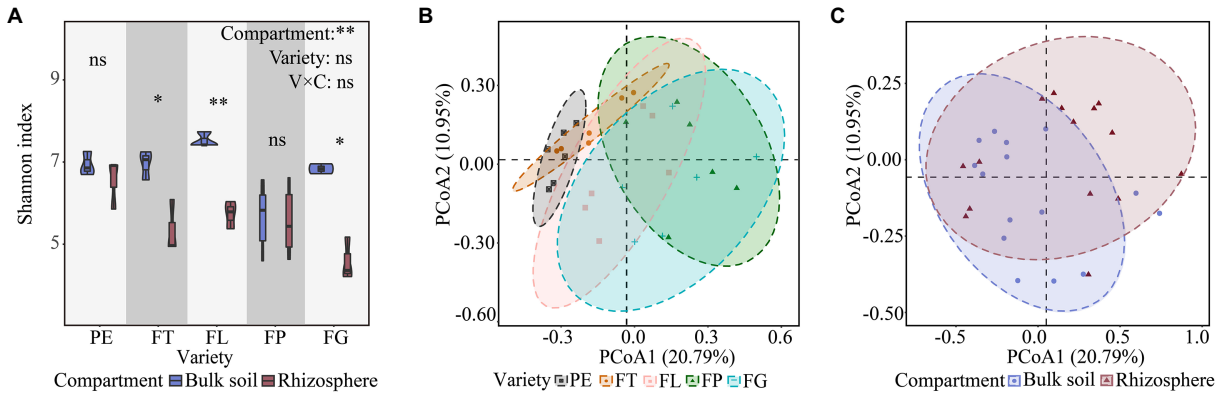

FIGURE 1
Effects of soil compartment, bamboo variety, and their interactions on fungal α **(A)** and β **(B,C)** diversity. "*"p<0.05, "**"p<0.01. PE, *Phyllostachys edulis*; FT, *P. edulis* f. *tao kiang*; FL, *P. edulis* f. *luteosulcata*; FP, *P. edulis* f. *pachyloen*; FG, *P. edulis* f. *gracilis*.

function based on relative abundance at the OTU level (Nguyen et al., 2016). This step was performed on the FUNGuild website.[1] According to the results of the confidence assessment of the FUNGuild database, only the confidence levels "highly probable" and "probable" were used for subsequent analysis. The heat map of fungal functions was visualized using the pheatmap (v1.0.12) R package. The effects of soil compartment and bamboo variety on fungal functions (trophic modes and functional guilds) were assessed using two-way ANOVA.

Fungal networks were constructed for both the rhizosphere and bulk soil. The complexity of the fungal community was evaluated using network analysis, and potential key taxa in the two soil compartments were identified. To reduce the complexity of the networks, only genera with a relative abundance > 0.1% were selected for the analysis. The R packages psych and igraph were used for Spearman's correlation analysis to construct the fungal co-occurrence networks. To ensure network robustness, only networks with a Spearman's correlation coefficient of $r > 0.80$ or $r < -0.80$ and a corrected $p < 0.01$ were retained (Benjamini and Hochberg false discovery rate, FDR; Langfelder and Horvath, 2012; Gao et al., 2019). The nodes and edges in the constructed networks represented genera and the correlations between pairs of genera, respectively. Following the method by Hough et al. (2020), keystone species were identified based on PageRank scores that quantify connectivity between nodes. The R packages psych and igraph and the software Gephi (v0.9.0; Mendes et al., 2014) were used to calculate node correlations and for network visualization (Bastian et al., 2009).

3. Results

3.1. Diversity and composition of the soil fungal community

Soil compartment had a significant effect on the Shannon index of the fungal community ($p < 0.05$), whereas bamboo variety and the compartment × variety interaction effects were not significant (Figure 1A). The Shannon index of the fungal community in the bulk soil was higher than that in the rhizosphere soil for all bamboo varieties. For rhizosphere soil, the Shannon indices of the fungal communities under the five bamboo varieties decreased in the order PE > FL > FP > FT > FG. For bulk soil, the Shannon index was highest under variety FL and lowest under FP (Figure 1A).

The first two axes in the PCoA accounted for 31.74% of the variance in the data matrix, with PCoA1 and PCoA2 representing 20.79% and 10.95%, respectively (Figures 1B,C). PERMANOVA indicated that there was a significant separation of fungal communities between the different soil compartments and bamboo varieties, which differed significantly in distance ($p < 0.05$, Supplementary Table S1). Further, there was an interaction effect between soil compartment and bamboo variety ($p < 0.05$, Supplementary Table S1), and rhizosphere fungal samples showed the highest degree of dispersion.

The dominant fungal phyla detected included Ascomycota (53.99%–21.74%), Basidiomycota (48.96%–2.39%), and Mortierellomycota (45.69%–1.82%; Figure 2A; Supplementary Table S2). The dominant fungal genera detected included *Mortierella* (53.01%–2.83%), *Umbelopsis* (23.74%–5.11%), and *Trichoderma* (24.21%–5.61%; Figure 2B; Supplementary Table S3). At the phylum level, the effect of soil compartment on the relative abundance of the dominant fungal taxa was stronger than that of bamboo variety. However, bamboo variety significantly affected the relative abundance of dominant fungi at the genus level. Except for Basidiomycota, there was no significant interaction between soil compartment and bamboo variety for the dominant fungal taxa (Figure 3; Supplementary Table S4).

3.2. Co-occurrence networks and potential keystone species of the soil fungal communities

Overall, rhizosphere soil (average degree, 20.7) had larger, more connected, and more complex fungal networks than bulk soil (average degree, 14.1), and the fungal communities of the different soil compartments were dominated by collaborative relationships (Figure 4; Supplementary Table S5). Moreover, PageRank screening indicated two keystone species in the rhizosphere (*Simplicillium*, *Nectria*) and in the bulk soil (*Penicillium*, *Laetisaria*; Supplementary Table S6).

1 https://github.com/UMNFuN/FUNGuild

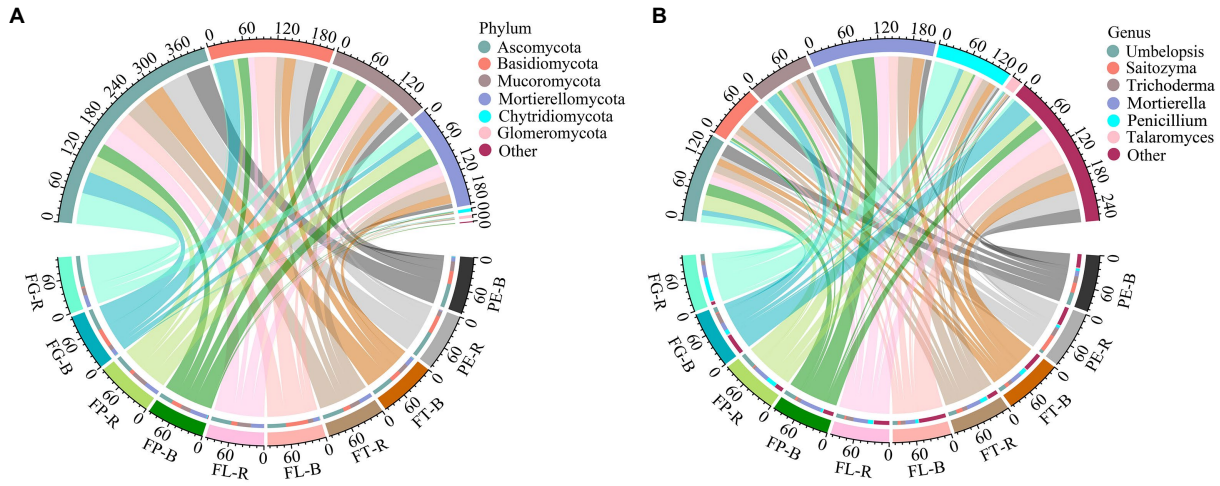

FIGURE 2
Composition of major fungal taxa at the phylum **(A)** and genus **(B)** level. Rhizosphere soil (R): PE-R, *Phyllostachys edulis*; FT-R, *P. edulis* f. *tao kiang*; FL-R, *P. edulis* f. *luteosulcata*; FP-R, *P. edulis* f. *pachyloen;* FG-R, *P. edulis* f. *gracilis*; Bulk soil (B): PE-B, *P. edulis*; FT-B, *P. edulis* f. *tao kiang;* FL-B, *P. edulis* f. *luteosulcata*; FP-B, *P. edulis* f. *pachyloen;* FG-B, *P. edulis* f. *gracilis*. The first six dominant fungal phyla (genera) with a relative abundance > 1% are shown in figure.

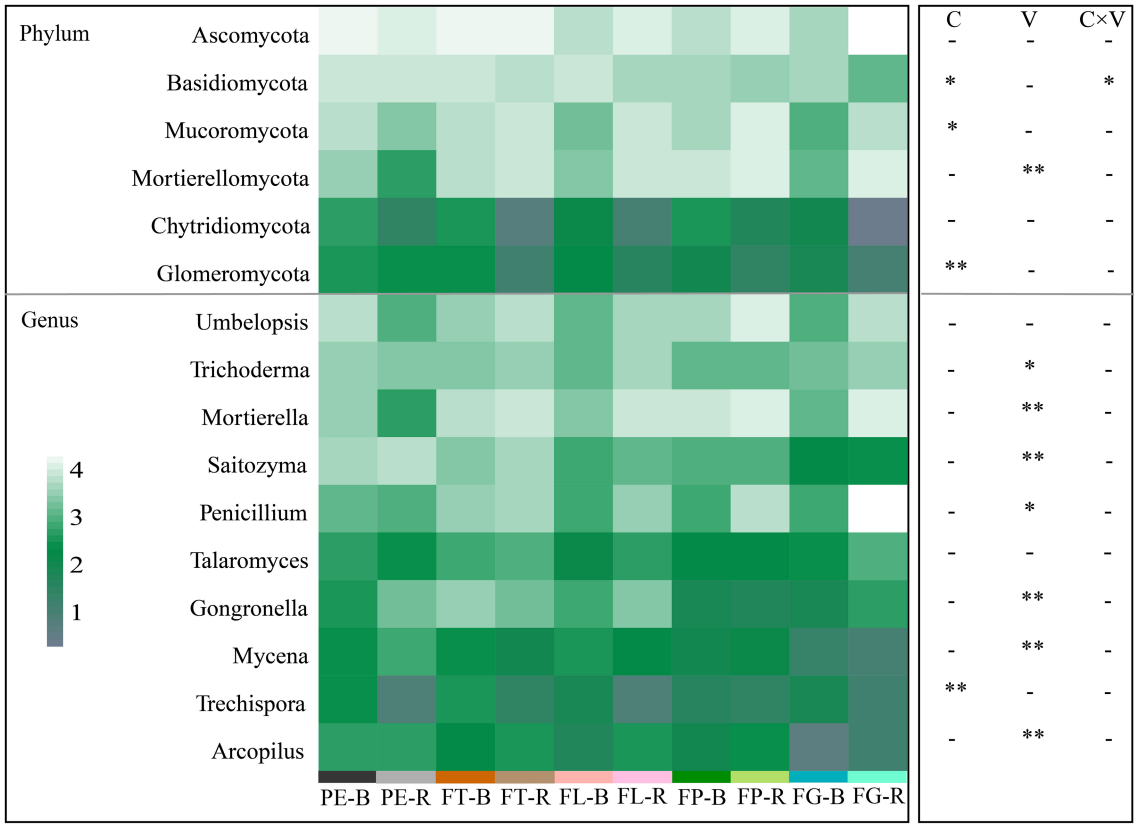

FIGURE 3
Effects of soil compartment, bamboo variety, and their interactions on the major fungal phyla (top 6) and genera (top 10). C, soil compartment; V, bamboo variety. "*" $p<0.05$, "**" $p<0.01$. Rhizosphere soil (R): PE-R, *Phyllostachys edulis*; FT-R, *P. edulis* f. *tao kiang*; FL-R, *P. edulis* f. *luteosulcata;* FP-R, *P. edulis* f. *pachyloen;* FG-R, *P. edulis* f. *gracilis*. Bulk soil (B): PE-B, *P. edulis;* FT-B, *P. edulis* f. *tao kiang;* FL-B, *P. edulis* f. *luteosulcata;* FP-B, *P. edulis* f. *pachyloen;* FG-B, *P. edulis* f. *gracilis*.

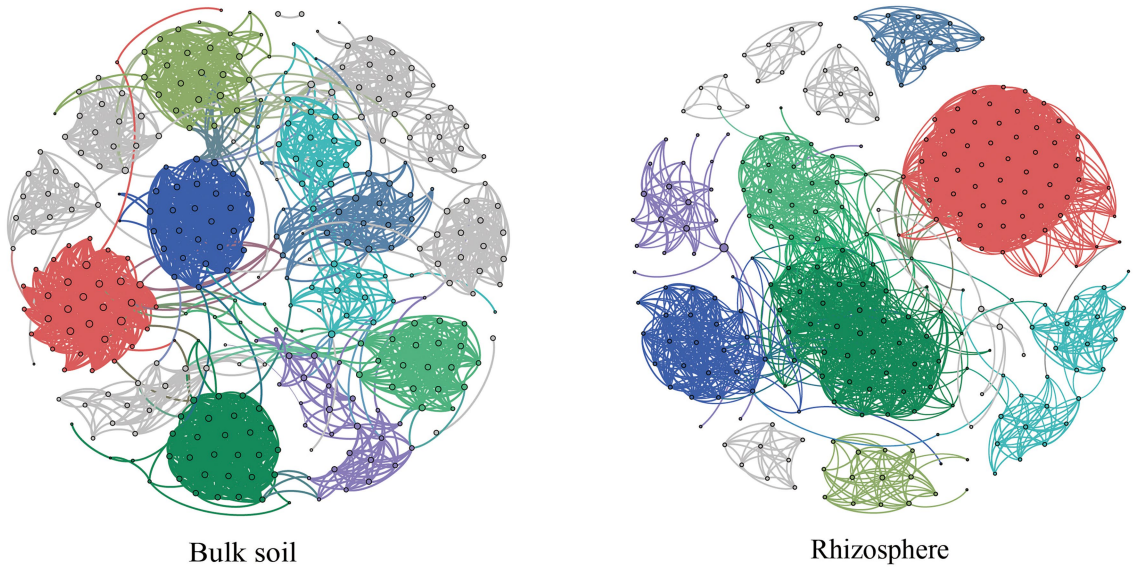

FIGURE 4
Effect of soil compartment on the co-occurrence network of fungal communities. Nodes and edges indicate genera and the strength of connections between genera, respectively, and different modules are represented by different colors.

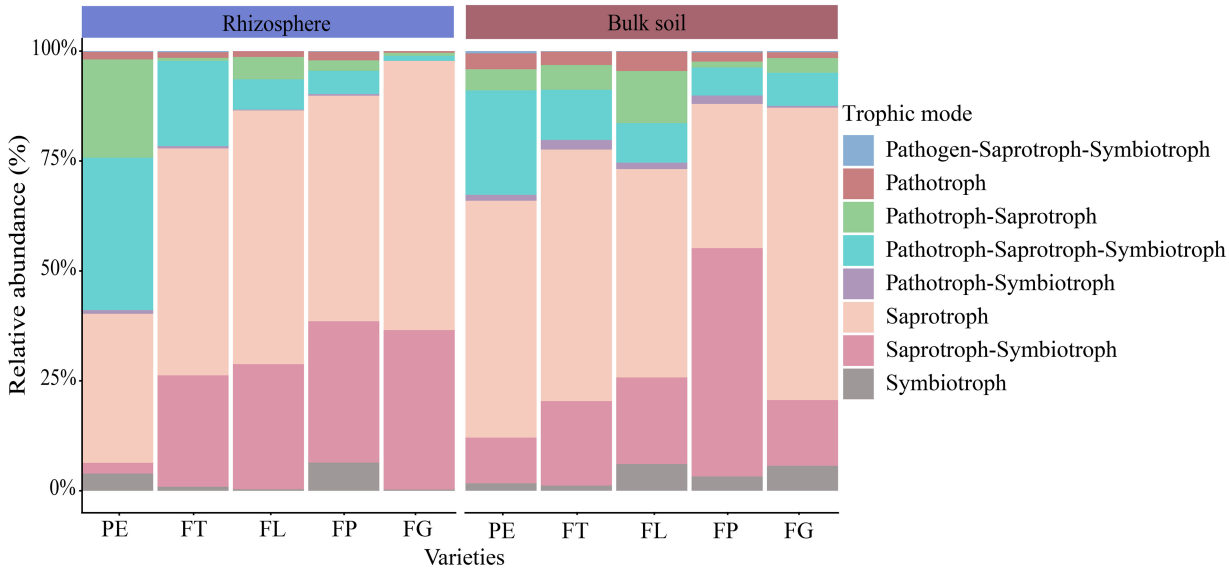

FIGURE 5
Relative abundance of fungal trophic modes in different soil compartments and bamboo varieties. PE, *Phyllostachys edulis*; FT, *P. edulis* f. *tao kiang*; FL, *P. edulis* f. *luteosulcata*; FP, *P. edulis* f. *pachyloen*; FG, *P. edulis* f. *gracilis*.

3.3. Fungal functional guild in relation to soil compartment and bamboo variety

The trophic mode of the soil fungi under the different varieties of moso bamboo was mainly saprotroph (51%), saprotroph-symbiotroph (24%), pathotroph-saprotroph-symbiotroph (13%), or pathotroph-saprotroph (6%), while the relative abundance of other trophic modes was <5% (Figure 5). Soil compartment significantly affected the relative abundances of the trophic modes pathogen-saprotroph-symbiotroph, pathotroph, pathotroph-symbiotroph, and symbiotroph, and bamboo variety significantly affected pathotroph-saprotroph and pathotroph-saprotroph-symbiotroph, but the interaction between compartment and variety was not significant. Bamboo variety and soil compartment both significantly affected the relative abundances of different fungal functional guilds, but their interaction was not significant (Supplementary Tables S7, S8; Supplementary Figure S1). Moreover, undefined saprotrophs, followed by wood saprotrophs, had the highest relative abundance (Figure 6).

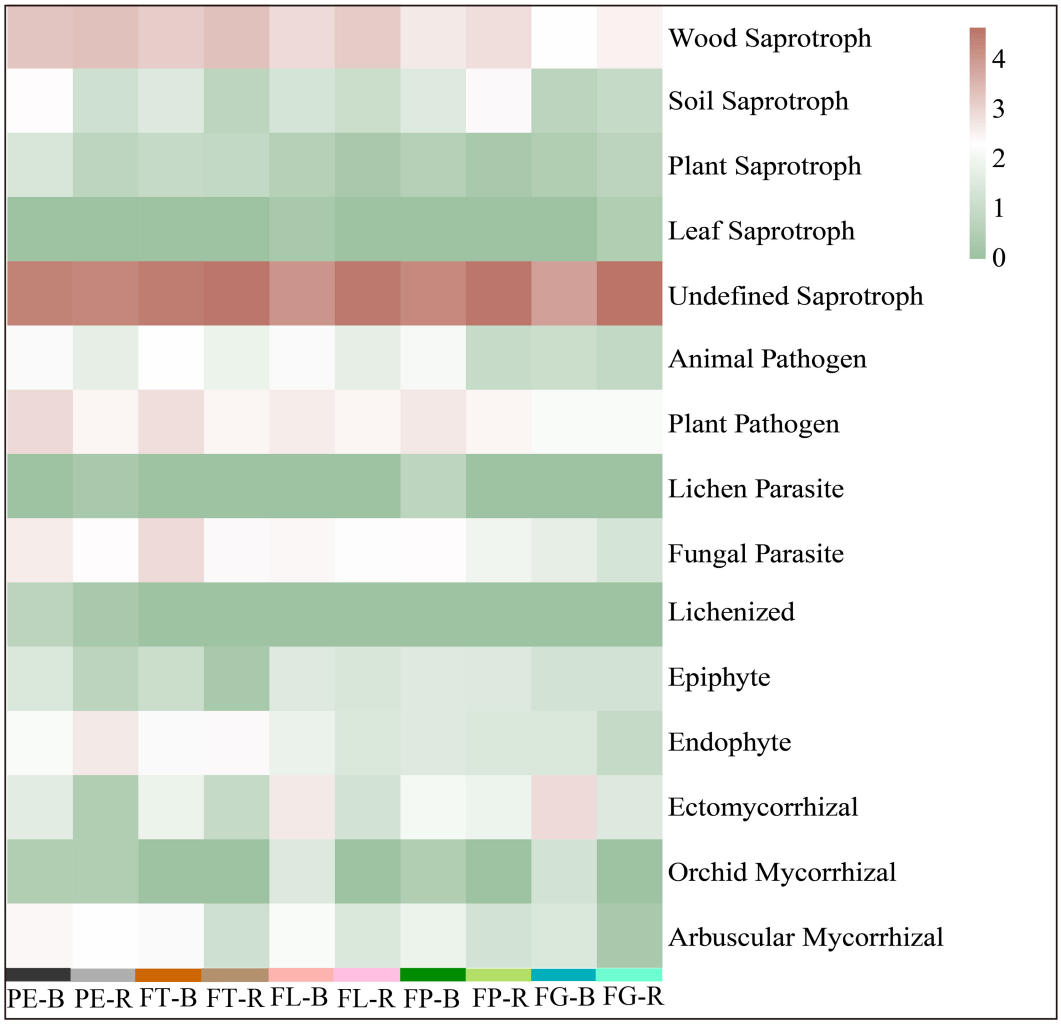

FIGURE 6
Prediction of the fungal functional guild based on the FUNGuild database. Rhizosphere soil (R): PE-R, *Phyllostachys edulis*; FT-R, *P. edulis* f. *tao kiang*; FL-R, *P. edulis* f. *luteosulcata*; FP-R, *P. edulis* f. *pachyloen*; FG-R, *P. edulis* f. *gracilis*. Bulk soil (B): PE-B, *P. edulis*; FT-B, *P. edulis* f. *tao kiang;* FL-B, *P. edulis* f. *luteosulcata;* FP-B, *P. edulis* f. *pachyloen*; FG-B, *P. edulis* f. *gracilis*.

4. Discussion

4.1. Effects of soil compartment on the characteristics of soil fungal communities

Consistent with our first hypothesis, the diversity of the fungal community differed significantly between the two soil compartments (Figure 1). In this study, the α diversity of fungi decreased from bulk soil to rhizosphere soil, suggesting allelopathic effects of bamboo root exudates on soil fungi. The fungal community associated with the rhizosphere may therefore be influenced by the host, leading to divergent fungal community characteristics in the different soil compartments (Figure 1A), as supported by findings from other recent studies (Urbina et al., 2018; Qin et al., 2022). However, Kong et al. (2020) found that the fungal α diversity in the rhizosphere soil of various maize varieties was higher than that in bulk soil, which could be related to factors such as species specificity and soil substrate (root exudates, cell debris; Nannipieri et al., 2007; Broeckling et al., 2008). In our study, soil compartment additionally significantly affected the relative abundance of fungal phyla (*Basidiomycota*, *Mucoromycota*, *Glomeromycota*) and genera (*Trechispora*; Figure 3; Supplementary Table S4), which may be due to the differentiation of fungal ecological niches across soil compartments. Findings from previous studies support these results (Schöps et al., 2018; Veach et al., 2019). For instance, ectomycorrhizal fungi of the genus *Trechispora* can supply water and nutrients to plant hosts, improve plant resistance to pathogens, and promote seedling growth and development, which may contribute to differences between soil compartments (Van der Heijden et al., 2015; Li Y. et al., 2022).

Also in line with our first hypothesis, the fungal network of the rhizosphere was significantly more complex and connected than that of bulk soil, implying that bulk soil may be more susceptible to external environmental disturbances (Figure 4; Supplementary Table S5). However, Li et al. (2021) reported opposite results to those from our study. It is worth noting that about 20% of plant photosynthesis products can be transported to the soil around the roots through exudation patterns, and the availability of resources to the fungal community, as well as the ecological niche, may alter their interactions, leading to different results (Nehls et al., 2016; Vishwakarma et al., 2017; Fan et al., 2018).

In our study, the keystone species of the rhizosphere and bulk soil were clearly different (Supplementary Table S6). *Simplicillium* and *Nectria* were the keystone taxa in bulk soil, and *Penicillium* and *Laetisaria* in the rhizosphere. Similar findings were reported in previous studies (Su et al.,

2020; Liu L. et al., 2021; Varsadiya et al., 2021; Zheng et al., 2021). For instance, *Penicillium* plays a key role in rhizosphere soil, it can generate products that promote plant health (e.g., soluble phosphorus, iron carriers, plant hormones) and affect plant adaptability through a series of biochemical processes (Altaf et al., 2018; Park et al., 2020). Further, *Simplicillium* has a wide range of hosts and substrates, which are associated with bioactive compounds and phytopathogens, and long-term continuous cultivation increases the relative abundance of *Simplicillium* (Wei et al., 2019; Liu Q. et al., 2021). Moreover, keystone species have been found to be involved in synergistic relationships, to change the community structure and function by altering the abundance of synergistic fungi, and even to affect plant growth and its productivity (Van Der Heijden et al., 1998; Banerjee et al., 2018). In this study, soil compartment significantly affected fungal functional diversity, supporting findings from a previous study (Liu et al., 2019). Interestingly, there were differences in resource availability and competition intensity between soil compartments. Fungi can flexibly adapt to changes in microenvironmental conditions, for example by transferring resources to restricted areas, which may be one of the reasons for the functional differences observed between soil compartments (Strickland and Rousk, 2010; Schöps et al., 2018).

4.2. Effects of bamboo variety on soil fungal community characteristics

In support of our second hypothesis, our results indicate that bamboo variety significantly affects fungal community β diversity (Figures 1, 3) but not α diversity. A previous study demonstrated that different *Zea mays* varieties had no significant effect on the α diversity of soil fungal communities, but that variety did affect their β diversity to some extent (Kong et al., 2020). Moreover, in our study bamboo variety significantly affected the relative abundance of major fungal genera with important functions (*Trichoderma*, *Mortierella*, *Saitozyma*, *Penicillium*, *Gongronella*, *Mycena*, *Arcopilus*; Supplementary Table S4). For instance, *Trichoderma*, which can promote root growth, regulate nutrient supply, and improve plant health, has been reported to be more efficient and competitive than other soil fungi (Harman et al., 2004; Vinale et al., 2008). Consistent with our findings, different *Solanum tuberosum* varieties have previously been observed to significantly affect fungal community composition (Hannula et al., 2010; Kong et al., 2020). Further, root secretions contain carbon substrates used by fungi, such as primary and secondary metabolites, and different plants can therefore maintain certain resident soil fungal taxa by mediating root secretions (Jones et al., 2004; Broeckling et al., 2008). There were differences in the effects of bamboo variety on the fungal communities, which may be caused by variety-dependent root secretions. Previous studies have indicated that different plant species can maintain resident soil fungal taxa through the mediation of root secretions (e.g., primary and secondary metabolites; Jones et al., 2004; Broeckling et al., 2008).

Our study supports the view that bamboo variety influences the function of soil fungi (Figure 5; Supplementary Table S7). Earlier studies have also shown that fungi are plant-dependent and that plant variety can influence the function of fungi (Berg and Smalla, 2009). However, the extent of this influence also depends, e.g., on the distance of the fungi from the root system and the morphology, biomass, and age of the roots (Marschner et al., 2004; Zhang et al., 2017). For instance, thinner roots may release more readily degradable carbohydrates than coarser roots (Kuzyakov et al., 2000), enhancing interactions with saprophytic fungi in the rhizosphere. This in turn may alter the proportion of strict symbionts and free-living saprotrophic fungi (Lozano et al., 2021), leading to changes in the functioning of the fungi in the community.

In conclusion, in the present study soil fungal community characteristics were significantly affected by both soil compartment and bamboo variety, in line with our two hypotheses. These results may be associated with the amount, chemical properties, and function of the root exudates and soil microenvironment. Our study emphasizes the important roles of soil compartment and plant species, including variety, in shaping soil fungal communities.

Data availability statement

The data presented in the study are deposited in the NCBI repository, accession number PRJNA771417.

Author contributions

WG and LQ conceived the experimental design. WG and JZ contributed to the field and indoor experiments. WG and M-HL contributed to the data analysis and manuscript writing. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Xin Sui,
Heilongjiang University,
China

REVIEWED BY
Wentao Luo,
Institute of Applied Ecology (CAS), China
Minghui Liu,
Northeast Forestry University,
China
Jiaming Liu,
Chinese Academy of Agricultural Sciences,
China

*CORRESPONDENCE
Dan Wei
✉ wd2087@163.com
Lei Wang
✉ yzswanglei@163.com

†These authors have contributed equally to this work

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Associations of soil bacterial diversity and function with plant diversity in *Carex* tussock wetland

Yan Li[1†], Chuanqi Shi[2†], Dan Wei[1*], Junnan Ding[2], Nan Xu[2], Liang Jin[1] and Lei Wang[1*]

[1]Institute of Plant Nutrition, Resources and Environment, Beijing Academy of Agriculture and Forestry Sciences, Beijing, China, [2]Heilongjiang Province Key Laboratory of Cold Region Wetland Ecology and Environment Research, Harbin University, Harbin, Heilongjiang, China

Some species of *Carex* can form tussocks, which are usually distributed in valleys and flood plains. The soil microbial community diversity and function of micro–habitats formed by tussocks are associated with plant diversity, and research on these associations can guide *Carex* tussock wetland restoration. In this study, we selected tussock wetlands dominated by *Carex appendiculata*, including natural wetlands (*NW*), artificially restored wetlands (*ARW*), and naturally restored wetlands (*NRW*), and investigated plant diversity. Soil samples were collected from the quadrats of each sample plot with the maximum (*ma*), median (*me*), and minimum (*mi*) plant Shannon index values, and high-throughput sequencing was used to analyze the bacterial community composition, diversity, and functions. The plant diversity indexes of neither *ARW* nor *NRW* significantly differed from that of *NW*, but the companion species in *NRW* were hygrophytes and mesophytes, in contrast to only hygrophytes serving as companion species in *NW* and *ARW*. The soil bacterial communities at the operational taxonomic unit level of the nine quadrats with different plant Shannon index values significantly ($p<0.01$) differed. The relative abundances of the dominant phyla (Proteobacteria, Chloroflexi, and Bacteroidetes) and the dominant genera (*Geobacter*, *Sideroxydans*, and *Clostridium* except for unassigned genera) significantly ($p<0.05$) differed under the different levels of plant diversity. The plant Shannon index, soil moisture content, total organic carbon, N, and P were significantly ($p<0.05$ or $p<0.01$) correlated with the bacterial Shannon index. The phylogenetic diversity of the bacterial community in *NW* was significantly ($p<0.0001$) different from those in *ARW* and *NRW*, and that in *ARW* was also significantly ($p<0.05$) different from that in *NRW*. The functional groups of bacterial communities associated with plant diversity. In the *NWme*, *ARWme*, and *NRWme* bacterial communities, the relative proportions of functional groups related to soil N cycle were higher, but those related to soil S and C cycles were lower. Considering the rehabilitation of both plant and microbial communities, the methods used for establishing the *ARW* are recommended for *Carex* tussock wetland restoration.

KEYWORDS

soil bacteria, bacterial diversity, bacterial function, plant diversity, tussock wetland

Introduction

Wetlands are areas with permanent or seasonal water bodies dominated by emergent vegetation and/or with permanently waterlogged soil (Janse et al., 2019). They play important roles in storing C and N, protecting species diversity, and ensuring regional ecological security (Hefting et al., 2013). At the global scale in particular, tussocks, defined as individuals of graminoid species growing in clumps, tufts, hummocks, or bunches, are often distributed within inland freshwater marshes, river wetlands, and plateau wetlands in the north temperate zone (Levine, 2000; Crain and Bertness, 2005). For example, in the alternately wet–dry regions of wetlands in Northeast China, individuals of *Carex appendiculata*, *C. meyeriana*, and *C. schmidtii* can form tussocks, or dense hummocks; after their roots grow and rot repeatedly, tiller nodes are elevated, and long–term condensates form peat (Wang et al., 2018; Zhang et al., 2020; Qi et al., 2021). A tussock is usually cylindrical, with an average height of 25.3 cm, sometimes up to 1 m. The diameter of a tussock is 30–40 cm, and their density is generally 2–22 tussocks per 9 m^2 (Tsuyuzaki and Tsujii, 1992; Zhang et al., 2020). Tussock species are often associated with *Deyeuxia purpurea*, *Glyceria spiculosa*, and *Polygonum hydropiper*, among others, forming tussock wetland vegetation (Wang M. et al., 2021). Owing to the morphological characteristics of tussocks, tussock wetlands can form unique surface micro–landforms, which can effectively increase the wetland surface area and environmental heterogeneity, provide sufficient growth space for wetland plants, further improve plant photosynthesis efficiency, and maintain high plant diversity (Peach and Zedler, 2006; Qi et al., 2019; Wang M. et al., 2021).

As important components of wetland ecosystems, microorganisms are crucial to substance transformation and energy flow, which promote the differentiation and succession of wetland ecosystems. Changes in wetland environment will cause changes in soil microbial communities (Xu et al., 2004; Lyons and Lindo, 2020). Especially in the micro-habitat formed by tussocks, the different hydrothermal conditions present affect the composition of the soil microbial community, and the soil microbial community thus exhibits heterogeneity. The number of bacteria in the topsoil of tussock wetland will gradually increase with decreases in water content and increases in temperature (Xu et al., 2004; Liu et al., 2018). Plant diversity and composition are associated with soil microbial community (Zak et al., 2003; Shi et al., 2021). In the rehabilitated wetland, vegetation type can effect on soil microbial dynamics and carbon emissions (Bonetti et al., 2021). Low wetland plant diversity and litter loss lead to the reductions of nutrients available to microorganisms, the number of microorganisms and microbial diversity (Chen et al., 2021). Therefore, revealing plant–microorganism association is fundamental to studying wetland functions, protection and restoration.

The formation of tussock wetland is the result of long-term comprehensive plant–microorganism–soil environment interaction (Mark et al., 1985; Peach and Zedler, 2006). Once damaged, it is difficult to restore tussock wetland in a short time (Qi et al., 2021). At present, researchers have evaluated a variety of restoration methods. However, which method is more suitable requires in-depth study of this comprehensive interaction. In particular, a specific assessment of the soil microbial community of *Carex* tussock wetlands is still insufficient. Therefore, the present study had the following aims: (1) to reveal the characteristics of plant diversity and soil bacterial community diversity of *Carex* tussock wetland, (2) to analyze the associations of soil bacterial diversity and function with plant diversity, (3) to provide suggestions for the restoration of *Carex* tussock wetland based on both plant and soil bacterial community rehabilitation.

Materials and methods

Study sites description

The study sites were in natural wetland (*NW*), artificially restored wetland (*ARW*), and naturally restored wetland (*NRW*), habitats located in Jinhewan Wetland Botanical Garden, Taiyangdao Park, and Alejin Wetland Park, respectively, in Harbin, Northeastern China. It has a semi–humid continental monsoon climate in the middle temperate zone. The dominant species of the three tussock wetlands was *C. appendiculata*. The *ARW* establishment began in 2008. After *in situ* longitudinal cutting of native tussock, root cloning and transplanting were conducted to expand the area of tussock wetlands (Qi et al., 2019, 2021). Naturally restored wetland establishment started from the returning farmland to wetland project in 2008, and the vegetation was naturally restored on the basis of the original tussock wetland. The water levels of *ARW* and *NRW* sites were controlled to avoid seasonal flooding. The three sample plots have peat soil.

Plant community diversity determination and soil sampling

In June, 2018, the fruiting period of *C. appendiculata*, the quadrat method (with 3 × 3 m quadrats) was used to investigate plant diversity. Nine quadrats were set up along a line transect for every sample plot. The minimum distance between any two quadrats was 10 m. Based on the plant diversity records, the three quadrats with the maximum (*ma*), median (*me*), and minimum (*mi*) plant Shannon index value in each sample plot were selected, respectively, to represent the plant diversity characteristics of each sample plot, and 500 g samples of the 10–20 cm layer soil were obtained from each quadrat using the five-point (i.e., apexes and center) method. Additionally, the *mi* of the each sample plot was located in an area with 5–15 cm of surface ponding.

Soil physicochemical indexes determination

In the laboratory, moisture content (MC) was determined using the drying method (at 105°C); soil bulk density (BD) was determined using a cutting ring (volume, 100 cm^3); pH value was determined [water:soil, 2.5:1 (*v*:*w*)] using the composite electrode method (INESA PHS-3C, Shanghai, China); total organic C (TOC) content was determined using a TOC analyzer (Multi N/C 2100; Analytik Jena, Jena, Germany); total N (TN) content was determined by the semimicro Kjeldahl method; alkali-hydrolyzed N (AN) content was determined by the alkaline diffusion method; total P (TP) content was determined by the molybdenum antimony colorimetric method; available P (AP) content was determined by the sodium bicarbonate

extraction-molybdenum antimony anticolorimetric method; total K (TK) content was determined by the flame spectrophotometry method; available K (AK) content was determined by the ammonium acetate extraction-flame photometric method (Shi et al., 2021). All index measurements were repeated three times.

Soil sample DNA extraction

From 0.5 g soil samples, the total DNA was extracted using a PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA, United States) following the manufacturer's instructions. A NanoDrop™ 2000 UV–Vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States) was used to detect the DNA concentration and purity. Then 1% agarose gel electrophoresis was used for DNA quality testing.

Polymerase chain reaction amplification and sequencing

Polymerase chain reaction amplification was performed using bacterial 16S rRNA V4–V5 primers (515F, 5′-GTGCCAGCMG CCGCGGTAA-3′, 926R: 5′-CCGTCAATTCMTTTGAGTTT-3′). In the first step, the volume of the PCR system was 50 μl, containing 10 μl of 5× Buffer, 1 μl of dNTP (10 mmol L^{-1}), 1 U of Phusion® High–Fidelity DNA Polymerase, 0.4 μl of bovine serum albumin (BSA; 10 mg mL^{-1}), 1 μl of each F and R primer (10 μmol L^{-1}), 25 ng of template DNA, and sufficient ddH_2O to complete the 50 μl volume. The PCR thermocycling protocol was as follows: 94°C denaturation for 2 min; 25 cycles of 94°C for 30 s, 56°C annealing for 30 s, and 72°C elongation for 30 s; 72°C final extension for 5 min. The PCR products were detected by 2% agarose gel electrophoresis and recovered using a DNA gel recovery kit (Axygen Biosciences, Union City, CA, United States). In the second step, the volume of the PCR system was 40 μl, containing 8 μl of 5× Buffer, 1 μl of dNTP (10 mmol L^{-1}), 0.8 U Phusion® High–Fidelity DNA Polymerase, 0.4 μl of BSA (10 mg mL^{-1}), 1 μl of each F and R primer (10 μmol L^{-1}), 5 μl of PCR products of the first step (for DNA templates), and sufficient ddH_2O to complete the 40 μl volume. The PCR parameters were the same as the first step, though with 8 cycles instead of 25. Finally, the PCR products were also detected and recovered as described for the first step and then quantified using the Quanti Fluor™-ST Blue Fluorescence Quantification System (Promega, Madison, WI, United States). The samples were sent to Genesky Biotechnologies Inc. (Shanghai, China) for sequencing using the NGS Illumina MiSeq high–throughput 2×300 bp sequencing platform (Illumina, San Diego, CA, United States). Each sample was analyzed in triplicate.

Statistical analysis

MiSeq sequencing generated paired–end reads, and optimized data were obtained using the method of Liu et al. (2019). All data were submitted to the NCBI Sequence Read Archive database (Accession number: PRJNA921719). Following Edgar (2013), operational taxonomic unit (OTU) clustering of nonrepetitive sequences was performed at a 97% similarity threshold using the UPARSE pipeline. The taxonomic analysis of OTUs was performed by applying the Ribosomal Database Project Classifier (Wang et al., 2007) and the Bayes algorithm with a 0.7 confidence level, and the taxonomic identification database was the SILVA 138/16 s bacteria database (Quast et al., 2013). The relative abundance of each OTU was determined.

According to the quadrats records, plant diversity indexes, including the Shannon index, Pielou index and Simpson's index, were calculated following Palaghianu (2016). The Chao1 index, Shannon index, and Simpson's index of the bacterial community diversity of each sample were analyzed using Mothur v1.39.5 software (Schloss, 2020). Null model analysis was conducted using the "picante" package in R v4.2.1 (Kembel et al., 2010) to classify the relative importance of stochastic processes and deterministic processes in the phylogenetic diversity of bacterial communities. The β–nearest taxon index (βNTI) score was used to measure the variation in the relative importance of these processes, with $|\beta NTI| < 2$ indicating stochastic processes were dominant, and $|\beta NTI| > 2$ indicating deterministic processes were dominant (Liao et al., 2022), and Bray–Curtis dissimilarity was used to measure significant differences among the three sample plots. Using the "vegan" package (2.6–4) in R v4.2.1, nonmetric multidimensional scaling (NMDS) analysis was performed based on Bray–Curtis distance at the OTU level, and the significance of differences was assessed by analysis of similarities (ANOSIM) with 999 permutations. Analysis of variance (ANOVA) was performed using SPSS version 17.0 software (SPSS Inc. Chicago, IL, United States) to analyze the significance of differences in diversity indexes, soil physicochemical indexes, and the relative abundance of taxa among different treatments. Additionally, Pearson correlation analysis was performed to analyze the correlation between the diversity index and soil physicochemical index. Bar plots were drawn using Office Excel 2016 (Microsoft Corp., Redmond, WA, United States) based on the relative abundance of dominant taxa. Linear discriminant analysis of effect size (LEfSe) was performed to detect taxa with significant differences in relative abundance among treatments using the nonparametric factorial Kruskal–Wallis sum-rank test, and linear discriminant analysis (LDA) was used to estimate the size of the effect of each taxon on the difference in relative abundance.[1] Soil bacterial functions were predicted using FAPROTAX (Louca et al., 2016).

Results

Plant community diversity

In each of the three sample plots, the dominant species of the plant community was *C. appendiculata*. Its coverage was above 70% in every quadrat. The companion species of all the quadrats spanned 24 families, 56 genera, and 81 species of herbaceous plants. Cyperaceae (14 species), Poaceae (10 species) and Asteraceae (9 species) were the dominant families among companion species, and the species of these families, for example, *Cyperus orthostachyus*, *Echinochloa crus-galli*, and *Bidens maximowicziana*, were highly abundant. Most of the companion species in *NW* and *ARW* quadrats were hygrophytes,

1 https://huttenhower.sph.harvard.edu/galaxy/

belonging to Cyperaceae and Poaceae, while the companion species in *NRW* quadrats were hygrophytes and mesophytes, belonging to Cyperaceae, Poaceae and Asteraceae.

Based on the ANOVA results (Table 1), the differences in the plant diversity indexes, including the Shannon index, Pielou index, and Simpson's index, among the three sample plots were not significant ($p > 0.05$). Specifically, there was no significant difference in plant diversity between *NW* and either *ARW* or *NRW*. The plant diversity index values in Table 1 show the Pielou and Shannon indexes of plants had the same trend, contrasting with the trend in Simpson's index.

Soil physicochemical properties

Soil physicochemical indexes are shown in Table 2. Among the three sample plots, the differences in MC were not significant ($p > 0.05$), except for *NWme* being significantly ($p < 0.05$) lower than *ARWme*. However, among the different quadrats of each sample plot, the MC of *mi* was significantly higher than those of *ma* and *me*. The BD of *NW* was higher than those of *ARW* and *NRW*, although the differences among *mi* were not significant. In each sample plot, the BD values of *ma* and *me* were significantly higher than those of *mi*, because the surface ponding in *mi* led to an increase in MC but a decrease in BD.

The soil was weakly acidic to neutral. The pH value of *NWme* was significantly higher than that of *ARWme*, and pH values of *mi* were significantly higher than those of *ma* and *me*, i.e., the soil pH value was closer to neutral in the quadrats with surface ponding.

The AN of *NWma* was significantly higher than those of *ARWma* and *NRWma*, but the TK of *NWma* significantly lower. The AP of *ARWme* was significantly lower than that of *NRWme*. The TOC, TP, and AK of the three sample plots did not significantly differ, respectively. Among the different quadrats of each sample plot, the nutrient contents of *mi* were lower than those of *ma* and *me*, namely, the lower the plant diversity, the lower the nutrient content.

Soil bacterial community structure

As shown in Figure 1, the soil bacterial communities of the different treatments were obviously distinct (ANOSIM test, $r = 0.9961$, $p < 0.01$) at the OTU level, indicating that the bacterial community structure was significantly associated with plant diversity. The three quadrats of *ARW* were more decentralized, i.e., the bacterial community structure of *ARW* was more strongly associated with plant diversity. In addition, the *ARWme* bacterial community was more similar to the *ARWmi* bacterial community than the *ARWma* bacterial community. In both *NW* and *NRW*, the *ma* bacterial community was closer to the *mi* bacterial community than the *me* bacterial community. Additionally, compared to the *NRWme* bacterial community, the *ARWme* bacterial community was more similar to the *NWme* bacterial community.

Soil bacterial community composition

At the 97% sequence similarity level, the entire soil microbial community (including bacteria and archaea) was identified to contain 47 phyla, 79 classes, 101 orders, 233 families, 645 genera, and 1,087 species in total. Excluding unassigned taxa, 35 bacterial phyla were obtained. The sum of relative abundances of the top four phyla was close to 70% of the total (Figure 2; Supplementary Table S1). The relative abundance of Proteobacteria was the highest, at above 20% in each sample, and it reached 44.65 and 21.95% in *NWmi* and *ARWma*, respectively. The relative abundance of Chloroflexi was significantly ($p < 0.05$) higher in *NWma* than that in *NWmi*, and the differences between *ARWma* and *ARWmi*, between *NRWma* and *NRWmi* were not significant ($p > 0.05$). The relative abundance of Bacteroidetes was significantly higher in *NWme* and *ARWme*, but did not significantly differ among *NRWma*, *NRWme*, and *NRWmi*.

At the genus level, excluding unassigned taxa, 626 bacterial genera were obtained, and the relative abundances of the top 20 genera are shown in Figure 3. The relative abundance of *Geobacter* (belonging to Proteobacteria) reached 6.07% in *NWmi* and close to 3% in *NRW* overall, but it was below 0.05% in both *NWme* and *ARWme*. The relative abundance of *Sideroxydans* (belonging to Proteobacteria) reached 6.09% in *NRWmi*, but was below 0.02% in both *NWme* and *ARWme*. The relative abundance of *Clostridium* (belonging to Firmicutes) reached 5.03 and 2.04% in *ARWma* and *NWmi*, respectively, but it was below 1% in the other samples. The relative abundance of *Opitutus* (belonging to Verrucomicrobia) reached 2.11% in *NWme*, but was only 0.1% in *ARWma*. The relative abundance of *Terrimonas* (belonging to Bacteroidetes) was above 3% in *NWme* and *ARWme*, but was below 1% in the other samples.

Therefore, the soil bacterial community composition at the phylum and genus levels was significantly associated with plant

TABLE 1 Plant community diversity indexes.

Index	*NW*			*ARW*			*NRW*		
	ma	*me*	*mi*	*ma*	*me*	*mi*	*ma*	*me*	*mi*
Shannon	0.980 ± 0.155 a			0.891 ± 0.105 a			0.951 ± 0.104 a		
	1.166	0.975	0.757	1.061	0.899	0.722	1.056	0.962	0.753
Pielou	0.708 ± 0.109 a			0.628 ± 0.080 a			0.681 ± 0.076 a		
	0.841	0.606	0.546	0.765	0.649	0.521	0.762	0.598	0.543
Simpson	0.460 ± 0.086 a			0.484 ± 0.046 a			0.447 ± 0.070 a		
	0.346	0.530	0.599	0.429	0.470	0.586	0.374	0.454	0.613

Data represent the means ± standard deviations. Analysis of variance (with Duncan's multiple comparison test) was used to test the significance of differences. Values labeled with the same lowercase letter were not significantly different ($p > 0.05$). *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

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TABLE 2 Physicochemical indexes of soil.

Index	Quadrat	Sample plot								
		NW			*ARW*			*NRW*		
MC %	*ma*	a	25.60 ± 1.84	b	a	26.52 ± 1.73	b	a	23.42 ± 2.75	b
	me	b	25.68 ± 2.64	b	a	28.32 ± 1.50	b	ab	26.94 ± 0.73	b
	mi	a	55.95 ± 5.61	a	a	60.10 ± 5.13	a	a	61.67 ± 3.99	a
BD ($g\,cm^{-3}$)	*ma*	a	1.15 ± 0.06	a	b	1.01 ± 0.10	a	b	0.94 ± 0.05	a
	me	a	1.18 ± 0.09	a	b	1.02 ± 0.11	a	b	1.00 ± 0.11	a
	mi	a	0.67 ± 0.07	b	a	0.59 ± 0.05	b	a	0.60 ± 0.05	b
pH	*ma*	a	6.50 ± 0.10	b	a	6.44 ± 011	b	a	6.46 ± 0.12	b
	me	a	6.54 ± 0.08	b	b	6.37 ± 0.06	b	ab	6.47 ± 0.13	b
	mi	a	6.73 ± 0.15	a	a	6.67 ± 0.03	a	a	6.75 ± 0.12	a
TOC ($g\,kg^{-1}$)	*ma*	a	53.16 ± 2.78	a	a	50.50 ± 1.82	a	a	52.72 ± 3.08	a
	me	a	54.10 ± 2.58	a	a	49.15 ± 2.28	a	a	50.46 ± 5.30	a
	mi	a	43.79 ± 1.59	b	a	43.36 ± 1.68	b	a	45.06 ± 1.63	b
TN ($g\,kg^{-1}$)	*ma*	a	3.59 ± 0.05	a	a	3.52 ± 0.07	a	a	3.50 ± 0.15	a
	me	a	3.53 ± 0.06	a	a	3.42 ± 0.12	a	a	3.37 ± 0.22	ab
	mi	a	3.17 ± 0.08	b	a	3.24 ± 0.16	b	a	3.21 ± 0.10	b
AN ($mg\,kg^{-1}$)	*ma*	a	321.25 ± 33.31	a	b	269.01 ± 22.34	a	b	271.26 ± 15.76	a
	me	a	209.05 ± 21.04	b	a	241.24 ± 34.35	a	a	228.98 ± 32.99	b
	mi	a	198.93 ± 20.58	b	a	194.16 ± 15.45	b	a	222.47 ± 23.52	b
TP ($g\,kg^{-1}$)	*ma*	a	0.84 ± 0.77	a	a	0.88 ± 0.10	a	a	0.83 ± 0.03	a
	me	a	0.77 ± 0.07	ab	a	0.80 ± 0.13	ab	a	0.84 ± 0.06	a
	mi	a	0.72 ± 0.04	a	a	0.70 ± 0.05	b	a	0.68 ± 0.14	b
AP ($mg\,kg^{-1}$)	*ma*	a	82.13 ± 5.25	a	a	77.30 ± 9.38	a	a	74.78 ± 10.19	ab
	me	ab	79.99 ± 7.39	a	b	69.38 ± 7.66	ab	a	83.87 ± 9.82	a
	mi	a	69.32 ± 5.11	b	a	65.00 ± 2.99	b	a	70.18 ± 7.36	b
TK ($g\,kg^{-1}$)	*ma*	b	1.01 ± 0.15	ab	a	1.35 ± 0.09	a	a	1.34 ± 0.18	a
	me	a	1.14 ± 0.30	a	a	1.25 ± 0.26	ab	a	1.31 ± 0.22	ab
	mi	a	0.83 ± 0.13	b	a	1.03 ± 0.25	b	a	1.09 ± 0.11	b
AK ($mg\,kg^{-1}$)	*ma*	a	309.19 ± 22.35	a	a	297.09 ± 29.31	ab	a	292.97 ± 40.81	a
	me	a	290.66 ± 17.47	a	a	319.65 ± 26.08	a	a	323.71 ± 29.83	a
	mi	a	254.65 ± 19.28	b	a	271.47 ± 20.46	b	a	276.82 ± 32.76	a

Data represent the means ± standard deviations. Analysis of variance (with Duncan's multiple comparison test) was used to test the significance of differences. Values labeled with different lowercase letters were significant different ($p < 0.05$). The left letters correspond to comparisons of the differences among the three sample plots, and the right letters correspond to comparisons of the differences among the three quadrats within each sample plot. MC, moisture content; BD, bulk density; TOC, total organic C; TN, total N; AN, alkali–hydrolyzed N; TP, total P; AP, available P; TK, total K; AK, available K. *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

diversity. Additionally, according to the LEfSe analysis ($p < 0.05$), when LDA > 2, the bacterial community biomarkers, including 32 phyla, 70 classes, 81 orders, 156 families, and 308 genera, significantly did differ among the different samples. When LDA > 3 (Figure 4), the bacterial community biomarkers included 22 phyla, 44 classes, 47 orders, 70 families, and 72 genera. There were 6 genera in *NWma*, 11 genera in *NWme*, 13 genera in *NWmi*, 8 genera in *ARWma*, 5 genera in *ARWme*, 5 genera in *ARWmi*, 3 genera in *NRWma*, 12 genera in *NRWme*, and 9 genera in *NRWmi*. For LDA > 4, the bacterial community biomarkers included 10 phyla, 18 classes, 18 orders, 11 families, and 5 genera, namely, *Ignavibacterium* (LDA = 4.106 in *NWma*), *Geobacter* (LDA = 4.398 in *NWmi*), *Clostridium* (LDA = 4.319 in *ARWma*), *Terrimonas* (LDA = 4.232 in *ARWme*), and *Sideroxydans* (LDA = 4.399 in *NRWmi*).

Soil bacterial community diversity

The total number of OTUs obtained by clustering was 16, 894, and the number of shared OTUs was only 618. In each sample plot, the number of observed OTUs of *ma* was significantly ($p < 0.05$) higher than those of *me* and *mi* (Table 3), namely, soil bacterial species were more abundant under higher plant diversity. Overall, the bacterial Chao1 index and Shannon index of *ma* were significantly higher than

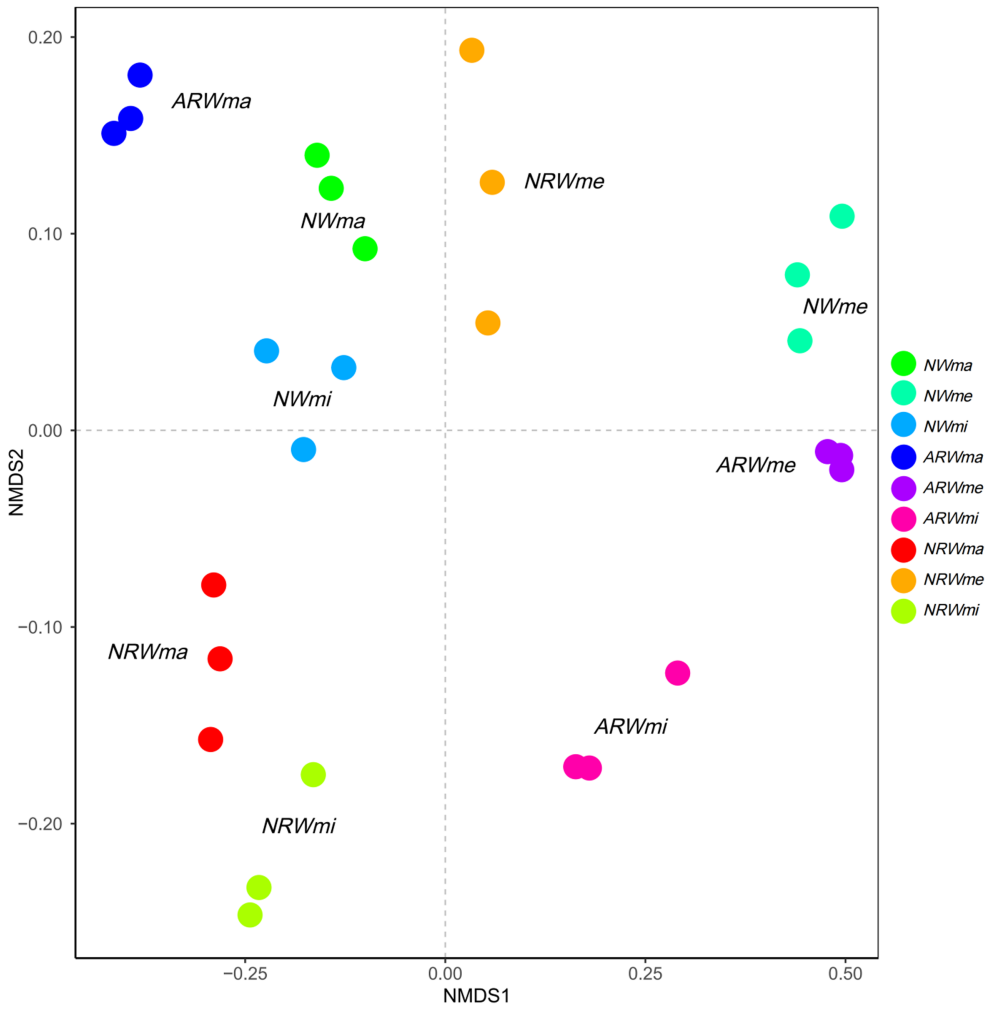

FIGURE 1
Nonmetric multidimensional scaling analysis based on Bray–Curtis distance at the operational taxonomic unit level (analysis of similarities test, r=0.9961, p<0.01). *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

those of *me* and *mi*, i.e., the higher the plant Shannon index, the higher the bacterial Shannon index. However, the difference in bacterial Shannon index between *me* and *mi* was not significant ($p > 0.05$). The difference in bacterial Simpson's index values between *ma* and *me* was not significant, and in *NRW*, the *mi* was not significantly different.

Among the three sample plots, *NRWma* OTUs significantly outnumbered *NWma* and *ARWma* OTUs, and the differences in OTU number between *me* and *mi* were not significant. The *ARWma* bacterial Chao1 index value was significantly lower than the *NWma* and *NRWma* bacterial Chao1 index values, and that of *ARWme* was significantly lower than that of *NRWme*, however, the difference was not significant among *NWmi*, *ARWmi*, and *NRWmi*. The bacterial Shannon index was lower in *ARW*, and the difference was not significant among *NWmi*, *ARWmi*, and *NRWmi*. The bacterial Simpson's index value among *NWme*, *ARWme* and *NRWme* showed no significant difference, but it was significantly higher and lower in *NWma* and *ARWmi*, respectively.

The difference in the phylogenetic diversity of the soil bacterial community between *ARW* and *NRW* was significant ($p < 0.05$), and both of them exhibited significant ($p < 0.0001$) differences from that of *NW* (Figure 5). All the |βNTI| scores of *NW* were below two (Supplementary Table S2), indicating stochastic processes largely determined the phylogenetic diversity of the bacterial community. Among *ARW* |βNTI| scores, 80.56% were below two, which indicated the dominant role of stochastic processes. However, the |βNTI| scores of *NRW* below and above two accounted for 52.78 and 47.22% of scores, respectively, indicating a balance between deterministic and stochastic processes. Thus, the stochastic processes underlying the formation of the phylogenetic diversity of bacterial communities were less important in *NRW*.

Correlations among soil physicochemical, plant diversity, and soil bacterial diversity indexes

The Pearson correlation analysis results are shown in Table 4. The bacterial Shannon index was significantly ($p < 0.01$) positively correlated with the plant Shannon index and the Pielou index and

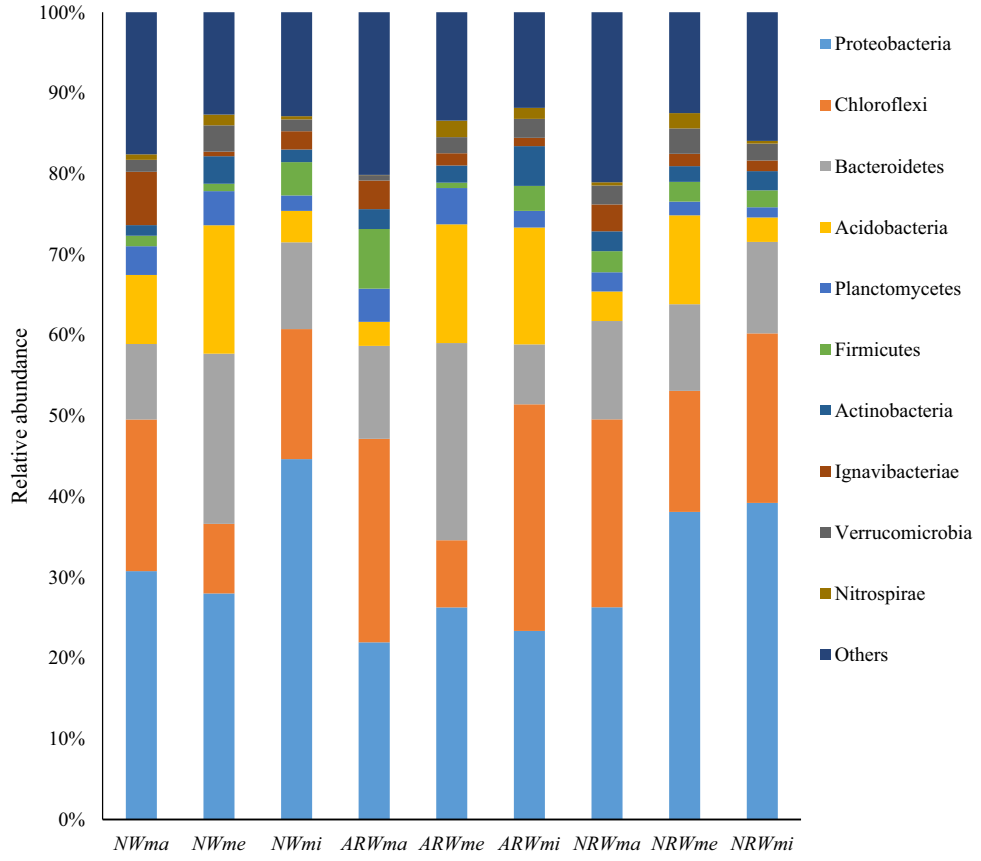

FIGURE 2
Top 10 phyla of the soil bacterial community. *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

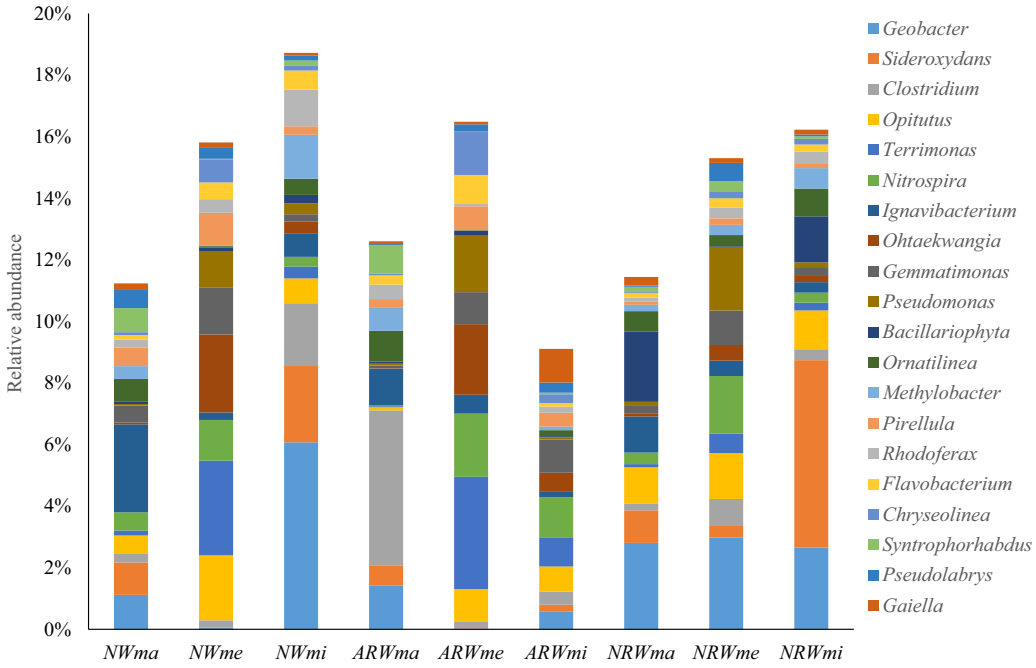

FIGURE 3
Top 20 genera of the soil bacterial community, excluding unassigned taxa. *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

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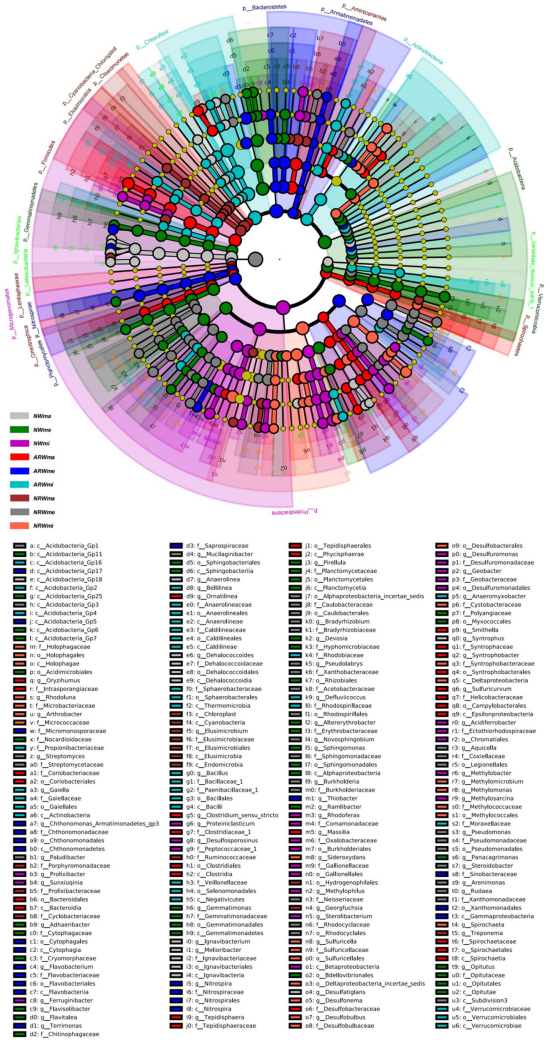

FIGURE 4
Linear discriminant analysis of effect size (LEfSe) analysis (LDA>3, p<0.05) of the soil bacterial community. The levels of phylum, class, order, family and genus are arranged from the outside to the inside. The yellow circle represents the taxa that did not significantly differ among the treatments. *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

significantly ($p < 0.01$) negatively correlated with the plant Simpson's index. Both the positive correlation between the Chao1 index and the Pielou index and the positive correlation between the bacterial Simpson's index and the plant Shannon index were significant ($p < 0.05$).

The bacterial Shannon index was significantly ($p < 0.05$) correlated with soil MC, TOC, TN, and P, and the correlation with AN was significantly ($p < 0.01$) positive. The Chao1 index was significantly ($p < 0.05$) positively correlated with AN. The bacterial Simpson's index was significantly ($p < 0.05$) negatively correlated with MC, and it was significantly ($p < 0.05$) positively correlated with BD and AP. The bacterial diversity indexes were not significantly ($p > 0.05$) correlated with pH, TK, and AK.

The plant Shannon index was significantly ($p < 0.05$ or $p < 0.01$) correlated with the soil physicochemical indexes except the soil K content. The Pielou index was not significantly correlated with pH, AP, and K. Additionally, the plant Simpson's index was not significantly correlated with AP and TK. MC and pH were positively correlated with the plant Shannon index and the Pielou index, but they were negatively correlated with the plant Simpson's index.

Soil bacterial community function prediction

FAPROTAX prediction identified 71 functional groups, and the relative proportions of the 19 functional groups were all above 1% (Figure 6). Chemoheterotrophy, aerobic chemoheterotrophy, fermentation, and iron respiration groups contained more OTUs, and the other functional groups were related to soil S, N, and C cycles. The relative proportion of chemoheterotrophic bacteria among the three sampled quadrats in each sample plot was not significantly ($p > 0.05$) different, and only that of *NRWmi* was significantly ($p < 0.05$) higher than that of *ARWmi*, but neither of them was significantly different from that of *NWmi*. Additionally, plant diversity was not significantly associated with chemoheterotrophy. The relative proportions of aerobic chemoheterotrophic bacteria in *ARWme* and *NRWme* were significantly higher than those in *ARWmi* and *NRWmi*, but there were non-significant differences compared to those in *ARWma* and *NRWma*, respectively.

The relative proportion of S cycle-related functional groups, such as respiration of S compounds and sulfate respiration, was lower in *NWme*, *ARWme*, and *NRWme*, and there was no significant difference in this relative proportion between *ma* and *mi* of each sample plot. The difference in this relative proportion among *NWma*, *ARWma*, and *NRWma* was not significant. That of *NRWme* was significantly higher than those of *NWme* and *ARWme*, and that of *ARWmi* was lower than those of *NWmi* and *NRWmi*.

The relative proportion of N cycle–related functional groups, such as nitrate reduction, nitrification, and aerobic nitrite oxidation, was higher in *NWme*, *ARWme*, and *NRWme* than in the other samples, and that in *ARWme* was significantly higher than that in *ARWma*. The difference in this relative proportion among *NWme*, *ARWme*, and *NRWme* was not significant. The relative proportion of nitrate-reducing bacteria in *NRWma* was significantly higher than that in *ARWma*. However, the relative proportions of nitrification and aerobic nitrite oxidation in *NRWma* and *ARWma* were significantly lower than that in *NWma*, and that in *ARWmi* was significantly higher than that in *NWmi*.

The relative proportion of C cycle-related functional groups, such as methylotrophy, hydrocarbon degradation, methanotrophy, and methanogenesis, was significantly higher in *ARWma* than those in *ARWme* and *ARWmi*. The relative proportions of hydrocarbon degradation and methanotrophy were higher in *NWmi* than those in *NWme*, and the *NWma* had a significantly higher relative proportion of methanogenesis functional groups. Except for the methanogenesis functional group, *NRWmi* had a significantly higher relative proportion of C cycle–related functional groups than *NRWma* and *NRWme*. Among the three sample plots, the difference in the relative proportion was not significant among *NWma*, *ARWma*, and *NRWma*, and it was significantly higher in *NRWme* and *NRWmi* than in the other samples, respectively.

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TABLE 3 Diversity indexes of the soil bacterial community.

Index	Quadrat	Sample plot								
		NW			*ARW*			*NRW*		
Observed	*ma*	b	4,604 ± 67	a	c	4,053 ± 82	a	a	5,083 ± 120	a
	me	a	3,353 ± 56	c	a	3,213 ± 121	b	a	3,470 ± 171	b
	mi	a	3,738 ± 218	b	a	3,428 ± 277	b	a	3,788 ± 439	b
Chao1	*ma*	a	6638.144 ± 166.951	a	b	5229.186 ± 226.644	a	a	6787.141 ± 143.728	a
	me	ab	4560.236 ± 211.105	c	b	4329.303 ± 117.189	b	a	4863.193 ± 197.837	b
	mi	a	5335.269 ± 297.842	b	a	4658.387 ± 286.579	b	a	5211.138 ± 623.290	b
Shannon	*ma*	a	6.946 ± 0.032	a	b	6.812 ± 0.086	a	ab	6.907 ± 0.033	a
	me	a	6.559 ± 0.056	b	b	6.400 ± 0.043	b	a	6.547 ± 0.081	ab
	mi	a	6.431 ± 0.223	b	a	6.175 ± 0.249	b	a	6.426 ± 0.366	b
Simpson	*ma*	a	0.997 ± 0.000	a	b	0.995 ± 0.000	a	b	0.994 ± 0.001	a
	me	a	0.995 ± 0.001	ab	a	0.994 ± 0.000	a	a	0.995 ± 0.001	a
	mi	a	0.993 ± 0.002	b	b	0.977 ± 0.009	b	a	0.990 ± 0.004	a

Data represent the means ± standard deviations. Analysis of variance (with Duncan's multiple comparison test) was used to test the significance of differences. Values labeled with different lowercase letters were significant different ($p < 0.05$). The left letters correspond to comparisons of the differences among the three sample plots, and the right letters correspond to comparisons of the differences among the three quadrats within each sample plot. *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

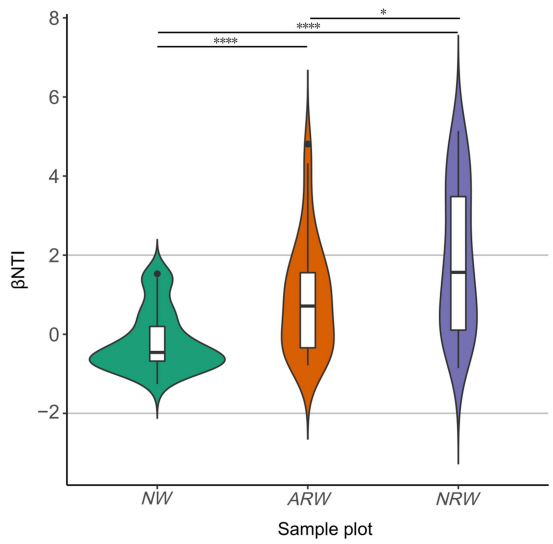

FIGURE 5
β−nearest taxon index (βNTI) score distribution of soil bacterial communities of *NW*, *ARW*, and *NRW*. βNTI, β−nearest taxon index; *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland. The significance measured using Bray–Curtis dissimilarity, **p*<0.05, *****p*<0.0001.

Discussion

Association of soil bacterial community diversity with plant diversity

The Shannon index is one of the most frequently used biodiversity indexes (Palaghianu, 2016). It was used to assess plant diversity in this study, and the maximum, median, and minimum Shannon index values were used to describe the plant diversity characteristics of the three sample plots, respectively. Based on the description and comparison of diversity indexes (Das et al., 2006; Palaghianu, 2016), the Pielou index and the plant Simpson's index were significantly ($p < 0.01$) positively and negatively correlated with the plant Shannon index, respectively in this study. Thus, when the dominance of *C. appendiculata* increased in *Carex* tussock wetland, the plant Simpson's index increased accordingly, but the Pielou index and the plant Shannon index instead decreased.

Kim et al. (2022) found that structurally and functionally distinct microbial communities develop under different plant species in wetlands, suggesting that it is important to consider the diversity of plant species, along with abiotic factors, when investigating the abundance and coexistence of different microbial species. Calheiros et al. (2009) demonstrated that high plant richness could increase bacterial abundance and community structure profiles in wetlands. Li et al. (2021) reported that wetland plants could enhance the soil microbial diversity. In *Carex* tussock wetland, the soil bacterial Shannon index was also significantly positively correlated with the plant Shannon index in this study, that is, a plant diversity increase could be associated with an increase in the diversity of soil bacteria.

The higher soil MC and the lower BD were consistent with surface ponding in the quadrats of *Carex* tussock wetland in this study. In these quadrats, the plant species and quantity of companion species decreased. Water is a key driver of plant diversity and community structure in wetland ecosystems, and it can change the vegetation and biomass (Li et al., 2015; Feng et al., 2020; Shan et al., 2020). In particular, in *Carex* tussock wetland, the growth and physiology of plants and species diversity respond to water level fluctuations (Wang et al., 2016; Zhang et al., 2020). Meanwhile, soil bacterial community structure is also strongly shaped by water content (Sui et al., 2021). Both the plant and bacterial Shannon indexes decreased in the *mi* with surface ponding in this study. Soil pH is a major factor driving variation

TABLE 4 Correlations among bacterial diversity indexes, plant diversity indexes, and soil physicochemical indexes.

		Bacterial diversity index			Plant diversity index		
		Chao1	Shannon	Simpson	Shannon	Pielou	Simpson
Plant diversity index	Shannon	0.565	0.917**	0.701*			
	Pielou	0.695*	0.923**	0.593	0.930**		
	Simpson	−0.617	−0.857**	−0.578	−0.938**	−0.931**	
Soil physico-chemical index	MC	−0.255	−0.717*	−0.713*	−0.895**	−0.746*	0.857**
	BD	0.147	0.644	0.726*	0.865**	0.685*	−0.745*
	pH	−0.053	−0.499	−0.511	−0.734*	−0.652	0.797*
	TOC	0.347	0.752*	0.654	0.903**	0.728*	−0.793*
	TN	0.364	0.779*	0.585	0.942**	0.847**	−0.849**
	AN	0.728*	0.874**	0.558	0.864**	0.959**	−0.890**
	TP	0.339	0.771*	0.643	0.890**	0.810**	−0.893**
	AP	0.295	0.683*	0.718*	0.793*	0.549	−0.642
	TK	−0.006	0.379	0.293	0.491	0.397	−0.545
	AK	−0.015	0.382	0.502	0.644	0.504	−0.701*

MC, moisture content; BD, bulk density; TOC, total organic C; TN, total N; AN, alkali–hydrolyzed N; TP, total P; AP, available P; TK, total K; AK, available K. *$p < 0.05$, **$p < 0.01$.

in bacterial diversity and community structure (Kang et al., 2021; Mod et al., 2021). In this study, the soil pH was negatively correlated with the bacterial diversity indexes, but the correlation was not significant; this may be owing to the small range of pH values observed.

Wang et al. (2019) and Wang M. et al. (2021) inferred that SOC, TN, and TP contents in *Carex* tussock wetlands were the main environmental factors affecting the plant community structure, abundance, and diversity. Soil nutrient content was positively associated with plant diversity. The present study also found that the soil nutrient (except for K) content was significantly ($p < 0.05$ or $p < 0.01$) positively correlated with the plant Shannon index. Chen et al. (2021) reported that when plant diversity is low, litter is reduced, and the nutrients available for soil microorganisms are reduced accordingly. This leads to the reduction of the microbial population and community diversity. The results of the present study also support this conclusion.

Soil microbial communities are closely related to plant diversity and composition, and changes in plant diversity and composition can affect bacterial community composition (Zak et al., 2003; Shi et al., 2021). In the present study, the soil bacterial communities under the different levels of plant diversity were obviously separated at the OTU level, indicating that the composition of the soil bacterial community was associated with plant diversity. Proteobacteria, Chloroflexi, and Bacteroidetes were shown to be the dominant phyla in the soil of plateau wetland and coastal wetland in China (An et al., 2019). These phyla also had higher relative abundance in the soil bacterial community in the present study, and under different plant diversity and composition conditions, the relative abundance significantly differed. Meanwhile, *Ignavibacterium*, *Geobacter*, *Clostridium*, and other genera, also significantly differed in relative abundance. In *Carex* tussock wetland, soil microbial community heterogeneity is promoted by variation among micro–habitats (Xu et al., 2004). Therefore, under the different levels of plant diversity and composition, soil bacterial community structure and species composition differed.

Association of the soil bacterial community function with plant diversity

FAPROTAX focuses on marine and lacustrine biogeochemistry, especially the S, N, and C cycles (Louca et al., 2016). In this study, chemoheterotrophy, aerobic chemoheterotrophy, and fermentation groups were represented at higher relative proportions, but were not significantly associated with plant diversity in *NW*; meanwhile, the relative proportions of chemoheterotrophic bacteria were not significantly different between *ARW* and *NRW*. Wetland ecosystems often experience year-round or seasonal flooding, resulting in poor soil aeration (Hu et al., 2021). The relative proportions of aerobic chemoheterotrophic bacteria in *ARWmi* and *NRWmi* were lower than those in *ARWme* and *NRWme*, although they were both non–significantly different from *ARWma* and *NRWma*, respectively, this might be owing to the water level control and higher MC in *mi*.

Soil S, N, and C are known to have important interactions in wetland ecosystems (Liu et al., 2021; Wang Q. et al., 2021). The S cycle in a constructed wetland microcosm was found to have electron mediating ability between C and N cycles (Guo et al., 2020). This study also showed that the S, N, and C cycles are interrelated, and the relative proportions of bacteria associated with S and C cycles were lower, though that of N cycle bacteria was higher. Bacteria (Proteobacteria, Chloroflexi, Nitrospirae, etc.) and archaea participate in the chemical cycle of wetland soil (Mellado and Vera, 2021). Liu et al. (2022) reported that the S and N cycles were significantly affected by wetland plant composition and coverage with the participation of bacteria. The results of the present study also indicated that the soil S, N, and C cycles were associated with plant diversity, likely through altering the microbial community.

Wetlands are an important source of methane emissions (Peng et al., 2022). Wetland plants can influence methane emissions by altering methane production, consumption and transport in the wetland soil (Koelbener et al., 2010), and the interaction between

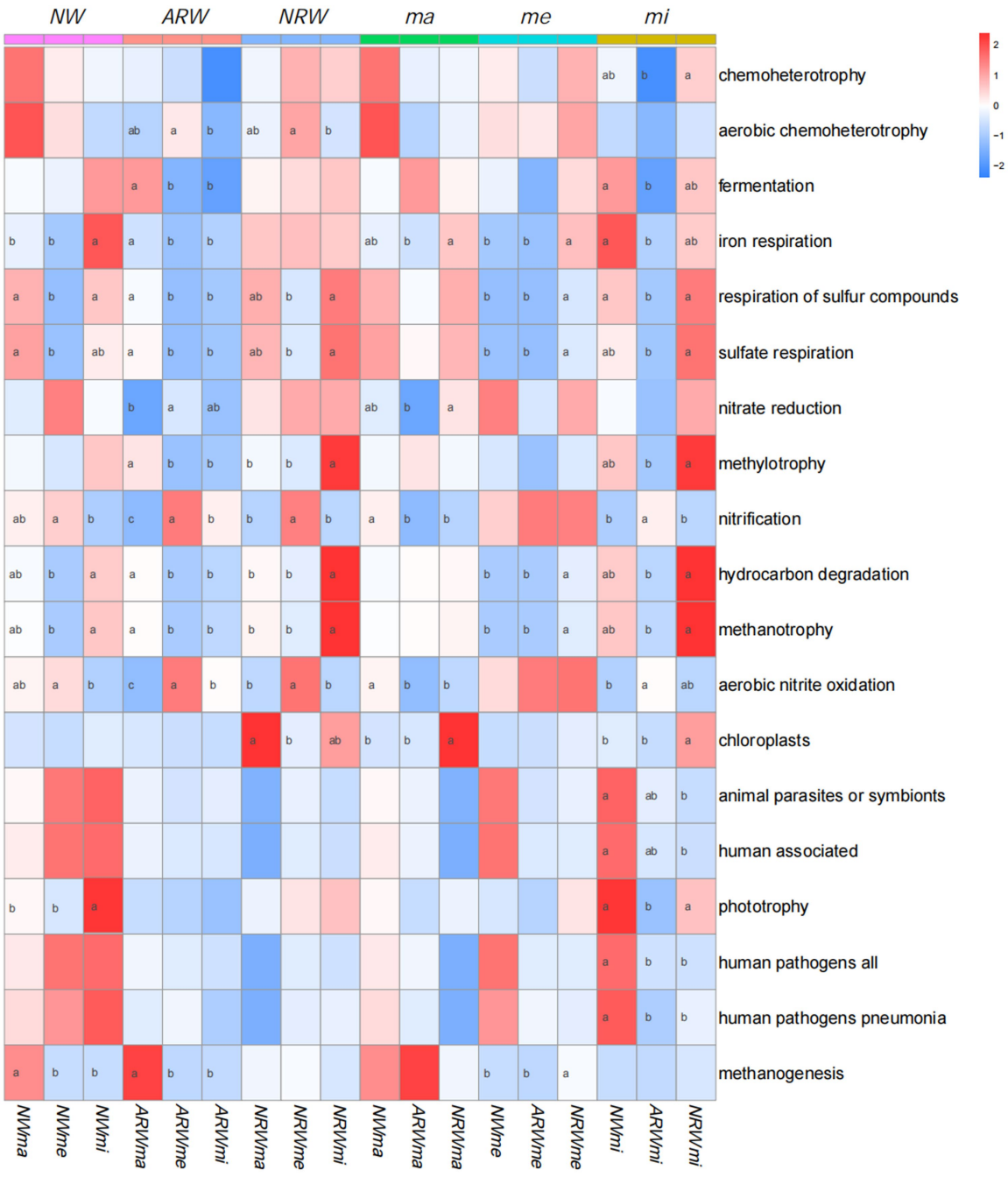

FIGURE 6
Functional prediction using FAPROTAX. The darker the red color, the higher the relative proportion is, and the darker the blue color, the lower the relative proportion is. Analysis of variance (with Duncan's multiple comparison test) was used to test the significance of differences. Values labeled with different lowercase letters were significant different ($p<0.05$). *NW*, natural wetland; *ARW*, artificially restored wetland; *NRW*, naturally restored wetland; *ma*, maximum plant Shannon index quadrat; *me*, median plant Shannon index quadrat; *mi*, minimum plant Shannon index quadrat.

wetland plants and soil microbes affects the soil C cycle (Schmid et al., 2021). In the present study, as shown in Figure 6, higher wetland plant diversity appears to increase methane emissions. In the *me* and *mi* samples, the relative proportion of C cycle-related functional groups in *NRW* was significantly higher than that in *ARW*. Therefore, the *NRW* appeared to have enhanced soil C cycles.

Carex tussock wetland restoration suggestion

Yu et al. (2010) reported that tussocks facilitate the establishment of plant species inside them and increase both diversity and reproduction. Thus, tussocks are of great importance to the stability of wetland ecosystems. Wang et al. (2018) noted that

tussock–forming *Carex* could be the preferred species for vegetation restoration of degraded peat bogs in view of their strong promotion of species diversity. Therefore, the restoration of *Carex* tussock wetland is very important for the protection of entire wetland ecosystems.

The genus *Carex* is comprised of more than 2,000 species (Bernard, 1990), however, only a few species can form tussocks, and *Carex* tussock formation requires a long time (Wang et al., 2018; Zhang et al., 2020; Qi et al., 2021). Qi et al. (2021) previously reported that root cloning and transplanting along with hydrological regulation can be used to accelerate plant diversity restoration in *Carex* tussock wetland. In the present study, on the whole, there was no significant difference in plant diversity among *ARW*, *NRW* and *NW* after 10 years of restoration, indicating that both types of restoration methods can achieve the desired results. However, in *NRW*, there were also mesophytes, while the species of companion plants in *ARW* were closer to those in *NW*, both of which were dominated by hygrophytes.

Soil microbial communities change along with the process of vegetation restoration in wetlands (Bonetti et al., 2021; Jeong and Kim, 2021). The present study indicated that there were significant differences in the phylogenetic diversity of soil bacterial communities among *NW*, *ARW*, and *NRW*. Stochastic processes played a significantly greater role in shaping the phylogenetic diversity of *ARW* relative to *NRW*. Therefore, *ARW* were closer to *NW* in terms of restoration indicators considering vegetation composition and the microbial community, such that the techniques used in *ARW* could be recommended for the restoration of *Carex* tussock wetlands.

Conclusion

In *Carex* tussock wetlands, different vegetation restoration methods did not differ in their effect on plant diversity, but did appear to change the plant species composition. The soil bacterial community compositions were also significantly different under different plant diversity conditions, and the relative abundance of phyla and genera was significantly associated with plant diversity. Surface ponding appeared to lead to an increase in soil MC, a decrease in BD and nutrient content, and decreases in both plant diversity and bacterial diversity. The phylogenetic diversity of bacterial communities in restored wetlands was significantly ($p < 0.0001$) different from that of *NW*, and compared to *NRW*, stochastic processes had a more similar role in shaping the phylogenetic diversity of *ARW* relative to *NW*. Different levels of plant diversity exhibited different relative proportions of functional groups comprising the bacterial community, especially affecting the relative proportions of bacteria affecting the wetland soil S, N, and C cycles. Higher plant diversity was likely associated with increased *Carex* tussock wetland soil methane emissions. According to both the plant and soil bacterial communities rehabilitated, the method of root cloning and transplantation coupled with hydrological regulation is recommended for *Carex* tussock wetland restoration.

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

YL and CS designed and performed the experiment and prepared this manuscript. DW and LW designed this experiment. JD, NX, and LJ helped to perform the experiment and process the data. All coauthors contributed to manuscript editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1142052/full#supplementary-material

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EDITED BY
Tengxiang Lian,
South China Agricultural University, China

REVIEWED BY
Jisong Yang,
Ludong University, China
Xue Sha,
Northwest A&F University, China

*CORRESPONDENCE
Wenxu Dong
✉ dongwx@sjziam.ac.cn

†These authors have contributed equally to this work

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Microbial gradual shifts during the process of species replacement in Taihang Mountain

Xiuping Liu[1†], Wangming Zhou[2†], Xinzhen Wang[1], Hongliang Wu[1] and Wenxu Dong[1*]

[1]Key Laboratory of Agricultural Water Resources, Hebei Key Laboratory of Soil Ecology, Center for Agricultural Resources Research, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Shijiazhuang, China, [2]School of Life Sciences, Anqing Normal University, Anqing, China

Introduction: Understanding microbial gradual shifts along species replacement can help elucidate the mechanisms driving secondary succession, and predict microbial responses to changing environments. However, how climate-induced species replacement alters microbial processes, and whether microbial shifts follow predictable assembly trajectories remain unclear.

Methods: Using space-for-time substitution approach, we studied shifts in bacterial and fungal communities in the succession from *Leptodermis oblonga* to *Vitex negundo* var. *heterophylla* shrubland in Taihang Mountain.

Results and Discussion: Species replacement, induced by climate related environmental change, significantly increased the above-ground biomass of shrublands, and TP and TK contents in topsoil. The succession from *L. oblonga* to *V. negundo* var. *heterophylla* communities resulted in the gradually replacement of cold-tolerant microbes with warm-affinity ones, and alterations of microbial communities involved in soil biogeochemical processes. Soil and plant variables, such as above-ground biomass, soil pH, total phosphorus, and total potassium, well explained the variations in microbial communities, indicating that the coordinated changes in plant communities and soil properties during secondary succession caused accompanied shifts in microbial diversity and composition.

KEYWORDS

species replacement, bacteria, fungi, microbial composition, indicator species, Taihang Mountain

1. Introduction

Soil microorganisms are an integral component of forest ecosystem, playing an essential role in regulating critical ecosystem processes such as primary production and nutrient cycling (de Gannes et al., 2016; Zhong et al., 2018). Changes in microbial communities occurring with secondary succession are closely related to shifts in ecological function (Heine et al., 2019; Zhong et al., 2019; Liu J. et al., 2020), and microbial diversity and composition are mainly mediated by plant traits and soil properties (Chai et al., 2019; Yan et al., 2020). Therefore, understanding shifts in microbial communities across successional stages could help illuminate the coupling relationships among plants, soil, and microbial community during the process of secondary succession (Zhong et al., 2018; Jiang et al., 2021).

Secondary succession, induced by replacement of dominant species, cause gradual shifts in plant community and soil properties, and thus resulting in microbial succession (Schmidt et al., 2014; Heine et al., 2019; Zhang et al., 2021). Previous studies have reported changes in and drivers of microbial diversity and composition along successional gradients in different

ecosystems (Nacke et al., 2016; Szoboszlay et al., 2017; Ding et al., 2020; Wang G. et al., 2022). However, due to the inherent heterogeneity among different successional environments, bacteria and fungi may perform different or even opposite shift and reassemble patterns (Gao et al., 2015; Zhou et al., 2017; Li C. et al., 2018). Therefore, how species replacement alters microbial processes, and whether microbial shifts follow predictable assembly trajectories remain unclear (Schmidt et al., 2014; Chai et al., 2019). Furthermore, current studies mostly focused on microbial succession in different vegetation types (forest, grasslands, farmland, etc.) (Cui et al., 2019; Liu Y. et al., 2020), but neglected microbial gradual shifts during the process of species replacement. Still, it remains unclear whether microbial shift and reassemble smoothly during the process of vegetation succession (Liu J. et al., 2020).

Climate and environment changes are expected to affect plant communities and consequently microbial composition (Cregger et al., 2012; Mou et al., 2022). Shifts in plant communities, such as plant diversity, composition, and biomass, can alter litter inputs and root exudates, which in turn influence microbial diversity and its related microbial process (Bakker et al., 2014; Lange et al., 2015; Xu et al., 2020; Wang G. et al., 2022). Moreover, soil properties such as pH and nutrient concentrations can regulate microbial composition or metabolic diversity through their effects on enzyme kinetics and nutrient diffusion (Zhang et al., 2016; Han et al., 2021). In turn, the shifts in microbial composition can also influence plant diversity by regulating nutrient bioaccessibility and then altering plant dominance (Van Der Heijden et al., 2008; Philippot et al., 2013; Kyaschenko et al., 2017). Thus, vegetation succession is essentially the interaction among plant, soil, and microorganisms (He et al., 2008; Kielak et al., 2008), a better understanding shifts in microbial composition along species replacement could help delineate the mechanisms that drive secondary succession and predict how microbes will respond to changing environments (Fierer et al., 2010; Jiang et al., 2021).

The current study was conducted in the hilly area of Taihang Mountain. In this region, before 1970s, large-scale human-induced deforestation such as clear-cutting, tilling, logging, and grazing has severely destroyed the original forest vegetation, and converted it into degraded shrub-herb communities (Calvo et al., 2002; Liu et al., 2012; Baudena et al., 2020). Starting from 1980s, a series of vegetation conservation projects such as banning grazing and returning marginal cropland to forest or grassland, were implemented to limit human disturbance, avoid soil degradation, and promote vegetation recovery (Liu et al., 2012; Guo et al., 2021). In addition, according to meteorological data of the study site, air temperatures exhibited a gradually increasing trend in the past 50 years (Figure 1). As affected by climate related environmental change, the vegetation in the area has experienced transition from perennial herbs to shrub-herb and then shrub communities, and shrublands have undergone gradual succession from *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* co-dominate to *V. negundo* var. *heterophylla* dominant communities (data not published). As succession progresses, community height, cover, and aboveground biomass increased gradually, while species diversity decreased generally (data not published). Thus, it offers an ideal landscape to investigate how the environmentally induced species replacement alters microbial processes. Here, we selected three successional stages (*V. negundo* var. *heterophylla* shrubland Orthodonic, *V. negundo* var. *heterophylla* and *L. oblonga* co-dominate shrubland (VLS), and *L. oblonga* shrubland

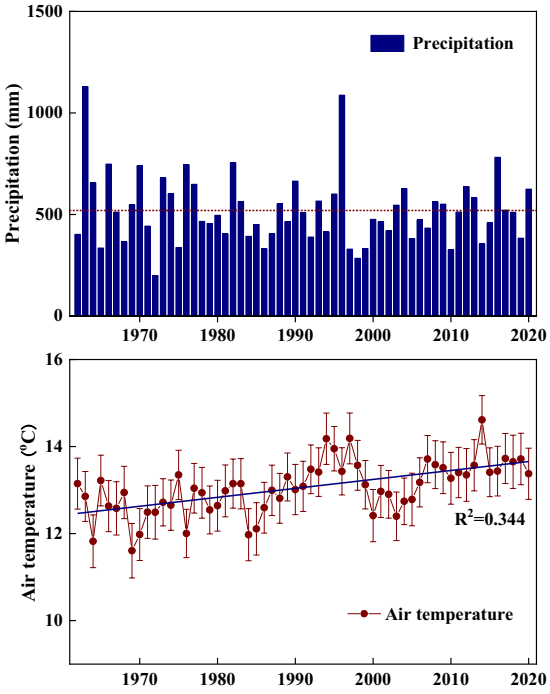

FIGURE 1
Annual variability of precipitation **(A)** and air temperature **(B)** in the hilly area of Taihang Mountain from 1962 to 2020. Precipitation is annual total and air temperature is annual average.

(LS)) to (1) elucidate how microbial diversity and composition shifts along species replacement; (2) identify the indicator species of shrubland during the process of secondary succession; and (3) determine which factors are closely related to shifts in microbial community.

2. Materials and methods

2.1. Site description

This study was conducted in the Niujiazhuang Catchment (area: 9.3 km^2) of middle Taihang Mountain, China (114°15′50″ E, 37°52′44″ N). Elevation across the study area ranges from 247 to 1,040 m a.s.l. and slope varies from 20 to 45°. This region has a typical temperate continental monsoon climate with wet warm summers and cool dry winters. Annual average precipitation from 1962 to 2020 is about 519 mm, 74% of which falling between June and September, and monthly mean air temperature ranges from −3.07°C in January to 26.8°C in July (Figure 1). The soil type is mainly highly-weathered mountainous cinnamon, classified as Ustalf (Liu et al., 2014). These soils are fertile and well-structured on shady slopes and skeletal and rocky on dry sunny slopes.

The original forests in the area have been severely destroyed and replaced by degraded shrub-herb communities. To better understand the succession processes of shrub-herb communities, we established 144 2 m × 2 m permanent plots in the Niujiazhuang Catchment, and conducted vegetation and soil census in 1986 and 2008, and in 2020, permanent plots were grouped by vegetation type and geographic

location, and 34 plots with expanded area of 16 m^2 were selected for re-census. In 1986, most plots were dominated by perennial herbs, with a high number of species composition and abundance, and low number of aboveground biomasses (Liu et al., 2011). From 1986 to 2008, along the decrease of herbaceous species, abundance, and biomass, the proportion of perennial herbs declined in favor of shrubs dominated by *V. negundo* var. *heterophylla* and *Ziziphus jujuba* var. *spinosa* on south-facing slope, and *L. oblonga* on north-facing slope (Liu et al., 2012). After a few years, in 2020, *V. negundo* var. *heterophylla* has become the most dominant species in the hilly area of Taihang Mountain, as it had highest importance value, height, cover, and biomass in all layers (data not published).

2.2. Vegetation investigation and soil sampling

Due to lack long-term monitoring of microbial dynamics, we used space-for-time substitution to analyze how microbial shifts as plants transitioned from LS to VLS and then VS. Along with vegetation census in September 2020, according to dominant species, soil properties, and spatial distribution, a total of 20 plots (8 VS, 6 VLS, and 6 LS) were selected to represent the typical successional stages during the process of species replacement. Except for slope aspect (VS often grows on sunny slope, while VLS and LS concentrate on shady slope), all plots have similar geographical distribution, including elevations, slope gradient, slope position, and soil type. In each plot, the species, number, height, cover, and aboveground biomass of shrubs and herbs were recorded, and topographic factors such as elevation, slope gradient, slope aspect, and slope position were also recorded. The aboveground biomass of shrubs and herbs were measured by expanding the sampling domain to neighboring areas of the plot. The plant samples were oven-dried at 80°C to a constant weight and weighed for dry matter.

In each plot, soil samples were collected from the top 20 cm using a 5 cm diameter soil auger. After removing visible plant roots, stones, litter, and debris, nine soil cores were collected along an S-shaped transect and then mixed together to form three composite sample. Thus, a total of 60 soil samples were collected (20 plots × 3 replicates). Each soil sample was divided into two subsamples after removing visible plant roots, stones, litter, and debris. One subsample was immediately stored at −80°C for DNA analysis, and the other was air-dried for physicochemical analysis.

2.3. Soil physicochemical properties

Soil density (SD) at 0–20 cm depth was estimated in undisturbed samples, collected with cylindrical stainless steel rings (100 cm^3), from three samples per plot. Soil pH was determined in 1:2.5 soil/water suspension using a pH meter (PHS-3C, Shanghai, China) (Bao, 2000). Soil organic matter (SOM) was determined by the Walkley-Black potassium dichromate oxidation method after H_2SO_4-$HCLO_4$ digestion (Nelson and Sommers, 1982). Total nitrogen (TN) was determined by the Kjeldahl method with an automated Kjeldahl apparatus (Kjeltec 8,400, Foss, Sweden) (Bremner, 1996). Total phosphorus (TP) was digested by perchloric acid and determined by the molybdate colorimetric method with a UV spectrophotometer (UV-2450, Shimadzu, Japan) (O'Halloran and Cade-Menun, 2006). Total potassium (TK) was determined by flame atomic absorption spectrophotometer (Analytikjena, Germany).

2.4. Soil DNA extraction, polymerase chain reaction amplification, and illumina sequencing

Soil DNA was extracted for three sub-samples from 0.5 g of soil using FastDNA Spin Kit (MP Biomedicals, Cleveland, United States) following the manufacturer's instructions. DNA concentration was determined with a NanoDrop ND-2000 spectrophotometer (NanoDrop Thermo Scientific, Wilmington, DE, United States), and DNA quality was evaluated by 1% agarose gel electrophoresis. Polymerase chain reaction (PCR) amplification of bacterial 16S rRNA V4-V5 region and fungal ITS1 region were conducted using the 515F/907R and ITS1F/ITS2R primer sets, respectively (Peng et al., 2019; Zhong et al., 2019). Detailed protocols for PCR amplification have been described in previous reports (Song et al., 2018; Zhao et al., 2019; Zhong et al., 2019). Each DNA extract was amplified in three replicates and then were mixed into one PCR product. After amplification, each mixed gene was detected by 2% agarose gel electrophoresis, purified with AxyPrepDNA purification kit (Axygen, United States), and quantified by Quantus™ Fluorometer (Promega, Madison, WI, United States) (Zhang et al., 2016; Liu et al., 2018). Subsequently, all amplicons were sequenced on the Illumina Miseq platform (Personal Biotechnology Co., Ltd., Shanghai, China). Approximately 50,884 and 14,868 high quality sequences per sample with an average length of 363 and 447 bp were obtained for bacteria and fungi, respectively.

Raw sequencing data were demultiplexed, quality-filtered, and analyzed using QIIME (Caporaso et al., 2010). After reads<50 bp and any unresolved nucleotides were discarded, noise filtering and chimera removal were performed using USEARCH (Edgar, 2010; Edgar et al., 2011), and high-quality sequences were assigned to operational taxonomic units (OTUs) using Silva and Unite databases with a similarity threshold of 97% (Caporaso et al., 2010). Finally, a total of 9,025 bacterial and 6,344 fungal OTUs were detected after trimming, assembly, and quality filtering.

2.5. Statistical analyses

Microbial community diversity (shannon), richness (Chao1), and rarefaction curve were performed using Mothur version 1.30.2. The Kolmogorov–Smirnov test was used to check the data distribution, and all data sets met the normality assumption for one-way analysis of variance (ANOVA). ANOVA followed by least significant difference (LSD) multiple comparison ($p < 0.05$) was used to assess the differences in plant traits (cover, height, and aboveground biomass), soil properties (pH, SOM, TN, TP, TK, and SD), and microbial alpha diversity (Shannon and Chao1 indices) and abundances among successional stages. Venn diagrams of shared and unique OTUs among different successional stages were performed using R-package VennDiagram. Indicator species analysis in the R indicspecies package was used to identify OTUs that were significantly associated with three successional stages, and discuss their potential as indicator species.

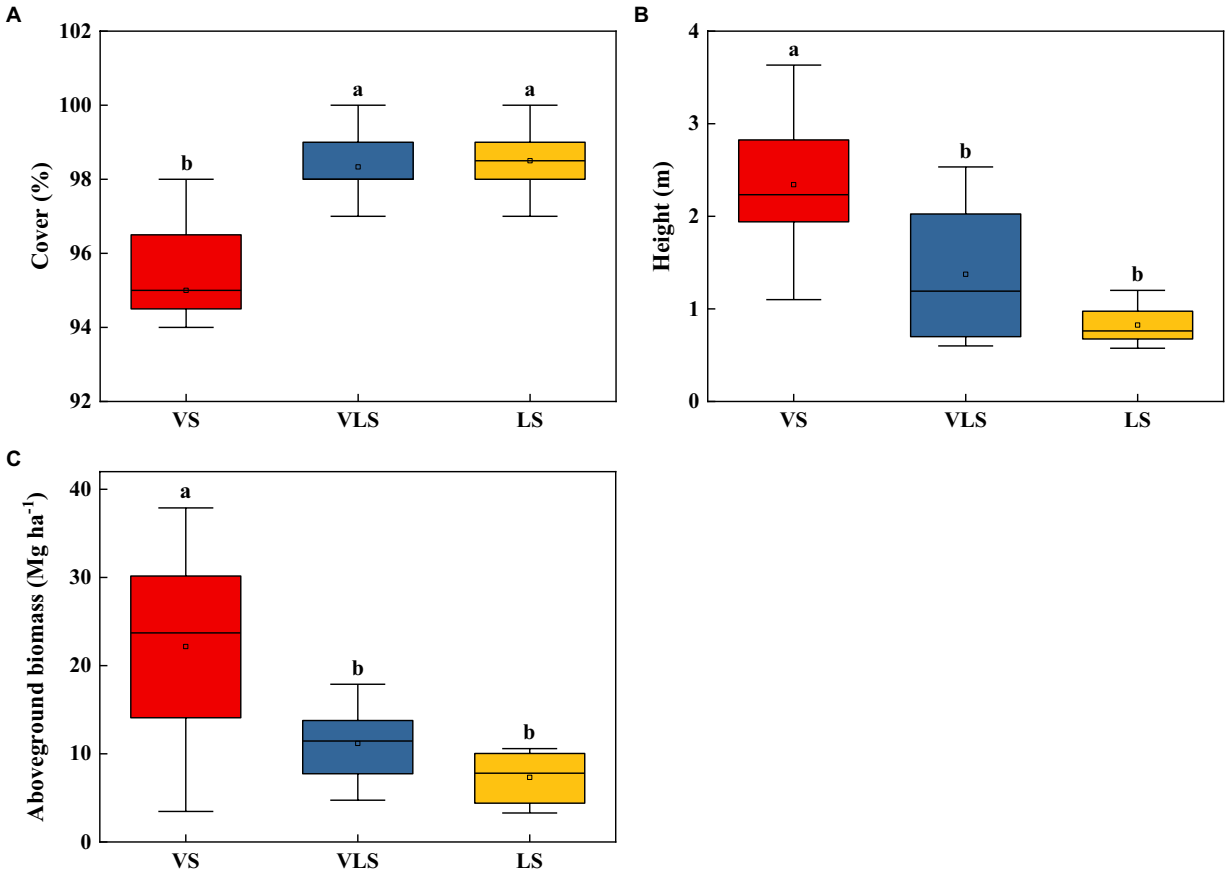

FIGURE 2
Changes in height **(A)**, cover **(B)**, and aboveground biomass **(C)** of shrub-herb communities across successional stages. Different letters indicate significant differences at $p<0.05$ according to ANOVA. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

Principal component analysis (PCA) was performed to explain the relationship between microbial genes (OTU) and environment variables (pH, SOM, TN, TP, TK, SD, cover, height, and aboveground biomass) during succession. ANOVA analysis was performed using SPSS 19 for Windows (SPSS Inc., Chicago, United States), diversity index and rarefaction curve were calculated using Mothur software,[1] PCA and indicator species analysis were conducted in R software package v4.2.1.[2]

3. Results

3.1. Plant and soil properties

In 2020, the mean height, cover, and aboveground biomass of VS, VLS, and LS were 2.19 m, 97.2%, and 15.7 Mg ha^{-1} (Figure 2). VS exhibited significantly higher community height and aboveground biomass compared with VLS and LS, who showed higher community cover ($p<0.05$). However, no significant differences in plant traits were detected between VLS and LS ($p>0.05$) (Figure 2).

Among three successional stages, there were no significant differences regarding soil pH, SOM, and TN ($p>0.05$) (Figure 3). VS and VLS showed significantly higher TP than LS, and VS exhibited higher SD relative to VLS and LS ($p<0.05$) (Figure 3). Compared to VS, VLS showed a lower TK, while compared to LS they displayed higher TK ($p<0.05$) (Figure 3).

3.2. Diversity and composition of microbial communities

Proteobacteria (30.1%) was the most dominant bacterial phylum in all samples, followed by Acidobacteriota (22.5%) and Actinobacteriota (18.1%) (Figure 4). The relative abundance of Proteobacteria and Acidobacteriota decreased, while that of Actinobacteriota increased with succession ($p<0.05$) (Figure 4). The fungal communities were dominated by Ascomycota (52.4%), Mortierellomycota (19.0%), and Basidiomycota (19.5%) across three successional stages (Figure 4). There were no significant differences in relative abundance of Mortierellomycota ($p>0.05$), but significant

1 https://mothur.org/wiki/calculators/
2 http://www.r-project.org/

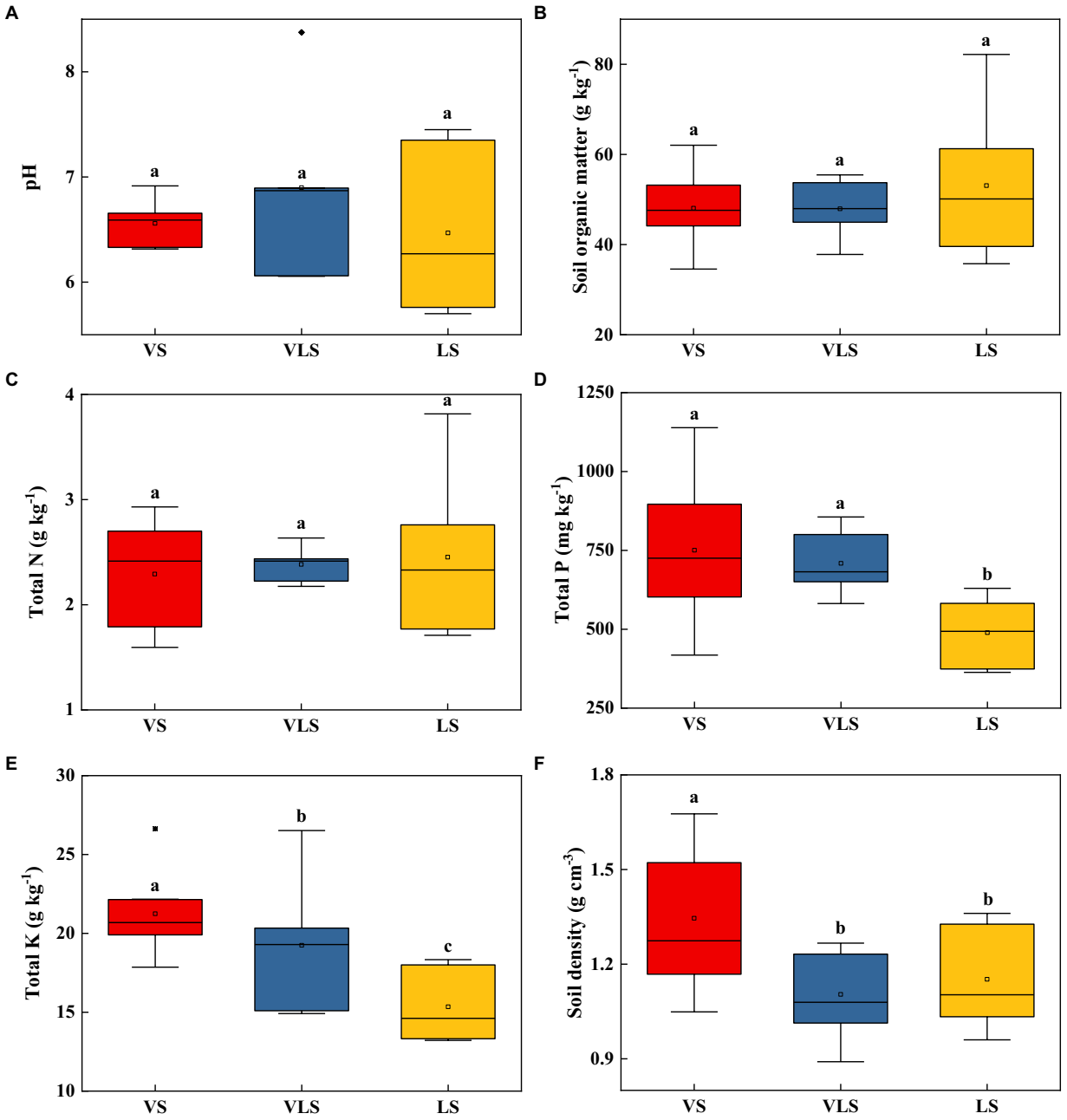

FIGURE 3
Changes in soil pH **(A)**, soil organic matter **(B)**, total N **(C)**, total P **(D)**, total K **(E)**, and soil density **(F)** in topsoil (0–20cm) across successional stages. Different letters indicate significant differences at $p<0.05$ according to ANOVA. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

increases in Ascomycota abundance and decreases in Basidiomycota abundance as succession progresses ($p < 0.05$) (Figure 4).

Rarefaction curves suggested that the overall bacterial and fungal OTUs were well captured at the current sequence depth (Figure 5). VS and VLS showed higher bacterial diversity than LS ($p < 0.05$), but there were no significant differences in fungal diversity among different successional stages ($p > 0.05$) (Figure 5). Soils in VS and VLS exhibited higher bacterial and fungal richness, compared to the soils in LS ($p < 0.05$) (Figure 5).

Venn diagrams indicated that the three successional stages shared 50.9% bacterial and 23.0% fungal OTUs (Figure 6). The core bacterial microbiome was dominated by Proteobacteria and Planctomycetota, among which Alphaproteobacteria (34.4%), Planctomycetes (30.6%), and Gammaproteobacteria (20.3%) were identified as the most predominant classes (Figure 6). Most of the core fungal microbiome belonged to Ascomycota, and 75.8% of these taxa belonged to Sordariomycetes, Dothideomycetes, and Eurotiomycetes (Figure 6).

3.3. Indicator species of successional stages

Indicator species of bacterial and fungal community varied among three successional stages (Figure 7; Supplementary Table S1). For bacteria,

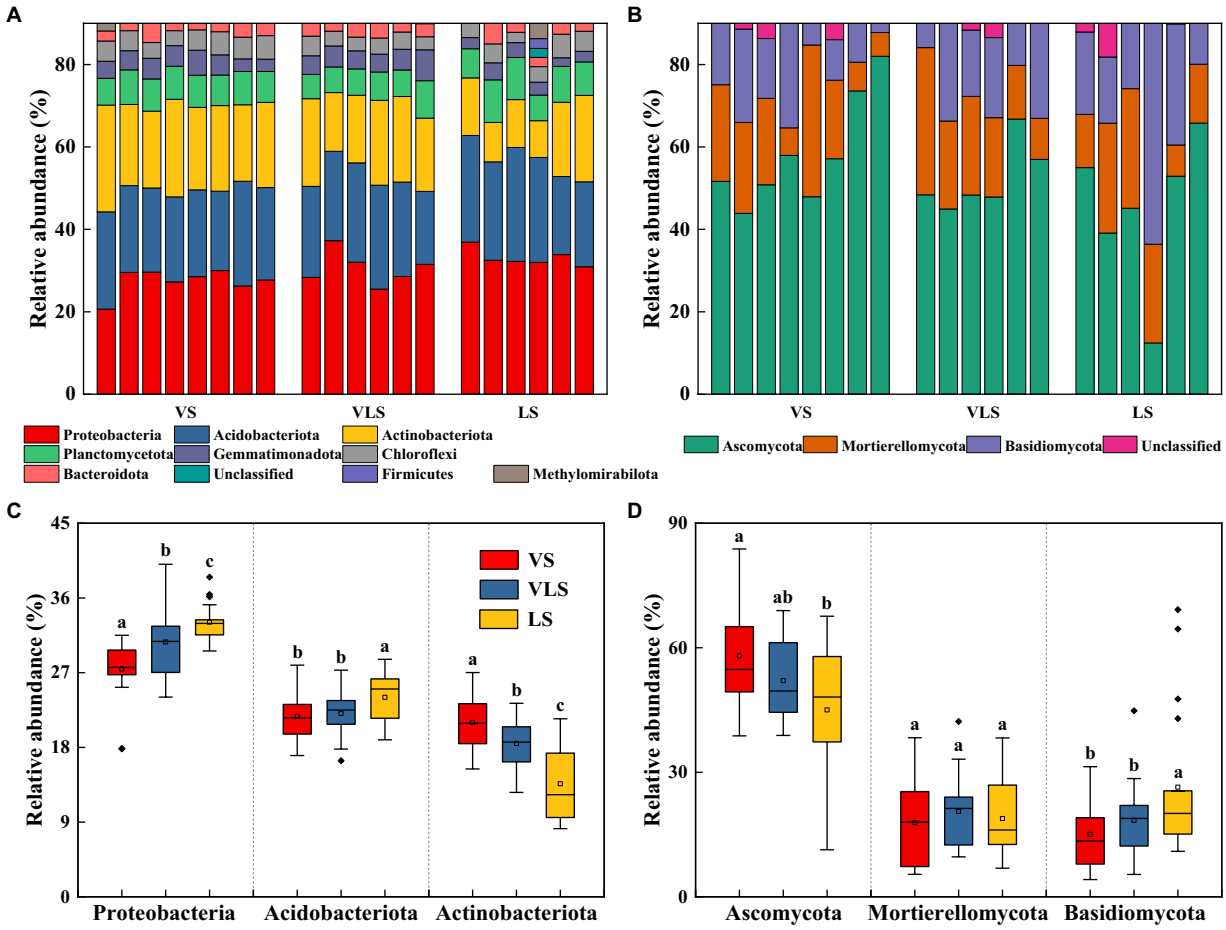

FIGURE 4
Relative abundance of bacteria **(A)** and fungi **(B)** at the phylum level, and relative abundance of dominant bacterial **(C)** and fungal **(D)** phyla. Phyla with low abundances (<2%) were not shown. Different letters indicate significant differences at $p<0.05$ according to ANOVA. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

the most abundant OTU in VS was assigned to Vicinamibacteraceae, a typically aerobic heterotroph that can survive in arid environments (Huber and Overmann, 2018; Wang K. et al., 2022). Whereas, the most abundant OTU in LS belonged to Acidobacteriales, an order comprising aerobic or facultatively anaerobic, and mostly acidophilic and mesophilic bacterium (Kuramae and de Assis Costa, 2019). In addition, OTU3915, together with OTU1302 and OTU3414, were the indicator species in VLS, and performed major roles in soil nitrogen and phosphorus cycles (Supplementary Table S1). Correspondingly, for fungi, the most abundant OTU in VS belonged to *Pleiochaeta*, a genus with pathogenic members that could cause leaf spots on legumes (Marin-Felix et al., 2017). However, one of the most abundant OTUs in VLS was classified as a member of the phylum Ascomycota with a relative abundance of 11.0%. Furthermore, the most abundant OTU in LS was OTU7420, and belonged to Basidiomycota, the major degraders of different components in wood (Taylor et al., 2015).

3.4. Shifts in microbial community

Eurythermal bacterial OTUs growing across a large temperature gradient increased in abundance due to species replacement, however, some stenothermal species with narrow growth temperature ranges declined with succession (Figure 8; Supplementary Figures S1, S2; Supplementary Tables S2, S3). Among indicator OTUs showing increased relative abundance following species replacement were acidophilic bacteria, such as *Acidibacter* and *Catenulispora*, pathogenic taxa, such as *Enterobacter* and *Legionella*, heat-tolerant taxa, such as *Legionella* and *Rhodovastum*, and some metabolism bacteria (Supplementary Table S2). The bacterial groups that declined with succession were mainly those associated with nitrogen cycling and biodegradation of organic pollutants, as well as a few psychrotolerant, acidophilic, and pathogenic bacteria (Supplementary Table S3).

Fungal indicator OTUs were more likely to decline with succession than bacterial ones (Figure 8; Supplementary Figures S3, S4; Supplementary Tables S4, S5). Ascomycota was the most abundant fungi phyla in all samples, and a majority of indicator OTUs in Ascomycota declined due to species replacement, while a minority expanded with succession (Supplementary Figures S3, S4; Supplementary Tables S4, S5). Among indicator OTUs showing declined relative abundance were endophytic taxa, such as *Apiospora* and *Aureobasidium*, saprobic taxa, such as *Chaetosphaeria* and *Endophragmiella*, and some pathogenic fungi (Supplementary Figure S3; Supplementary Table S4). The fungal groups that increased with succession were mainly rock-inhabiting, nematode-trapping, endophytic, and pathogenic species (Supplementary Figure S4; Supplementary Table S5).

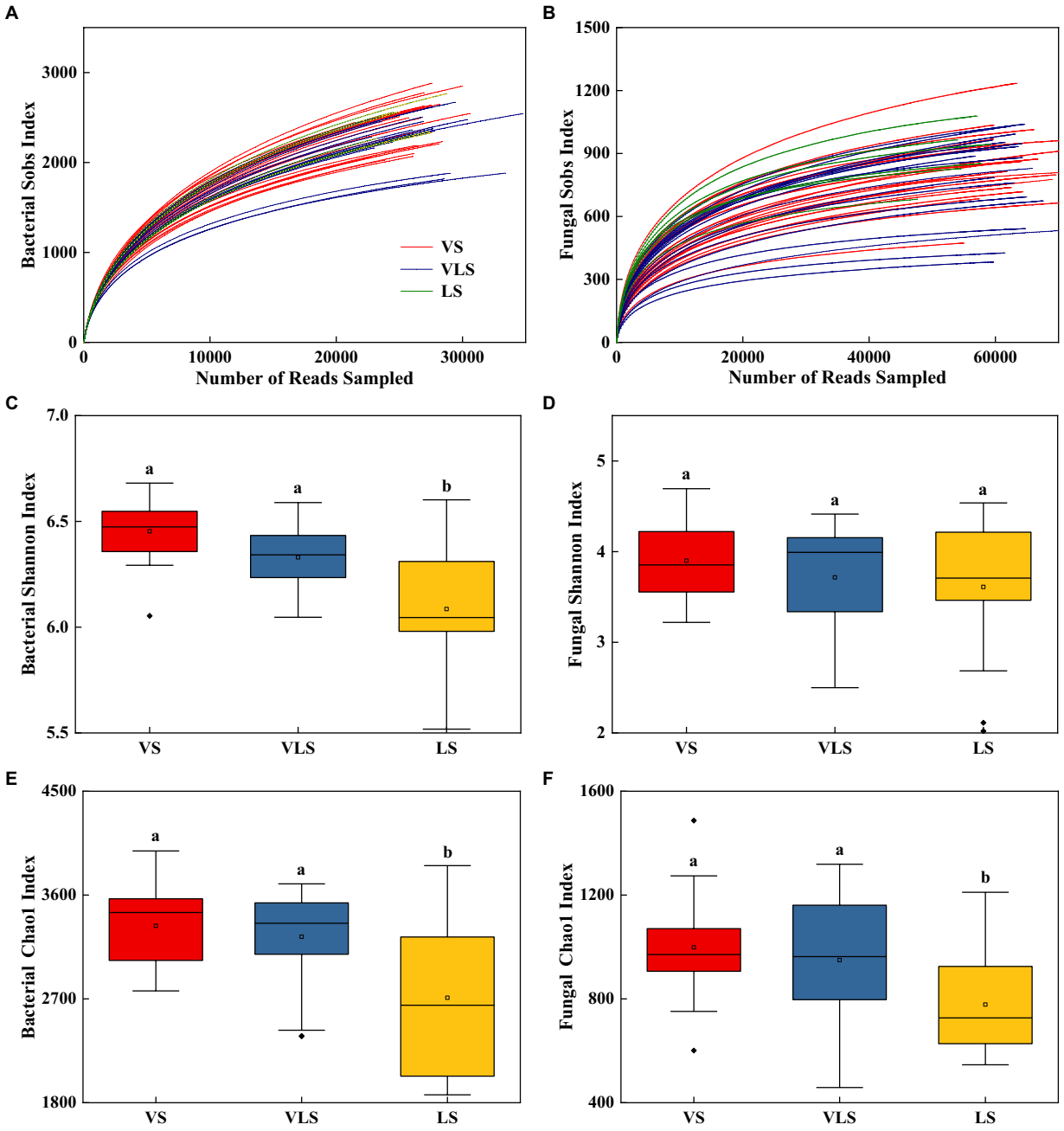

FIGURE 5
Changes in microbial diversity of bacteria and fungi across successional stages. **(A)** bacterial rarefaction curves, **(B)** fungal rarefaction curves, **(C)** Shannon index of bacteria, **(D)** Shannon index of fungi, **(E)** Chao 1 index of bacteria, **(F)** Chao 1 index of fungi. Different letters indicate significant differences at $p<0.05$ according to ANOVA. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

3.5. Key factors driving the shifts in microbial community

According to the PCA results, the shifts in microbial community composition across successional stages were strongly associated with soil properties and plant traits (Figure 9). Soil pH, TK, and aboveground biomass significantly affected bacterial community composition, it could explain 49.8% of bacterial community variation (Figure 9). For fungal communities, soil pH, TK, aboveground biomass, and TP were best suited to explain the shifts in microbial composition, and explained 27.9% of the total variability of fungal community dynamics (Figure 9).

4. Discussion

Secondary succession in shrub-herb communities following disturbance has commonly been shown to directly alter species composition and ecosystem functions (Heine et al., 2019; Yan et al.,

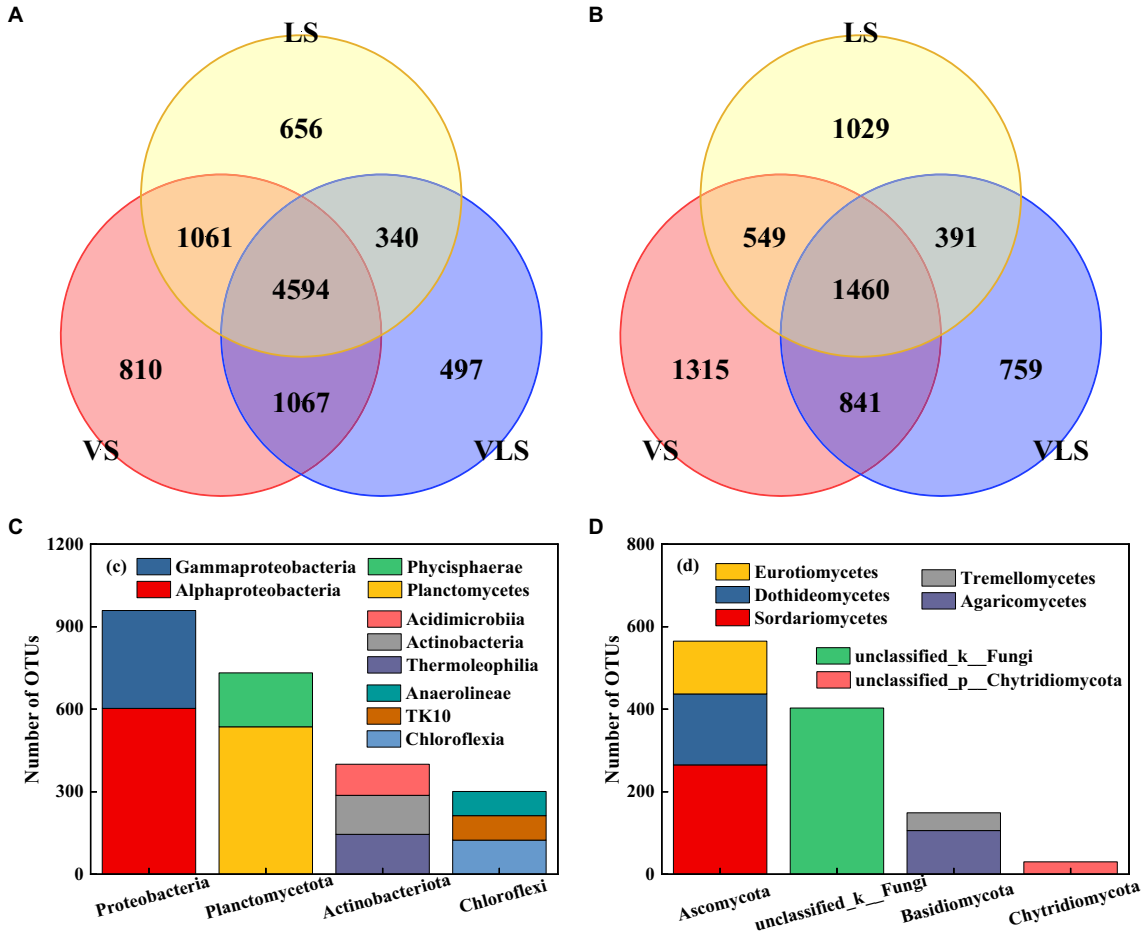

FIGURE 6
Venn diagrams and core OTUs of bacteria and fungi. **(A)** venn diagram of bacteria, **(B)** venn diagram of fungi, **(C)** core OTUs of bacteria, and **(D)** core OTUs of fungi. VS, Vitex negundo var. heterophylla shrubland; VLS, Vitex negundo var. heterophylla and *Leptodermis* oblonga shrubland; LS, *Leptodermis* oblonga shrubland. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

2020), and such changes in turn affect soil biochemical processes in degraded environments (Zhao et al., 2019; Jiang et al., 2021). In our results, during the process of species replacement, the aboveground biomass of shrubland increased over 3 times, from 7.3 Mg ha^{-1} in LS to 22.2 Mg ha^{-1} in VS (Figure 2), similar to the results of Li Q. et al. (2018) and Ma and Wang (2020). Aboveground biomass increased significantly in the presence of *V. negundo* var. *heterophylla*, indicating that these shrublands in the hilly area of Taihang Mountain are important carbon pools, and if all the established shrublands in this area are eventual replaced by *V. negundo* var. *heterophylla*, it will have great potential for carbon sequestration. These changes in plant community due to species replacement altered the distribution of soil nutrients, especially TP and TK (Figure 3; Feng et al., 2007; Sullivan et al., 2019; Segura et al., 2020). Possible explanations for the increased TP and TK with succession can be ascribed to plant species and local topographic features (Zhu et al., 2016; Jucker et al., 2018). The high morphological plasticity of *V. negundo* var. *heterophylla* (Moreira et al., 2003; Wang et al., 2017), allows it to use nutrients of fractured rocks on barren land, and facilitates P and K translocation from deeper soil layers to topsoil (Sullivan et al., 2019; Segura et al., 2020). Altogether, the coordinated changes in vegetation and soil highlight the importance of above- and belowground linkages as succession progresses (Roy-Bolduc et al., 2016).

The coordinated changes in plant and soil during secondary succession also caused accompanied shifts in microbial diversity and composition (Zhong et al., 2018; Chai et al., 2019; Zhao et al., 2019). As expected, our results showed that aboveground biomass significantly influenced microbial community composition across successional stages (Figure 9), consistent with previous findings (Chen et al., 2016; Zhang et al., 2016; Kyaschenko et al., 2017). This result may be ascribed to the fact that increased aboveground biomass with succession could accelerate accumulation of plant-derived resources and nutrients for microbial growth (Kardol et al., 2006; Lange et al., 2015). In addition, belowground soil properties, such as soil pH and nutrient concentrations, have also been identified as potential ecological drivers for shaping soil microbial processes (Figure 9; Kaiser et al., 2010; Hu et al., 2014). In this regard, large number of studies have confirmed the effects of soil pH on microbial community structure and function (Siciliano et al., 2014; Tripathi et al., 2015; Lu et al., 2022), and demonstrated that the differences in microbial community could be explained primarily by the

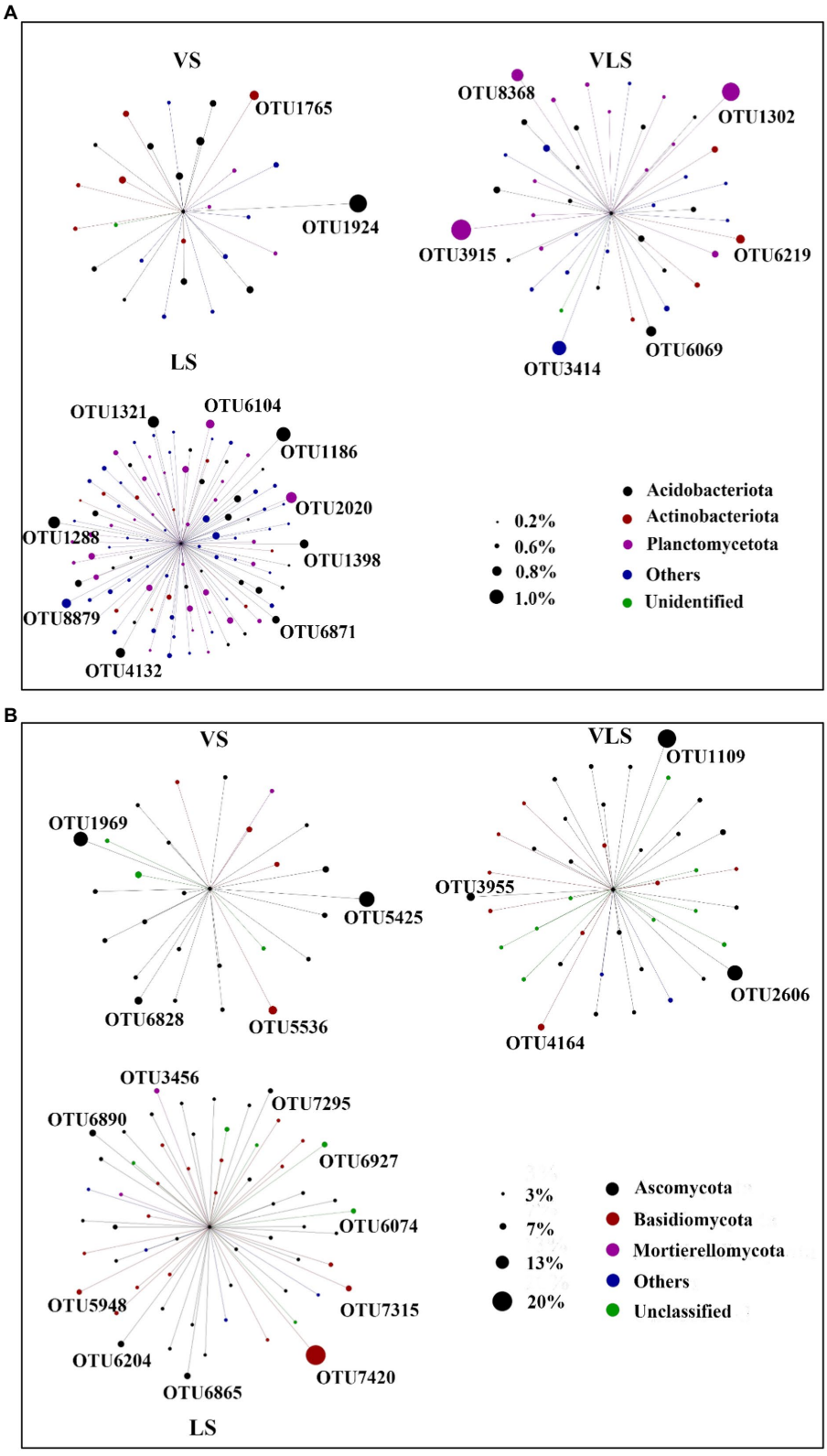

FIGURE 7
Indicator species by treatment regime. Circles represent OTUs, and the size of each circle represents its relative abundance. OTUs with low abundances (bacteria<0.1%, and fungi<0.3%) were not shown. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

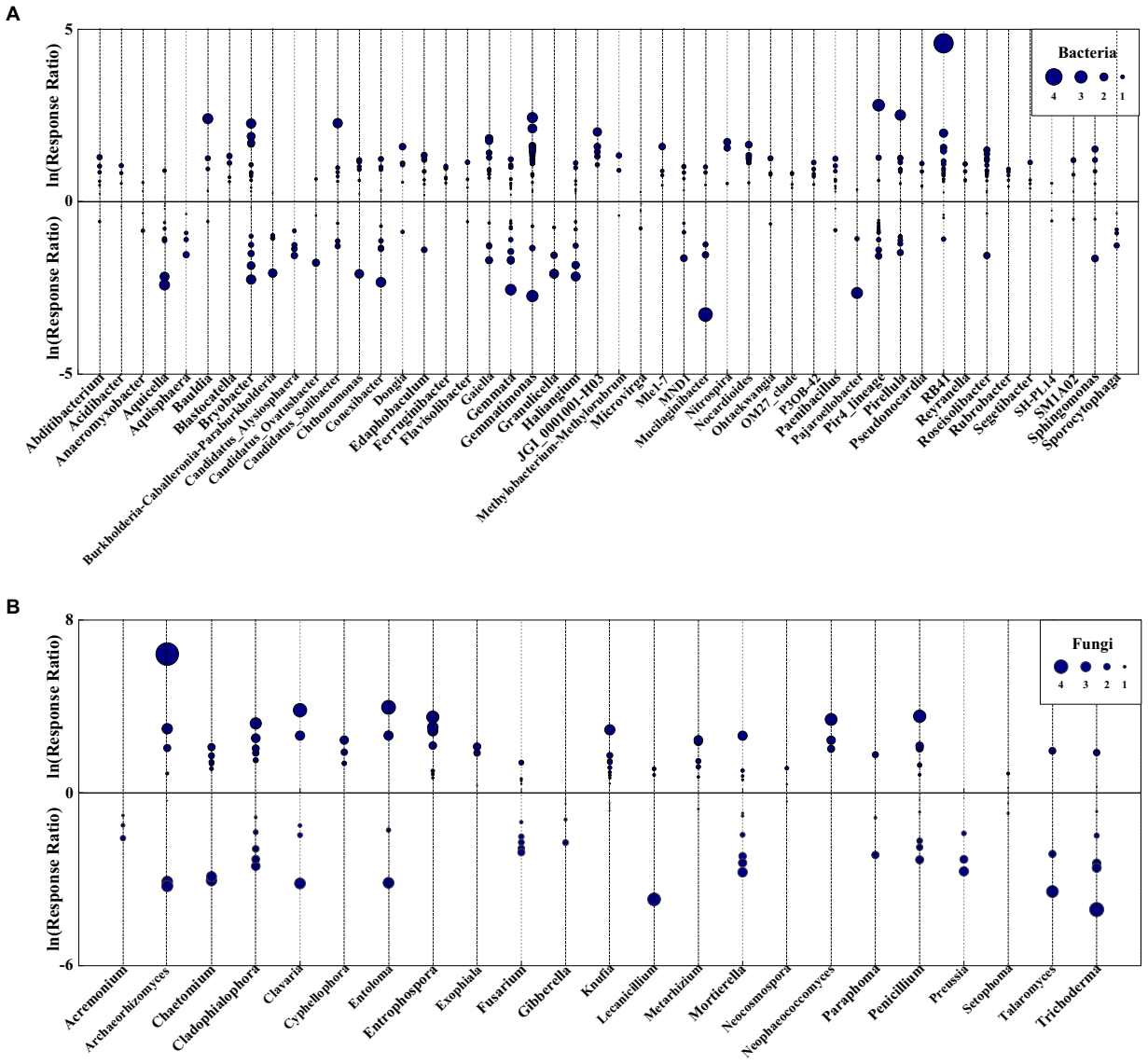

FIGURE 8
Log response ratio of bacterial **(A)** and fungal **(B)** genera in *VS* and VLS plots relative to LS plots. Circles represent OTUs with $p<0.05$, and the size of each circle represents its relative abundance. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

variation in soil pH (Zhang et al., 2018; Chai et al., 2019). Moreover, we also found that TP and TK were significantly correlated with microbial community structure (Figure 9). Soil phosphorus and potassium were mainly derived from parent material, along the depleted and fixed in plants and animal tissues, their concentrations may restrict microbial growth and metabolisms (Zhao et al., 2013; Hou et al., 2017; Song et al., 2018). Therefore, both plant and soil properties shift driven by vegetation succession could structure soil microbial communities (Koyama et al., 2018).

Species replacement, induced by climate related environmental change, resulted in a gradually replacement of cold-tolerant microbes with warm-affinity ones (Figure 8; Supplementary Figures S1, S2). The relative abundance of bacteria growing optimally above 30°C increased (Figure 8; Pagnier et al., 2010; Falagán and Johnson, 2014; Percival and Williams, 2014), and the indicator species in VS can survive in environments where water is extremely scarce (Huber and Overmann, 2018; Wang K. et al., 2022). The consistent increases in warm-affinity taxa along species replacement reflected a high tolerance for drought and heat stress of *V. negundo* var. *heterophylla* for survival in harsh environment, so it has potential to expand its range under climate warming (Du et al., 2014; Li et al., 2017; Wang et al., 2017). In addition, species replacement altered the relative abundance of several bacterial groups involved in soil biogeochemical processes (Supplementary Figures S1, S2). These included declining populations of *Asticcacaulis* (Poindexter, 2015) and *Dongia* (Liu et al., 2010), and increasing abundances of *Novosphingobium* (Kumar et al., 2022) and *Rhizobacter* (Goto, 2015), previously identified as major degraders of organic compounds. Moreover,

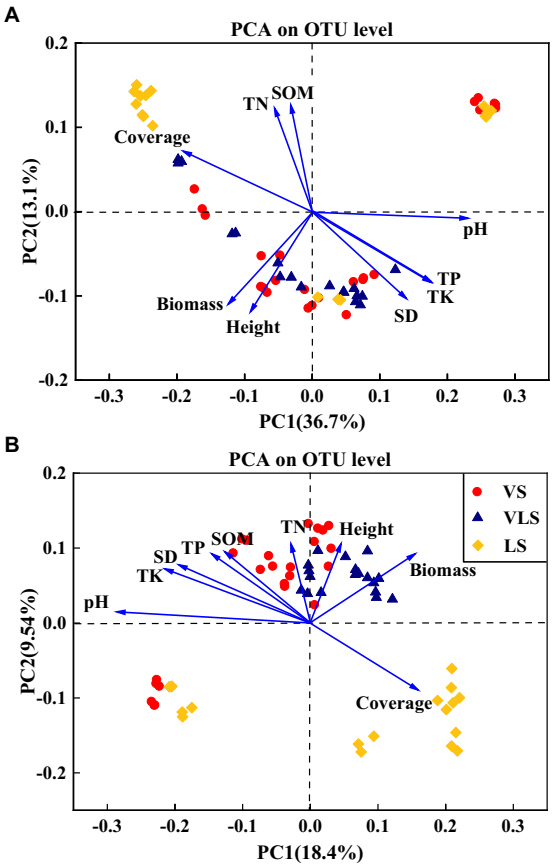

FIGURE 9
Principal component analysis (PCA) of the relationship between the bacterial **(A)**, fungal **(B)** community composition and environment variables. SOM: soil organic matter; TN: total N; TP: total P; TK: total K; SD: soil density; Biomass: community aboveground biomass; Height: community height; Cover: community cover. VS, *Vitex negundo* var. *heterophylla* shrubland; VLS, *Vitex negundo* var. *heterophylla* and *Leptodermis oblonga* shrubland; LS, *Leptodermis oblonga* shrubland.

species replacement also produced significant expansion in *Microlunatus* (Hanada and Nakamura, 2015) and *Rhodovastum* (Okamura et al., 2009), and decline in *Dyella* (Xie and Yokota, 2005) and *Rivibacter* (Stackebrandt et al., 2009), which was previously linked to soil nutrient cycles. However, populations of *Enterobacter* (Iversen, 2014) and *Rhizorhapis* (Francis et al., 2014) also increased following species replacement, a group of animal or plant pathogens. Therefore, secondary succession, induced by the replacement of dominant species, resulted in significant changes in soil environment and bacterial microbial community.

Ascomycota was the most abundant phylum of fungi, the decline of their dominant taxa was consistent with the finding of Zhang et al. (2018) and Chai et al. (2019). Generally, members of Ascomycota are dominant in stressful environments (Tripathi et al., 2016), the decline of dominant taxa indicated that soil ecological environments were improved through vegetation succession (Dong et al., 2016; Chai et al., 2019). In contrast, the overall relative abundance of Ascomycota increased with succession (Figure 4), similar to the observations in fire-affected and exposed soil environments (Taş et al., 2014; Wilhelm et al., 2017), this pattern likely reflected the increase of rock-inhabiting and some other extremotolerant fungi (thermophilic, desiccation-tolerant, etc.) during gradual expansion of *V. negundo* var. *heterophylla* toward harsh environment (Morgenstern et al., 2012; Egidi et al., 2014; Hubka et al., 2014). Notably, the nematophagous subset of Ascomycota, including *Dactylellina*, *Purpureocillium*, and *Pochonia*, that expanded in most plots following species replacement have all been reported in mountain environments and forest soils (Li et al., 2006; Deng et al., 2020; Gouveia et al., 2022). Numerous nematophagous fungi can immobilize and digest nematodes, and are thought to be important in regulating entomopathogenic nematode populations in the field (Wang et al., 2006; Willett et al., 2017). All these results indicated that various fungal microbial communities exhibited different adaptions to shifts in environmental conditions during secondary succession (Schmidt et al., 2014; Alfaro et al., 2017).

5. Conclusion

Secondary succession from *L. oblonga* to *V. negundo* var. *heterophylla* shrubland in Taihang Mountain significantly increased the aboveground biomass of shrublands, and TP and TK contents in topsoil. Species replacement, induced by climate related environmental change, resulted in the gradually replacement of cold-tolerant microbes with warm-affinity ones, and alterations of microbial communities involved in soil biogeochemical processes. Soil and plant variables, such as aboveground biomass, soil pH, TP, and TK, well explained the variations in microbial communities. Altogether, the coordinated changes in plant communities and soil properties during secondary succession caused accompanied shifts in microbial diversity and composition.

Data availability statement

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

Author contributions

XL and WZ: investigation, formal analysis, and writing–original draft. XW and HW: methodology, data curation, and writing–review and editing. WD: resources, supervision, and project administration. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1158731/full#supplementary-material

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EDITED BY
Tengxiang Lian,
South China Agricultural University, China

REVIEWED BY
Osama Abdalla Abdelshafy Mohamad,
Xinjiang Institute of Ecology and Geography (CAS), China
Evangelos Petropoulos,
Newcastle University, United Kingdom

*CORRESPONDENCE
Jingyang Bian
✉ nongkeyuanliukai@163.com

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Soil bacterial communities of paddy are dependent on root compartment niches but independent of growth stages from Mollisols of Northeast China

Kai Liu[1], Qiuju Wang[2], Minglong Sun[3], Shiwei Gao[4], Qing Liu[4], Lili Shan[5], Junxiang Guo[5] and Jingyang Bian[6]*

[1]Heilongjiang Academy of Agricultural Sciences, Harbin, China, [2]Heilongjiang Academy of Black Soil Conservation and Utilization, Harbin, China, [3]Crop Resources Institute, Heilongjiang Academy of Agricultural Sciences, Harbin, China, [4]Suihua Branch of Heilongjiang Academy of Agricultural Sciences, Suihua, China, [5]Rice Research Institute of Heilongjiang Academy of Agricultural Sciences, Jiamusi, China, [6]Daqing Branches of Heilongjiang Academy of Agricultural Sciences, Daqing, China

Introduction: Deep insights into adhering soil of root zones (rhizosphere and rhizoplane) microbial community could provide a better understanding of the plant-microbe relationship. To better understand the dynamics of these microbial assemblies over the plant life cycle in rhizodeposition along rice roots.

Methods: Here, we investigated bacterial distribution in bulk, rhizosphere, and rhizoplane soils at tillering, heading, and mature stage, from rice (*Oryza sativa*) fields of the Northeast China.

Results and Discussion: Our results revealed that soil bacterial α-diversity and community composition were significantly affected by root compartment niches but not by temporal change. Compared to rhizoplane soils in the same period, bulk in the heading and rhizosphere in the mature had the largest increase in Shannon's index, with 11.02 and 14.49% increases, respectively. Proteobacteria, Chloroflexi, Bacteroidetes, and Acidobacteria are predominant across all soil samples, bulk soil had more phyla increased across the growing season than that of root related-compartments. Deterministic mechanisms had a stronger impact on the bacterial community in the compartments connected to the roots, with the relative importance of the bulk soil, rhizoplane and rhizosphere at 83, 100, and 56%, respectively. Because of ecological niche drivers, the bacterial networks in bulk soils exhibit more complex networks than rhizosphere and rhizoplane soils, reflected by more nodes, edges, and connections. More module hub and connector were observed in bulk (6) and rhizoplane (5) networks than in rhizosphere (2). We also detected shifts from bulk to rhizoplane soils in some functional guilds of bacteria, which changed from sulfur and nitrogen utilization to more carbon and iron cycling processes. Taken together, our results suggest distinct bacterial network structure and distribution patterns among rhizosphere, rhizoplane, and bulk soils, which could possibly result in potential functional differentiation. And the potential functional differentiation may be influenced by plant root secretions, which still needs to be further explored.

KEYWORDS

bacterial microbiome, distribution, MiSeq sequencing, co-occurrence network, rice fields

Introduction

In nature, plant roots associate with several soil-derived bacterial microbiota, which impact plant development, nutrient uptake, and disease resistance positively or negatively (Edwards et al., 2018; Dang et al., 2022; Tspa et al., 2022). Previous studies using next-generation sequencing technologies have shown that root microbiota composition is predominantly controlled by environmental factors, plant genotype and soil type (Dang et al., 2022). In fact, due to the selective pressure imposed by the environment, which results in exchanges within a local population and migrations between other populations, microbial populations can experience brief alterations in their structure (Cavaglieri et al., 2009). It is well known that plant development has an impact on the production and diffusion of root exudates (Canarini et al., 2019). These exudates secreted by plants stimulate specific microorganisms, and the function of exudates is also influenced by the stage of plant growth (Wen et al., 2021). Therefore, the degree of adaptation of soil microorganisms may vary with the different stages of plant development.

Four distinct compartments from the outside to the inside of the root: bulk soil (unaffected by root activity), rhizosphere (the soil microenvironment immediately surrounding the root), rhizoplane (the root surface), and endosphere (the root interior), and microbial diversity follows a compositional transition along this gradient (Van der Heijden and Schlaeppi, 2015). Since root-associated microorganisms are mostly sourced from the local edaphic communities, the specific compositions of these compartments rely on the soil source. Roots maintain a complex microbial community at the soil-root interface, which can affect plant nutrition, growth, and health (Yang H. J. et al., 2022). Huang et al. found significant differences in the structure and function of bacterial communities among the three root-associated ecological niches (bulk soil, rhizosphere, and rhizoplane), such as bacterial community diversity and composition (Huang et al., 2022). Rhizocompartment were the dominant factors affecting bacterial assemblage, with bacterial community OTU numbers decreasing from bulk soil to rhizoplane and specific OTUs enriching from bulk soil to rhizoplane (Lang et al., 2019). In addition, Yamazaki et al. also pointed out that bacterial communities in rhizosphere environments differ from those in bulk soils through a multi-omics analysis (microbiome and transcriptome) of soybean plants in a field at Tokyo University of Agriculture and Technology, Japan (Yamazaki et al., 2021). However, most studies have focused on studying the effects of rhizocompartment on bacterial communities without considering the effects of plant life cycles and the interaction between the two on soil bacterial communities (Lucas et al., 2018; Yin and Yan, 2020; Li B. et al., 2022). Deterministic processes based on ecological niches and neutral stochastic processes are two processes that explain the changes in microbial community assembly, which may be influenced by the external environment (Beck et al., 2015; Zhou and Ning, 2017). Similarly, there are more and more studies to promote soil microbial interactions by constructing correlation network analysis (Wang et al., 2019; Xiao et al., 2022; Li et al., 2023). For example, by exploring the effect of tillage practices on soil bacterial communities, Liu et al. (2021) found that deep tillage leads to a tighter and more competitive network of better soil bacterial communities. Because of this, we attempted to construct microbial network analysis and community assembly to investigate the mechanisms of soil bacterial communities affected by rhizocompartment under different rice life cycles.

More than 65% of Chinese people use rice as their primary food source, and an area of 3×10^7 ha represents 20% of the total world cropping area (FAO, 2019). Consequently, it is crucial to investigate the changes of microorganisms in root environment of rice. The objective of the present study was to focus on two main questions (1) How variable are bacterial communities associated with different plant growth stages? and (2) Whether there are spatial variations in the soil rhizodeposition. We offer insights into the process of rice root bacteria development through dynamic investigations of the composition of the rice bacteria.

Materials and methods

Field experiment design

The experimental site was located at the experimental field of the Heilongjiang Academy of Agricultural Sciences in Minzhu village, Harbin, China (45°49′N, 126°50′E). The area has a temperate continental monsoon climate. The mean annual temperature in the region is 3.6°C, with a frost-free period of 141 days. The mean annual rainfall is 502 mm, and nearly 60% of the total rainfall is mainly concentrated from July to September. The mean annual precipitation is 1,032.5 mm (Li et al., 2019). The soil was a clay loam (Mollisols Udolls Paleudolls) according to the USDA soil Taxonomy system (Soil Survey, 2010). The soil properties are listed: organic matter, 35.1 g·kg^{-1}; total N, 1.42 g·kg^{-1}; total P, 0.38 g·kg^{-1}; total K, 21.37 g·kg^{-1}; available N, 148.2 mg·kg^{-1}; available P, 38.5 mg·kg^{-1}; available K, 249.1 mg·kg^{-1}; and pH 6.53.

A susceptible rice variety, Wuyoudao-4 was planted on March 16 and harvested on October 20 in 2019 using approximately 30 cm seedlings at an inter-row spacing of 0.20 m. The planting densities were maintained at 280,000 plants ha^{-1}. Three replicates of the treatments were set up in a randomized block design, with each plot size 5 m × 4.5 m each and being spaced apart by 2 m. During the growing season, weeds were manually pulled twice; no herbicide or rhizobium inoculant was used. Nitrogen fertilization (140 kg N ha^{-1}) was supplied as urea on June 7, 2019 and June 30, 2019, respectively.

Sample collection

Rice plants ($n = 10$–12) were harvested by digging around for maximum intactness of the roots at each site. This study has 3 replications for each treatment. Soil sampling procedure and compartment separation were described by Edwards et al. (2015) (Supplementary Table S1). Specifically, soil and root sample collection were at tillering, heading and mature stages, respectively. The sampling times are listed: July 8 (tillering stage), August 1 (heading stage), and September 25, 2019 (mature stage). All soil samples were placed in polyethylene bags and sent to the lab on ice packs. The soils were hand-selected to remove small stones, residues, and roots, and then sieved through 2 mm meshes. Storage of the samples was at −80°C for DNA extraction.

Sequencing of the 16S rRNA gene

The total DNA of each sample was isolated from 0.25 g of soils using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA, United States). Prior to amplification, the DNA concentration was standardized to be the same. The 16S rRNA gene's V3-V4 region was chosen to create the community library using the forward primers 338\u00B0F (Huse et al., 2008) and reverse primer 806 R (Li et al., 2019), using a six-base barcode that is particular to each sample. Supplementary Table S2 offers more information on the PCR conditions. PCR products from all samples were combined and identified by 2% agarose gel electrophoresis, and the PCR products were recovered by gel cutting using the AxyPrep DNA Gel Recovery Kit (AXYGEN). PCR products were quantitatively detected by QuantiFluor™-ST Blue fluorescence quantitative system (Promega), and the MiSeq sequencing library was constructed using TruSeqTM DNA Sample Prep Kit, which was sequenced based on PE300 strategy. The PCR products were purified, pooled in equimolar amounts, and paired-end sequenced (2 × 300) using an Illumina MiSeq platform at Shanghai Meiji Biological Medicine Technology Co Ltd., Shanghai, China.

Bioinformatics analysis

Low-quality raw data sequences (length < 250 bp and average base quality score < 20) were removed using Trimmomatic (Bolger et al., 2014) and merged using FLASH software (Magoc and Salzberg, 2011; Bolger et al., 2014). In total, 1,460,755 high-quality reads were generated in this study. Using the USEARCH v7.1 pipeline, operational taxonomic units (OTUs) were produced with at a ≥ 97% similarity level (Edgar, 2013), which yielded 6,513 OTUs. The remaining sequences were denoised (Schloss et al., 2009) and aligned against the bacterial 16S rRNA gene database in Mothur (Koljalg et al., 2013). The 16S rRNA gene sequences were submitted to the Sequence Read Archive (SRA) at the National Center for Biotechnology Information (NCBI) with accession number PRJNA738275.

Statistical analysis

Based on this output normalized data, subsequent analyzes of alpha and beta diversity were all carried out. Alpha-diversity indices were calculated with Mothur (version1.30.2). The effect of rhizospheric compartmentalization, temporal change, and their interaction on soil bacterial community composition was assessed using Permutational multivariate analysis of variance (PERMANOVA). Subsequently, beta-diversity of bacterial community was ordinated using principal coordinates analysis (PCoA). Beta regression was performed using the BetaReg package (Cribari-Neto and Zeileis, 2010). In order to evaluate the community assembling processes, a null modeling approach was used to calculate the beta Nearest Taxon Index (NTI) (Stegen et al., 2013). The βNTI between −2 and 2 range denotes stochasticity, while theβ NTI > 2 or < −2 indicates determinism in community assembly (Dini-Andreote et al., 2015). Additional, based on both βNTI and Bray–Curtis-based Raup-Crick Index (RC_{Bray}) values, five ecological processes were examined: homogeneous selection (βNTI < −2), heterogeneous selection (βNTI > +2), dispersal limitation (βNTI <2 and RC_{Bray} > 0.95), homogenizing dispersal (βNTI <2 and RC_{Bray} < −0.95), and undominated (βNTI <2 and RC_{Bray} < 0.95) were analyzed (Zhou et al., 2014; Jiao et al., 2020). To better comprehend the taxonomic and functional relationships within the microbial communities, network studies were carried out (Mendes et al., 2014). Bacterial OTUs that appeared in over 50% of the communities were included in the networks analysis so that we could concentrate exclusively on the abundant OTUs (Guan et al., 2020). Spearman's correlation coefficients were calculated between OTUs (Revelle, 2013). The correlations between OTUs with a Spearman's coefficient < 0.7 and a p value >0.01 were deleted in order to filter the data for reduced network complexity (Widder et al., 2014). Network visualisation was done using igraph package Csardi and Nepusz (2006). Then, in each network, the threshold values of Zi and Pi as given by Guimera and Amaral (2005) were used to evaluate the topological roles of each node. The bacterial functional groups were evaluated using Functional Annotation of Prokaryotic Taxa (FAPROTAX) after obtaining the OTU' identification and abundance information (Sansupa et al., 2021). All samples' distribution of function groups was evaluated using the PCA and heatmap. The analyzes indicated above were completed using R program (v.3.1.1) (R Core Team, 2013).

Two-way ANOVAs were used to assess the effects of rhizospheric compartmentalization, temporal change, and their interaction on soil bacterial diversity and richness indices. Statistical differences among rhizospheric compartmentalization and temporal change were measured by Tukey's HSD and Mann–Whitney non-parametric test with subsequent Bonferroni correction, with $p < 0.05$ considered significant.

Results

Soil bacterial diversity index

Soil bacterial Shannon diversity, Simpson diversity, Ace richness, and Chao 1 richness indices were significantly affected by rhizospheric compartmentalization but not by temporal change and their interaction (Supplementary Table S3). The Shannon index was significantly higher in bulk and rhizosphere soils (increased by 3.73 and 8.24% in the tillering, 11.09 and 12.19% in the heading, and 8.97 and 14.58% in the mature stage, respectively). However, the differences between bulk and rhizoplane soil were not significant ($p > 0.05$) in the tillering stage. The results of the Simpson index showed the opposite trend to that of the Shannon index only in heading stage (Table 1). The Ace and Chao 1 indices show the same trend; the Ace and Chao 1 indices were significantly higher in bulk (11.54 and 12.28%, respectively) and rhizosphere soils (17.14 and 16.86%, respectively) than that of rhizoplane soil in the heading stage; in the mature stage, rhizosphere soils showed an increase of 14.95 and 19.71% in Ace and 15.68 and 20.26% in Chao 1 indices compared with bulk and rhizoplane soils, whereas Ace and Chao 1 indices were not shown significantly differences among rhizospheric compartmentalization ($p > 0.05$) in the tillering stage.

Soil bacterial community composition

Using permutational multivariate analysis of variance (PERMANOVA), root compartment niches had a substantial impact

TABLE 1 Soil bacterial Shannon diversity, Simpson diversity, Ace richness, and Chao 1 richness indices among three compartments in the tillering, heading, and mature stage.

		Shannon	Simpson	Ace	Chao 1
Tillering	BS	6.67 ± 0.23 ab	0.0044 ± 0.0010 a	3103.93 ± 322.85 a	3117.76 ± 290.76 a
	R1	6.96 ± 0.14 a	0.0030 ± 0.0015 a	3424.43 ± 28.95 a	3424.77 ± 5.86 a
	R2	6.43 ± 0.18 b	0.0055 ± 0.0012 a	3221.06 ± 192.29 a	3202.29 ± 207.37 a
Heading	BS	7.11 ± 0.13 a	0.0024 ± 0.0010 b	3531.04 ± 144.03 a	3537.76 ± 136.26 a
	R1	7.18 ± 0.02 a	0.0017 ± 0.0001 b	3708.30 ± 66.64 a	3681.90 ± 43.56 a
	R2	6.40 ± 0.09 b	0.0063 ± 0.0006 a	3165.68 ± 129.61b	3150.80 ± 137.51 b
Mature	BS	6.80 ± 0.37 a	0.0056 ± 0.0058 a	3244.54 ± 322.40 b	3246.05 ± 320.94 b
	R1	7.15 ± 0.04 a	0.0018 ± 0.0002 a	3729.68 ± 51.30 a	3755.11 ± 74.49 a
	R2	6.24 ± 0.15b	0.0074 ± 0.0016 a	3115.66 ± 98.94b	3122.62 ± 45.58b

Values with different letters indicate significant differences (analysis of variance; $p < 0.05$). BS, bulk soil; *R1*, rhizosphere soil; *R2*, rhizoplane soil.

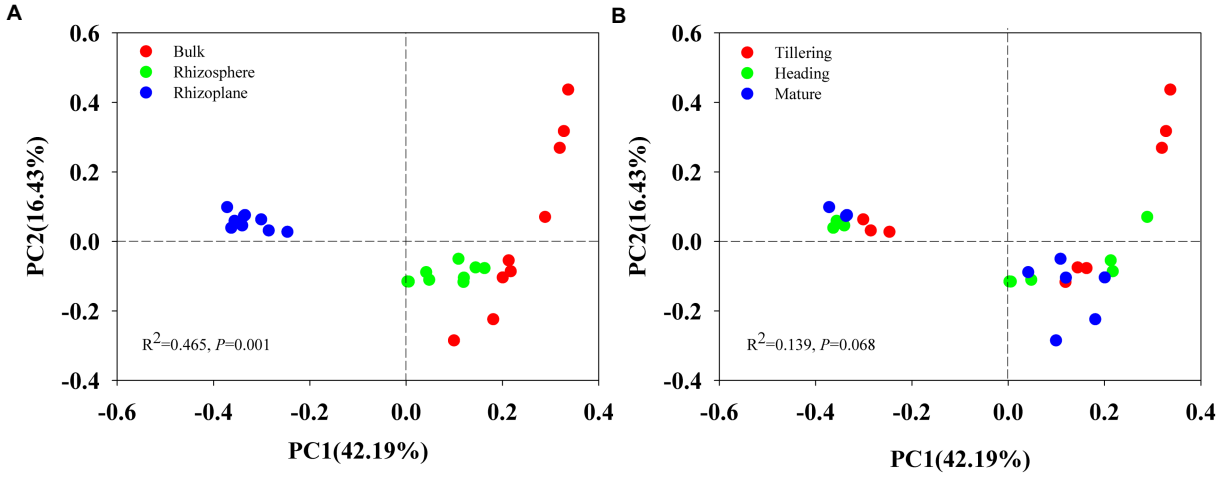

FIGURE 1
Principal Coordinates Analysis (PCoA) on Bray_Curtis dissimilarity demonstrated the differences of bacterial communities of 27 soil samples at two grouping patterns: **(A)** grouping pattern as bulk soil, rhizosphere and rhizoplane soil; **(B)** grouping pattern of tillering, heading, and mature stage.

on the composition of the soil bacterial population ($r^2 = 0.465$, $p = 0.001$) but had no effect on growth stage ($r^2 = 0.139$, $p = 0.068$). Principal Coordinates Analysis (PCoA) also showed distinct bacterial community composition in different soils (bulk, rhizosphere and rhizoplane; Figure 1A), but bacterial communities of the tillering, heading and mature stage overlapped (Figure 1B), indicating that the largest source of variation in root-associated microbial communities is proximity to the root.

Four bacterial phyla including Proteobacteria, Chloroflexi, Bacteroidetes, and Acidobacteria were predominant across all soil samples and 80.51% of the total bacterial sequences (Figure 2A). In addition, Actinobacteria, Nitrospirae, Verrucomicrobia, Cyanobacteria, Firmicutes, Gemmatimonadetes, Spirochaetae, and Ignavibacteriae were less abundant (relative abundance >0.1%) but were still identified in all soil samples. These bacterial phyla were significantly influenced by rhizocompartment throughout the growth stage of rice (Figure 2B). Of the 42 detectable phyla, we found 15 phyla with significantly different geographic distributions across the bulk and rhizoplane soils (Figure 2B). From the bulk to the rhizoplane soils, there were 9 phyla whose relative abundance considerably rose and 6 bacterial phyla whose relative abundance declined. Within each compartment, we conducted beta-regression to identify phyla whose relative abundance changed during the duration of the growth stage (Figure 2C). In rhizoplane soils, 3 bacterial phyla all increased, whereas 7 bacterial phyla consistently decreased during the growth stage. In rhizosphere soils, 2 bacterial phyla all increase, while 4 bacterial phyla consistently decreased during the growth stage. In bulk soils, 10 bacterial phyla all rose during the growth stage, while 3 bacterial phyla consistently decreased. In general, bulk soil had more phyla increased across the growing season than that of root related-compartments.

Assembly processes of the bacterial communities

We calculated the beta nearest taxon index (NTI) of three compartments in order to distinguish between the deterministic and stochastic processes in community assembly along spatial distribution from the exterior to the surface of the root. βNTI values of the three groups were mainly above 2 (Figure 3A), always bulk soil (83%),

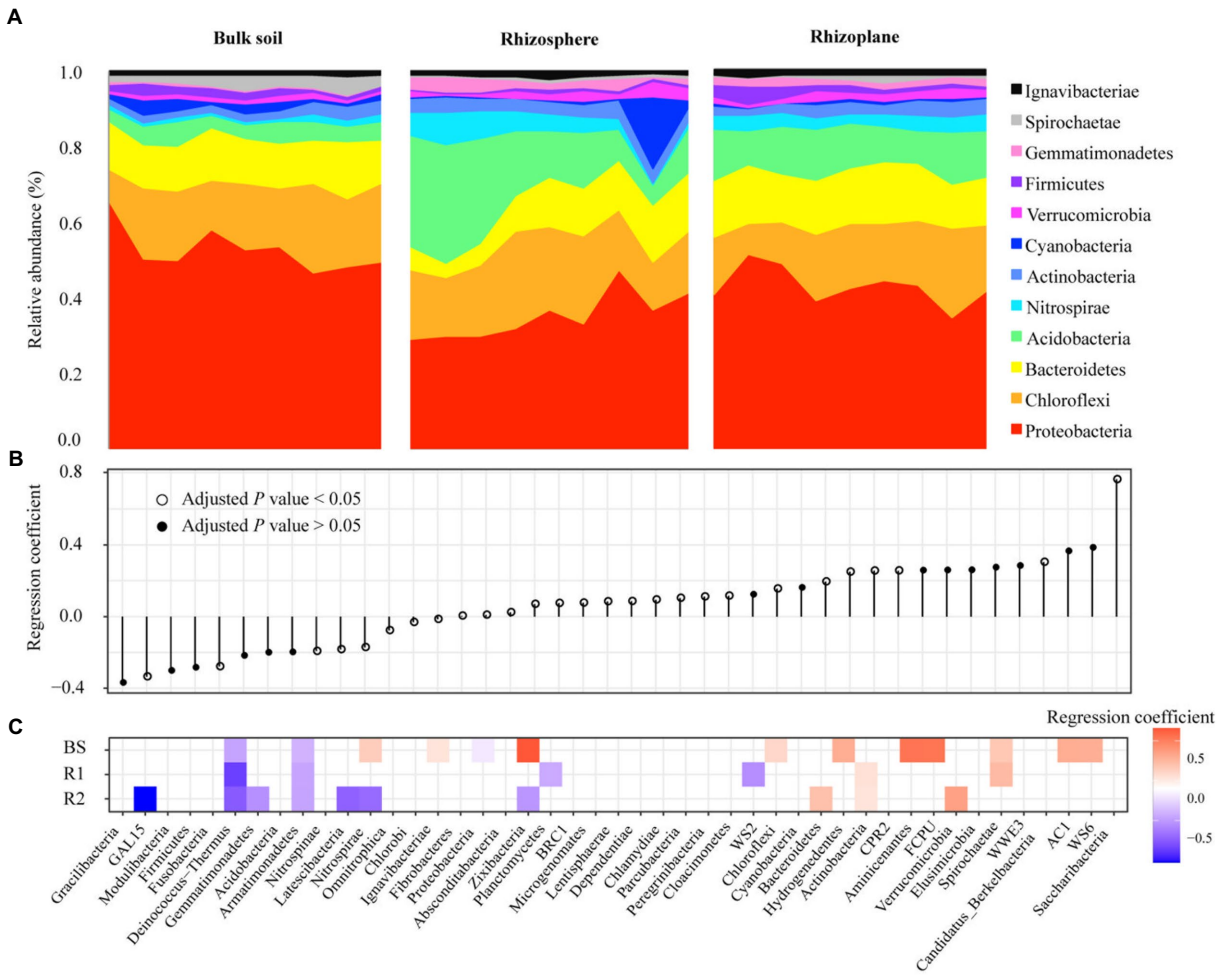

FIGURE 2
Shifts in the bacteria over time are associated with increasing and decreasing phyla. Bar plots of the top 12 phyla abundances over the course of the seasons in each compartment **(A)**. Beta regression coefficient estimates for bacterial phyla that are either increasing (above 0) or decreasing (below 0) in relative abundance in spatial distribution from the bulk to the rhizoplane soils **(B)**. Beta regression coefficient estimates for microbial phyla that are increasing (above 0) or decreasing (below 0) in relative abundance over the course of the growing stage in each compartment **(C)**.

rhizosphere (100%), and rhizoplane (56%). For the bulk and rhizosphere soils, higher relative deterministic process contribution primarily belongs to heterogeneous selection, while rhizoplane soil, the relative abundances of heterogeneous selection and undominated were similar (Figures 3B,C). Overall, deterministic mechanisms had a stronger impact on the bacterial community in the compartments connected to the roots.

Co-occurrence networks of soil bacterial communities

Three sections of the soil bacterial community's co-occurrence networks were examined Figures 4A–C and Table 2 lists the major topological characteristics. The network size was larger in bulk soils compared with rhizosphere and rhizoplane soils (Figures 4A–C), the number of nodes and links reflects this (Figures 4A–C; Table 2). The bulk soils networks were better connected and more complicated than rhizosphere and rhizoplane soils network (Figures 4A–C). The network topological qualities further supported this pattern, that is, the connectedness in bulk soils existed the highest, followed by rhizoplane and rhizosphere, while modularity exhibited opposite trend. The whole network is divided into many clusters according to phylum level features, rhizosphere soils was the one with the most clusters (186), considerably greater than bulk soils (135) and rhizoplane soils (130) (Table 2). Additionally, compared to rhizosphere and rhizoplane soils, bulk soil networks had a larger ratio of positive to negative links (Figures 4A–C; Table 2).

From the plot of Zi (a value measuring within-module connectivity) and Pi (a value measuring among-module connectivity), more module hub and connector were observed in bulk and rhizoplane networks than in rhizosphere (Figures 4D–F). Six keystone taxa were identified in bulk soils, belonged primarily to Proteobacteria, Verrucomicrobia, Chloroflexi, and Acidobacteria. Two keystone taxa were identified in rhizosphere soils, belonged primarily to Proteobacteria and Nitrospirae. Five keystone taxa were identified in rhizoplane soils, belonged primarily to Proteobacteria, Chloroflexi, Firmicutes, and Bacteroidetes (Figures 4D–F; Supplementary Table S4). Supplementary Table S4 shows the evolutionary classification of each module hub and connection.

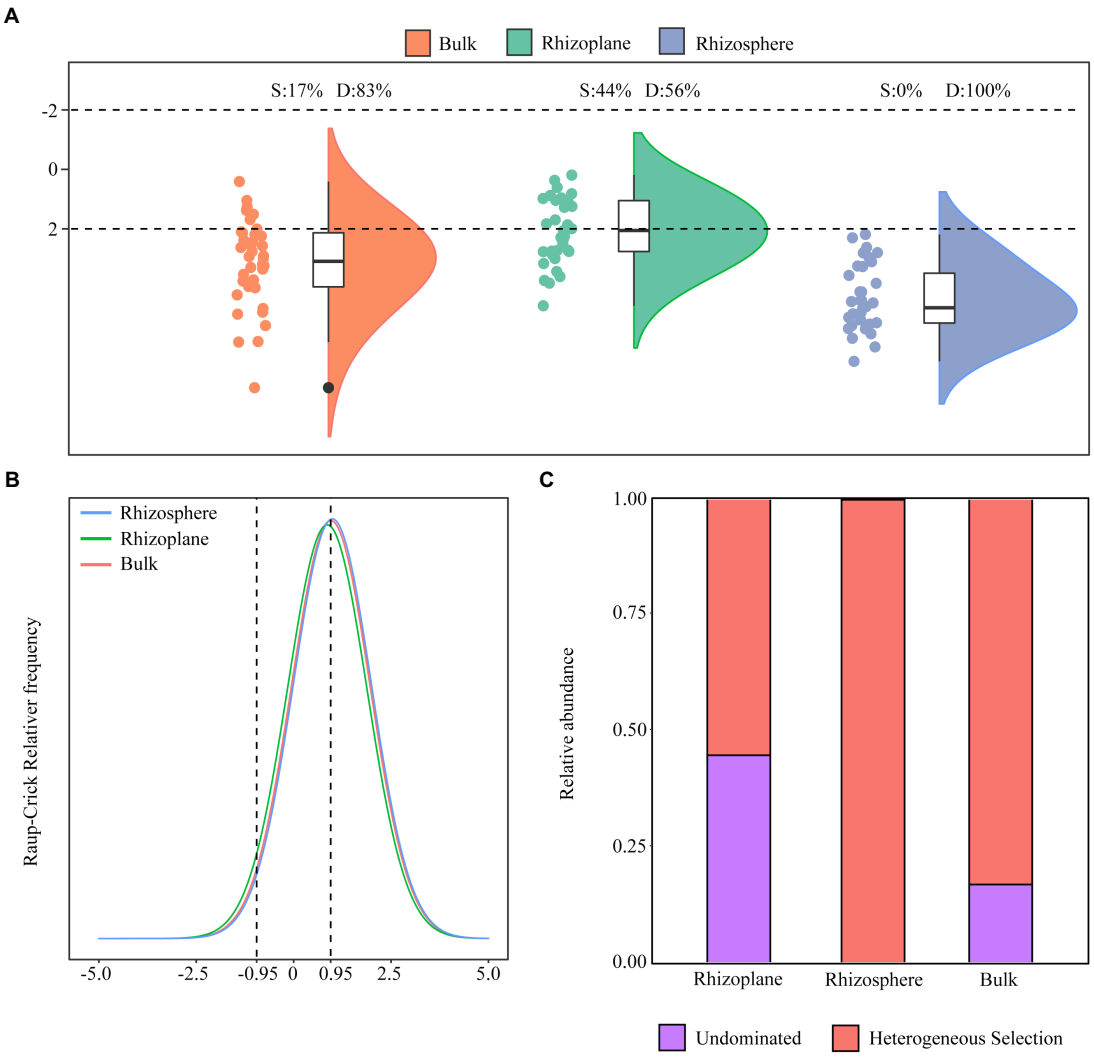

FIGURE 3
Deterministic and stochastic processes in bacterial assembly. Relative contribution of determinism and stochasticity on bacterial assembly based on the β-Nearest Taxon Index (βNTI) values **(A)**, Raup-Crick Relative frequency **(B)** and the relative importance of ecological processes **(C)**.

Soil bacterial community functional groups

The bacterial community in the soil was evaluated using FAPROTAX analysis, and 58 ecological types were predicted, with chemoheterotrophy, aerobic chemoheterotrophy, nitrification, and fermentation as the dominant functions. The PCA analysis was applied to visualize the differences among samples showed distinct bacterial functional profiles in different rhizocompartments (Figure 5A), demonstrating distinct communities with some overlap. Regarding the functional profile, the functional groups belonging to S and N cycle were more abundant in bulk and rhizosphere soils ($p < 0.05$), while functional groups belonging to C and Fe cycle were more abundant in rhizoplane soils (Figure 5B; Supplementary Table S5; $p < 0.05$).

Discussion

Several studies indicate that microbial communities associated with plants have been discovered to be advantageous for plant growth and resistance against biotic and abiotic stresses (Van Syoc et al., 2022; Zhang et al., 2022). Therefore, understanding the spatial pattern and temporal evolution of the root-associated microbiota is essential for agroecosystem function (Edwards et al., 2015). Our findings in this study showed that root compartment niches, but not temporal variation, significantly impacted bacterial α-diversity indices. Although it has been repeatedly shown that the root microbiome changed as the plant shifted throughout its life cycle (Chaparro et al., 2014; Edwards et al., 2015; Dombrowski et al., 2016). Soil environments have been considered as a hot spot for studying the biodiversity of plant-associated microbiota (Compant et al., 2010). Sanaullah et al. (2016) showed that different enzyme systems exist at the plant–soil interface including the rhizosphere, rhizoplane soil, and bulk soil. Soil bacteria be more sensitive to changes in soil enzyme activity than changes in the rice life cycle (Gong et al., 2019). Root compartment niches appears to influence soil microbial diversity through its effect on soil enzymes. Mollisols is commonly fertile and productive with high content of organic materials (Liu et al., 2012), and the SOM content was

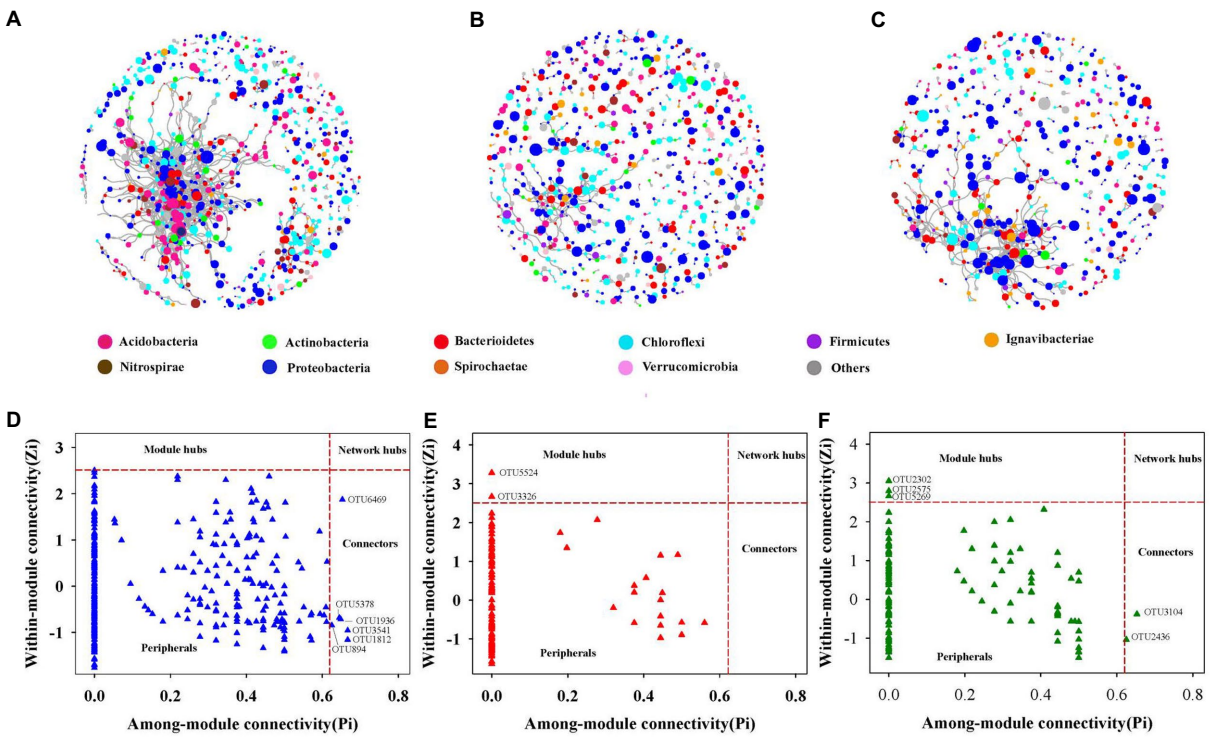

FIGURE 4
The co-occurrence network of soil bacterial community in bulk **(A)**, rhizosphere **(B)** and rhizoplane soils **(C)**, the size of each node is proportional to its relative abundance. Zi-Pi plots showing the distribution of soil bacterial OTUs based on their topological roles in bulk **(D)**, rhizosphere **(E)** and rhizoplane soil **(F)**.

TABLE 2 Major topological properties of the co-occurrence networks of soil bacterial communities in BS, R1, and R2 treatments.

Topological properties	BS	*R1*	*R2*
No. of original OTUs	2,697	3,070	2,295
Total nodes	982	749	577
Total edges	3,633	725	729
Average degree	7.40	1.94	2.53
No. of clustersa	135	186	130
Average clustering coefficient	0.50	0.36	0.37
Average path distance	5.54	5.91	6.74
Modularity	0.61	0.93	0.80
Connectedness	0.28	0.03	0.17
Positive links/negative links	1.40	1.19	1.37

BS, bulk soil; *R1*, rhizosphere soil; *R2*, rhizoplane soil.

35.1 g·kg^{-1} in the present study. Moreover, nitrogen fertilizer applied in green and jointing stage, respectively. Consequently, the high SOM and N supplement could potentially lessen how plant growth stages affect bacterial diversity. Overall, we noticed similarities in patterns in alpha-diversity at tillering and heading stages (Table 1). The alpha-diversity was significantly lower in in rhizoplane soils than that of bulk and rhizosphere soils (Table 1). Cui et al. (2019) found that plant selects microorganisms and as a result, rhizosphere microbial community diversity is less than that of bulk soils.

Similar soil bacterial diversity, community structure responded to root compartment niches over the course of the growth stage. This finding verified the outcomes of Edwards et al. (2015) who demonstrated that the largest source of variance in the sampled microbial communities was described by the rhizocompartments. The root "filtration effect," which allows plants to choose a microbiome in two steps, was assumed to be the cause of the variable alpha-diversity and community structure with root proximity (Dibbern et al., 2014). The transition from external to internal occupancy in the root comes first, followed by a general recruitment to the area around the root

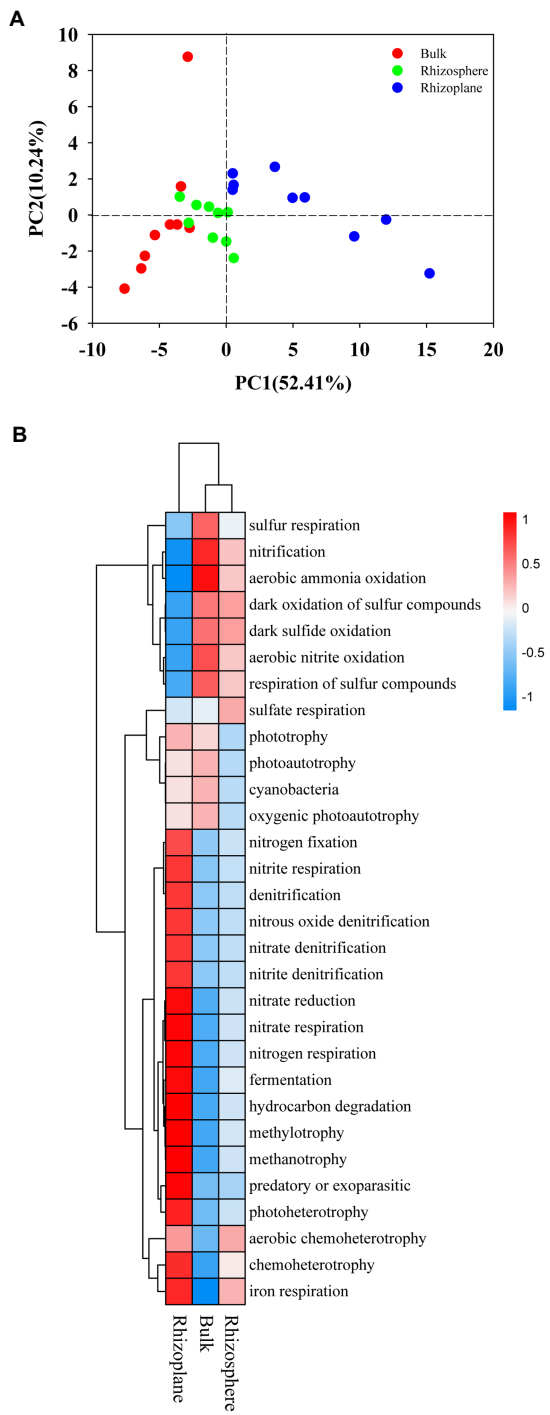

FIGURE 5
Principal Component Analysis (PCA) **(A)** and heatmap **(B)** of the soil bacterial community upon the functional groups predicted by FAPROTAX among all samples. BS, bulk soil; R1, rhizosphere soil; R2, rhizoplane soil.

(Edwards et al., 2015). It is possible that the host plant will select against the declining variety of the bacteria associated with the roots or that they will finally be outcompeted by other taxa (Edwards et al., 2018). In our study, rhizoplane had a lower relative abundance of Acidobacteria (Figure 2B), which decreased monotonically by soil pH changes. Fan et al. (2017) demonstrated that Acidobacteria were depleted in rhizospheric soil. Conversely, advantageous microbes that enhance nutrient acquisition and combat pathogenic taxa (Bull et al., 2005; János, 2005), e.g., Bacteroidetes and Actinobacteria, had enriched in rhizosphere and rhizoplane soils.

Our findings demonstrating the same patterns of βNTI values of various root compartment niches suggest that deterministic processes tend to dominate community assembly (Figure 3). Regarding this project, Fan et al. (2017) reported that most bacterial community assemblages in bulk soil, loosely bound soil, and tightly bound soil in wheat crop fields tended to be dominated by deterministic mechanisms. Moreover, it is also interesting to note that when the bacterial Shannon diversity indices level is low, deterministic mechanisms frequently predominate during community assembly (Xun et al., 2019). Recent research proposed that with decreased biomass and a smaller population, the community is more susceptible to drift (a stochastic process) or founder effects (Evans et al., 2017). Nevertheless, dispersal, which is dependent on the local environment, can have a significant impact on the local dominance of stochastic or deterministic processes in an established community with saturated population or community size (Vellend et al., 2007). Hence, the decreased bacterial diversity from the exterior to the surface of the root may be attributed to environmental selection.

Biological communities' ecological networks were lately widely used in plant and soil microbial ecology (Bastolla et al., 2009; Guan et al., 2020). Previous study has shown that microbial network complexity was impacted by farming systems, soil type, and ecological niches (Fan et al., 2017). Root compartment niches had an impact on the structure of the bacterial population in the soil as well as the pattern of bacterial co-occurrence. Our study showed that the network was more complexity in bulk soils compared with rhizosphere and rhizoplane soils (Figure 4), as evaluated by more nodes and stronger connections. This suggests that bacterial communities in bulk soils are more likely to be able to establish mutually beneficial communities and enhance resistance to external disturbances (Scheffer et al., 2012). In addition, more connected networks can use carbon more efficiently and improve nutrient exchange between various species (Morriën et al., 2017). This could mean that bacterial communities in bulk soil have more carbon-related energy sources, which is also reflected in bulk soils having more phyla increased. These findings are in line with those of Fan et al. (2017), who discovered that in wheat crop fields, bulk soil is more connected than tightly bonded and loosely bound soils. The distinction of topological among three compartments may be attributed the niche differentiation (Ma et al., 2015). In this sense, the interaction of soil physical–chemical properties and root-derived products, which shapes the niches and exerts niche pressures in the community assembly, has a significant impact on the observed niche-based selection of the rhizosphere microbiome (Mendes et al., 2014). Moreover, from the perspective of plants, community assembly in the rhizosphere is frequently seen as an increasingly plant-driven selection of microbial taxa from bulk soil to the rhizosphere and rhizoplane (Rüger et al., 2021). Therefore, a less complex network is anticipated in our study since the rhizosphere community is a subset of the bulk soil community (Mendes et al., 2014). Notably, rhizosphere soils had a considerably higher Shannon index than that of rhizoplane soils at all growth stages, and yet we observed a more complex network structure in rhizoplane soils. It has been demonstrated that the increase in the Shannon index of the bacterial community is accompanied by a more complex network structure (Pereira et al.,

2021; Yang F. et al., 2022). Microbial taxa in the rhizoplane soil ecotone have strong interactions with each other, which may contribute to the complexity of the bacterial network in rhizoplane soils (Li F. Q. et al., 2022). In addition, rhizoplane soils are also capable of recruiting enriched stress-resistant bacteria to combat external environmental stresses (Huang et al., 2022). Indeed, the number of associations that the community's taxa have with one another rather than the number of taxa in the community generally determines the complexity of the microbiome (Banerjee et al., 2019). Moreover, compared to bulk soils, the complexity of the co-occurrence network dropped considerably, while the number of negative co-occurrences in rhizosphere and rhizoplane soils increased, possibly indicating that these bacterial species are competing for resources (Fuhrman, 2009).

Keystone species are crucial to the microbiome and their absence would significantly change the composition of the microbiome (Berry and Widder, 2014; Banerjee et al., 2018). The Zi-Pi plot (Figures 4D–F) revealed that generalists inhabited a small percentage of modules, frequently less than 2% of all modules in soil bacterial networks (Barberan et al., 2012; Jiang et al., 2015). In the three networks, nodes' roles also changed. For example, OTU 1936, OTU 894, OTU 5378, OTU 1812, and OTU 6469 were found to be specialists in rhizoplane and rhizosphere soils networks but generalists in bulk soils networks, indicating that networks were well organized and that root compartment niches could change the topological roles of specific OTUs and important microbial populations Moreover, of the taxa identified as keystone taxa in rhizosphere soils, *Nitrospira* (OTU3326) might be related in nitrifying, indicating to a possible increase in nitrification in the rhizosphere inches (Ehrich et al., 1995; Ahmed, 2011). These generalists bridged various nodes between various modules or inside their own modules (Ling et al., 2016), As a result, these generalists might be able to form relationships with other species and control the nitrogen cycle in Mollisols.

Like the community structure, distinct functional cluster were found among three compartments. Changes in bacterial community structure may be a reflection of modifications in how that structure functions (Qu et al., 2020). According to our findings, the group capable of chemoheterotrophy was the primary predictors of soil bacterial functional structure, then followed by the groups of aerobic chemoheterotrophy, nitrification, and fermentation. These functional groups were associated with the carbon and nitrogen cycle in the soil (Liang et al., 2019), suggesting that changes in the soil's carbon and nitrogen pools may be the primary factor influencing the functional organization of soil bacteria (O'Donnell et al., 2001). Carbon and nitrogen cycles in the soil nutrient cycle are essential for sustainable agroecosystems (Enebe and Babalola, 2021). Our study found that the bacterial functional groups associated with the nitrogen cycle were enriched in bulk and rhizosphere soils, this may be due to variations in soil enzyme activity caused by changes in ecological niche. Yang et al. (2017) found that the activities of enzymes related to the nitrogen cycle (urease and protease) showed significant differences in bulk and rhizosphere soils by comparing the enzymatic activities of heavy metal contaminated bulk and rhizosphere soils in the Qinling region. Furthermore, the rhizoplane has been proved to be a specialized niche for some taxa (Edwards et al., 2015). The rhizoplane appears to play a key gating role in the process of microbiome acquisition in the root, as the initially recruited microorganisms are subjected to physically selective binding at the rhizoplane to the root interstitial (Edwards et al., 2015). Since rice cultivation is responsible for some degree of carbon loss (CH_4 emission) from farmland, rhizoplane may recruit more functional groups related to the carbon cycle to maintain plant growth requirements. The functional groups sulfur and nitrogen cycling processes in the bulk soils had considerably higher abundances than was discovered in the rhizoplane soils. However, the functional groups involved in the cycling of carbon and iron were more abundant in rhizoplane soils, suggesting that niches shift structure of the bacterial community from sulfur and nitrogen utilizing bacteria to carbon and iron cycle involving bacteria of rice in Mollisols.

Conclusion

Our work demonstrated that root compartment niches is the main factor affecting the structure of soil bacterial communities rather than rice life cycle, as evidenced by changes in bacterial diversity and composition. Proteobacteria, Chloroflexi, Bacteroidetes, and Acidobacteria may be the core taxa in bacterial communities and may be important in maintaining the networks of bacterial community interactions. In all three compartments, deterministic processes dominated the bacterial community assemblage, and all were dominated by heterogeneous selection. In comparison to rhizosphere and rhizoplane soils, bulk soils had a more sophisticated bacterial co-occurrence network, as evidenced by more nodes, more hubs, and stronger connections, which was mainly driven by compartment niches. In bulk soils, sulfur-and nitrogen-using bacteria predominated; in rhizoplane soils, however, there were greater abundances of bacteria involved in the cycling of carbon and iron. Overall, our data demonstrate the importance of the mechanism of ecological niche changes caused from the outside to the inside of roots in the structure of bacterial communities in Mollisols of Northeast China, rather than the rice life cycle. The rice life cycle may lead to changes in plant and soil metabolites, which requires the application of soil metabolomics to establish mechanistic links between soil metabolites and microbial communities, which still needs further exploration.

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

KL, MS, and QL: methodology. QW and SG: data processing. KL, LS, and JG: literature review. KL and MS: writing–original draft preparation. JB and SG: writing–review and editing. JB: supervision. KL and JB: funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1170611/full#supplementary-material

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EDITED BY
Xin Sui,
Heilongjiang University, China

REVIEWED BY
Yuanying Ma,
The University of Queensland, Australia
Lianjun Sun,
China Agricultural University, China

*CORRESPONDENCE
Lianxia Wang
✉ wlx0427@163.com
Dongwei Han
✉ handongwei126@126.com

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Salt altered rhizosphere fungal community and induced soybean recruit specific species to ameliorate salt stress

Ming Yuan[1], Di Zhang[1], Zhen Wang[1], Zhijia Zhu[1], Haoyue Sun[1], Wei Wang[2], Dezhi Han[3], Zhongcheng Qu[1], Bo Ma[1], Junqiang Wang[1], Lianxia Wang[1]* and Dongwei Han[1]*

[1]Qiqihar Branch of Heilongjiang Academy of Agricultural Sciences, Qiqihar, China, [2]Institute of Soil Fertilizer and Environmental Resources, Heilongjiang Academy of Agricultural Sciences, Harbin, China, [3]Heihe Branch of Heilongjiang Academy of Agricultural Sciences, Heihe, China

Different crop genotypes showed different adaptability to salt stress, which is partly attributable to the microorganisms in the rhizosphere. Yet, knowledge about how fungal communities of different genotypes in soybean respond to salt stress is limited. Here, qPCR and ITS sequencing were used to assess the response of rhizobial fungal communities of resistant and susceptible soybean to salt stress. Moreover, we isolated two fungal species recruited by resistant soybeans for validation. The assembly of fungal community structure might be strongly linked to alterations in fungal abundance and soil physicochemical properties. Salt stress derived structural differences in fungal communities of resistant and susceptible genotypes. The salt-resistant genotype appeared to recruit some fungal taxa to the rhizosphere to help mitigating salt stress. An increase of fungal taxa with predicted saprotrophic lifestyles might help promoting plant growth by increasing nutrient availability to the plants. Compared with the susceptible genotypes, the resistant genotypes had more stronger network structure of fungi. Lastly, we verified that recruited fungi, such as *Penicillium* and *Aspergillus,* can soybean adapt to salt stress. This study provided a promising approach for rhizospheric fungal community to enhance salt tolerance of soybean from the perspective of microbiology and ecology.

KEYWORDS

soybean, salt stress, fungal structure, network, recruitment

Introduction

It is well known that soil salinization is a considerable problem in agricultural system, and that soil salinity can greatly reduce plant productivity and yield value (Khasanov et al., 2023). Due to an increase in global population and the ever-increasing demand for food quality, the issue of how to alleviate the pressure of soil salinity, improve plant resistance to salt stress and eventually increase crop yields is an urgent need to be addressed. Soybean [*Glycine max* (Linn.) Merr.], an important source of protein and oil in the world, is very sensitive to salt stress, which can severely restrict nutrient use and growth and development, ultimately reducing yields (Phang et al., 2008). In the last few years, traditional breeding techniques combined with beneficial microorganisms have been widely used to improve the salt resistance of soybeans (Pathan et al., 2007; Hanin et al., 2016).

Different soybean varieties have different root exudates, which determines the composition of the plant-specific root and microbial communities in rhizosphere area (Bulgarelli et al., 2013; Lian et al., 2019a). Under salt stress, the amount and type of root exudates secreted by different species are different (Lian et al., 2020). It has been demonstrated that salt-resistant soybeans have a much greater salicin, arbutin 6-phosphate, phosphoglycolate, and 1-methlseleno-N-acetyl-dgalactosamine than salt-susceptible soybeans in soils, which may increase the salt adaptation of soybeans (Lian et al., 2020).

Microorganisms have the benefit of promoting health and increasing productivity in plants (Mendes et al., 2013; Li et al., 2014a,b). Different types and amounts of metabolites from plants or microorganisms could alter the diversity and structure of rhizosphere microbes, which could assist the host to become more resistant to stress (Wu et al., 2006; Qin et al., 2016; Hu et al., 2018; Lian et al., 2020). It is well known that plant growth promoting rhizobacteria (PGPB) have certain functions that can promote plant growth (Bhatt et al., 2022). For example, a variety of metabolites produced by *Pseudomonas* can lead to salt stress-relieving, including exopolysaccharides, ACC deaminase and hormones (indoleacetic acid and gibberellins; Etesami and Glick, 2020; Li et al., 2021). However, studies in recent years have largely emphasized on bacteria, neglecting fungal species, with the improvement in nutrient cycling and the resistance to environment, which can also assist plants to mitigate damage caused by abiotic stresses (Kawai et al., 2000; Peltoniemi et al., 2012). *Penicillium* and *Aspergillus*, which were reported to increase nitrogen and phosphorus to plant roots, stimulate the growth of host plants by increasing the accumulation of nutrient under unfavorable conditions (Kiers et al., 2011), and thus might help plant alleviate the biotic and abiotic stresses. Thus, to understand how salt-resistant soybean better adapt to salt stress, it is necessary to investigate how rhizosphere microbes of salt-resistant soybean genotypes respond to salt stress.

In this study, we selected the resistant soybean (Qinong7) or susceptible soybean (Hefeng50), growing at soils under salt and non-salt stress. Then, we analyzed the fungal community structure in rhizosphere through ITS high-throughput sequencing. Moreover, the fungal community structure was investigated in relation to its physicochemical properties. We hypothesized that (1) Salt-R genotype possesses higher fungal diversity compared to Salt-S genotype, and (2) Salt-R genotype will enrich particular Salt-R fungal taxa to the rhizosphere that help mitigating salt stress.

Materials and methods

Pot experiment and rhizosphere soil collection

Two different soybean (*Glycine max L.*) genotypes were shown to be resistant (Qinong7) or susceptible (Hefeng50) to salt stress. Soil collection was conducted in an agricultural field in Qiqihar (110°25′N, 21°32′E), Heilongjiang Province, China in June 2022. We conducted a pot experiment with six replicates in a greenhouse at Heilongjiang Academy of Agricultural Sciences, Qiqihar Branch, Qiqihar, China, in a completely randomized block design. A 4 mm mesh was used to sieve the soil. Eight seeds were sown in each pot and then two better seedlings were kept after the ninth day of sowing. The temperature range of the greenhouse was 16–20°C night-time temperature and 25–30°C daytime temperature. Each treatment was watered with 150 mM NaCl solution, with equal amounts of pure water as a control. Soil moisture content was 85% of field capacity.

Soil samples were collected with a shaking of the root at the flowering stage. Each replicate for each treatment, a microcentrifuge tube containing 8 g of soil was placed at −80°C for DNA extraction after shaking for 3 min. Soil physicochemical property analysis were performed on the remaining soil that stored at 4°C.

Soil properties admeasurement

Soil pH was measured using a pH metre. A VarioEL III elemental analyzer (Germany) was used to measure TN and TC contents in soil (Jones and Willett, 2006). Inductively coupled plasma atomic emission spectrometry (ICPS-7500, Shimadzu, Japan) was used to determine soil TK. A continuous flow analysis system (SKALAR SAN++, The Netherlands) was used to measure NH_4^+ -N and NO_3^- -N, TP and Olsen-P.

Molecular genetic analyses

Following the manufacturer's instructions, DNA was extracted using a E.Z.N.A. DNA Kit for Soil (Omega, United States). qPCR was conducted through the ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3′) and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3′) primers to measure the fungal abundance (Jobst et al., 1998). PCR amplification system were as follows, with 15 μL of 2 × KAPA HiFi Mix, the forward and reverse primers (0.2 μM), and 0.5 ng of template DNA in a volume of 30 μL. PCR reaction cycling conditions were followed by 3 min at 95°C for one cycle, 30 s at 95°C, 30 s at 55°C, 15 s at 72°C for twenty-five cycles, and then 5 min at 72°C for thermal extension.

For next-generation sequencing, the hypervariable ITS region of fungal was amplified by ITS1F/ITS2R primers. Using an Illumina MiSeq platform, standard protocols were followed to paired-end sequence the pooled-purified in equimolar amounts of amplicons. PCR products were used to create sequencing libraries and then paired-end sequences were carried out on the Illumina MiSeq platform. Raw sequences were uploaded in the NCBI with accession number of PRJNA918498.

Bioinformatic processing

Raw FASTQ files obtained by sequencing were subsequently used for processing in the QIIME Pipeline (version 1.19.1; Pauvert et al., 2019). To be brief, each sample was allocated to obtain a certain number of sequences reads, which were then quality filtered and chimeras were removed by UCHIME (Edgar et al., 2011). Based on the best match for the sequences in the RDP database, sequences were assigned phylogenetically by the RDP classifier (Wang et al., 2007). Amplicon sequence variants (ASVs) were classified using CD-HIT with 97% of sequence similarity (Li and Godzik, 2006). α-diversity (Chao1 richness and Shannon diversity) was done using QIIME.

Principal coordinate analysis (PCoA, Bray-Curtis's dissimilarities), nonparametric multivariate analysis (PERMANOVA), were performed using the *aMDS* and *adonis* functions of the "vegan" package in R (version 4.0.2), respectively. Canonical correspondence analysis (CCA) and Mantel test were performed using *cca* and *mantel* functions of the "vegan" package in R, respectively (Anderson, 2001; Tierney, 2012; Oksanen et al., 2015). Fungal phyla relative abundance was indicated by the "circlize" package (Gu et al., 2014). Differential abundance analysis of 438 ASVs was carried out using generalized linear models and likelihood ratio tests to identify significant ASVs that caused the structural segregation of fungal communities across genotypes. ASVs that were co-enriched and unique across different salt treatments and genotypes were identified using Venn analysis (Yin et al., 2022). Statistical analyses were done by SPSS with Duncan tests at 95% confidence level ($p < 0.05$) (version 24.0, IBM, United States).

To understand the relationships in the fungal communities for each ASV, co-occurrence network analysis was performed in this study. ASVs with relative abundance exceeding 0.05% were to be selected for calculating the Spearman's rank correlation coefficient between them. The standard for the determination of statistically significant correlation between ASVs was Spearman's correlation coefficient more than 0.8 and $p < 0.05$ (Shi et al., 2020). Those nodes in the network were assigned at the phylum level of the fungus, and correlations were indicated by different colored edges, i.e., red was positive and green was negative. A series of indices were employed to evaluate the stability and complexity of the network, including graph density, the average weighted degree (avgK), the number of positive correlations, and modularity (M). The ASVs that with high betweenness centrality and high degree were consider as keystone species (Shi et al., 2020). After the statistical analyses completed, the Gephi was used for visualizing (Parente et al., 2016).

Isolation of fungal species and their effect on the soybean

Microorganisms enriched in soybean grown under salt stress were considered to be salt-resistant. Samples of the rhizosphere were obtained by resuspension with 1 x phosphate buffer saline (PBS) (2.0 g of sample per 10 mL of PBS). The rhizosphere soil suspension was diluted to 10^{-6} and 100 μL dilutions and was plated to the fungal medium. 0.1 M NaCl was then added to the homogenate as an inoculum for microbial enrichment cultures via R2A liquid medium (at 30°C, 150 rpm on a rotary shaker for 48 h). After incubation for no more than 2 weeks, single colonies were selected from plates with no more than 20 colony forming units and subjected to ITS gene analysis. Then the single colonies were inoculated into susceptible soybeans at an inoculate level of 2×10^5 per plant and soybean growth was observed 30 days after sowing.

Results

Biomass in soybean, fungal abundance, and diversity

Salt stress limited the increasement of soybean biomass in both genotypes and was more suppressive to Salt-S biomass than Salt-R (Figure 1A). In terms of fungal abundance, varying from 5.03 to 11.14×10^7 copies g/dry soil, salt stress showed a significant decrease in the abundance of both genotypes and a higher abundance in Salt-R (Figure 1B, $p < 0.05$). Interestingly, salt stress exhibited no significantly effect on fungal Shannon diversity and Chao1 richness of Salt-R and Salt-S genotypes (Figure 2, $p > 0.05$).

Fungal community structure in the rhizosphere

There were 985,254 high qualities filtered fungal ITS1 sequences with a read number range of 52,531 ~ 96,735. Clustering yielded a total of 438 fungal ASVs. The fungal community structure was significantly dissimilar at the four treatment levels, but had a stronger tendency to detach under salt stress conditions rather than genotypes (Figure 3A). The fungal community members were assigned into six dominant phyla (Figure 3B). Ascomycota and Basidiomycota, followed by Chytridiomycota, Glomeromycota, Mortierellomycota and Rozellomycota were the main phyla across the treatments. In detail, for these six fungal phyla, relative abundance of Ascomycota was decreased with the salt stress, while the relative abundance of Mortierellomycota and Basidiomycota were increased in both Salt-R and Salt-S. Moreover, the relative abundance of Mortierellomycota was markedly higher in Salt-R than in Salt-S under salt stress.

Using differential abundance analysis, each 12 ASVs were significantly enriched in Salt-R and Salt-S genotypes under salt stress, when compared to the control (Figure 4). Among them, four ASVs that ASV1 (*Saitozyma*), ASV6 (*Idriella*), ASV29 (*Talaromyces*) and ASV80 (*Cladosporium*) were specially enriched to Salt-R genotype soybean, while four ASVs that ASV44 (*Saitozyma*), ASV63 (*Cladosporium*), ASV75 (*Candida*) and ASV96 (*Gliomastix*) were specially enriched to Salt-S genotype soybean (Supplementary Tables S1, S2). Furthermore, eight ASVs that ASV7 (*Talaromyces*), ASV9 (*Saitozyma*), ASV17 (*Wallemia*), ASV19 (*Aspergillus*), ASV21 (*Wallemia*), ASV24 (*Saitozyma*), ASV32 (*Candida*) and ASV38 (*Papiliotrema*) were significantly enriched at both Salt-R and Salt-S genotypes (Supplementary Table S3).

The correlation linkages with soil physicochemical properties and fungal community structure were evaluated using CCA and Mantel test (Figure 5). The results indicated that the fungal communities of Salt-R and Salt-S genotypes showed significant correlations with certain soil physicochemical properties, including Na^+, Olsen-P, pH, NH_4^+ and NO_3^-.

FUNGuild analysis of rhizosphere soil fungi

All filtered ASVs were categorized into 10 guilds by FUNGuild (Table 1). With the exception of "Ectomycorrhizal," "Fungal Parasite," and "Animal Pathogen," the remaining guilds differed significantly in terms of salt stress and genotypes. "Wood Saprotrophs" were the largest guild with 251 ASVs affiliated to Ascomycota and Basidiomycota, accounting for 32.6–51.8% in different genotypes. "Wood Saprotroph," "Plant Pathogen," "Endophyte" and "Soil Saprotroph" were significantly increased in the salt R genotype under the salt stress.

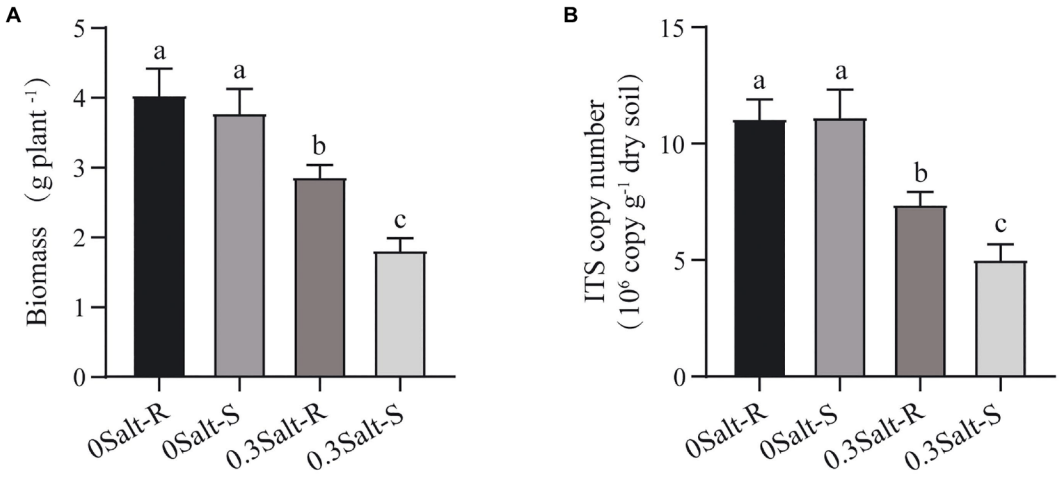

FIGURE 1
Effects of salt stress on soybean biomass **(A)**, and rhizospheric fungal ITS copy number **(B)** Salt-S and Salt-R were significant differed by one way ANOVA using Student's t-tests (p <0.05). The standard error of the biological repetition mean is shown through error bars (n =6). Salt-R: salt-resistant soybean genotype; Salt-S: salt susceptible soybean genotype.

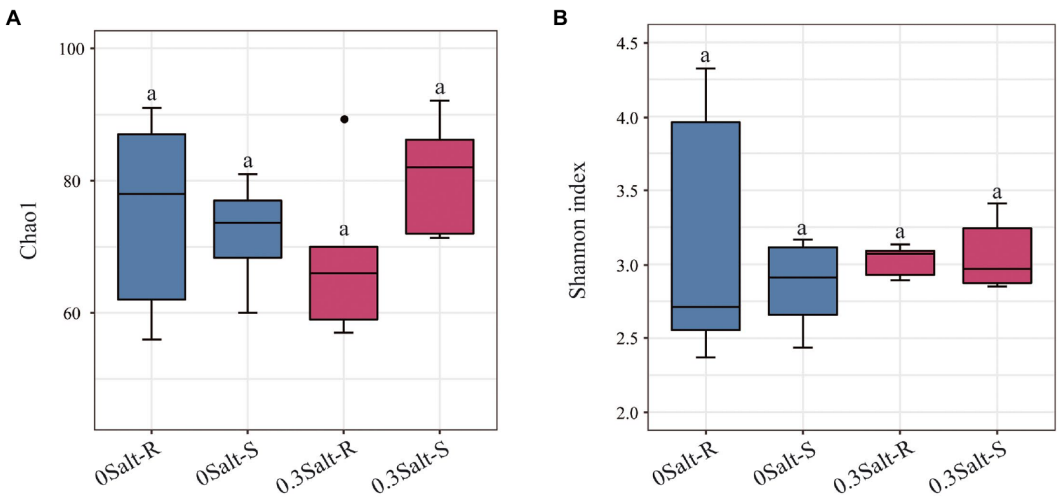

FIGURE 2
Effects of salt stress on Chao1 value **(A)** and Shannon index **(B)** of soybean rhizosphere. Salt-S and Salt-R were significant differed by one way ANOVA using Student's t-tests (p <0.05). The standard error of the biological repetition mean is shown through error bars. (n =6). Salt-R: salt-resistant soybean genotype; Salt-S: salt susceptible soybean genotype.

Effect of salt on the fungal networks

The co-occurrence network analysis of fungi in both genotypes showed that the network structure was significantly different by salt stress conditions (Figure 6; Table 2). Specifically, the number of nodes and positive correlations and average weighted degree (avgK) decreased in Salt-T genotype under salt stress, while the opposite trend was observed in Salt-S genotype. Interestingly, modularity (M) and average path length (APL) increased in Salt-T genotype and decreased in Salt-S genotype. Taken together, the salt-S genotype had a more intricate and stable network structure with respect to the salt-R genotype under salt stress. Degree of node, closeness centrality and betweenness centrality were the main indicators for identifying key ASVs (Table 3). For example, ASV45 (*Talaromyces*) and ASV8 (*Aspergillus*) were determined as keystones for the Salt-R genotype when exposed the salt stress.

Effect of *Talaromyces* and *Cladosporium* on the soybean under salt stress

More than 100 fungal clones were isolated and 15 strains were identified from the Salt-tolerant rhizosphere soil. Further comparing these 15 strains with the sequencing results, we found two strains, i.e., *Talaromyces* and *Cladosporium,* whose relative abundance was increased in the resistant genotpye. Pouring the fungal solution on the roots of soybean significantly increased the shoot and root biomass of soybean. In general, *Talaromyces*

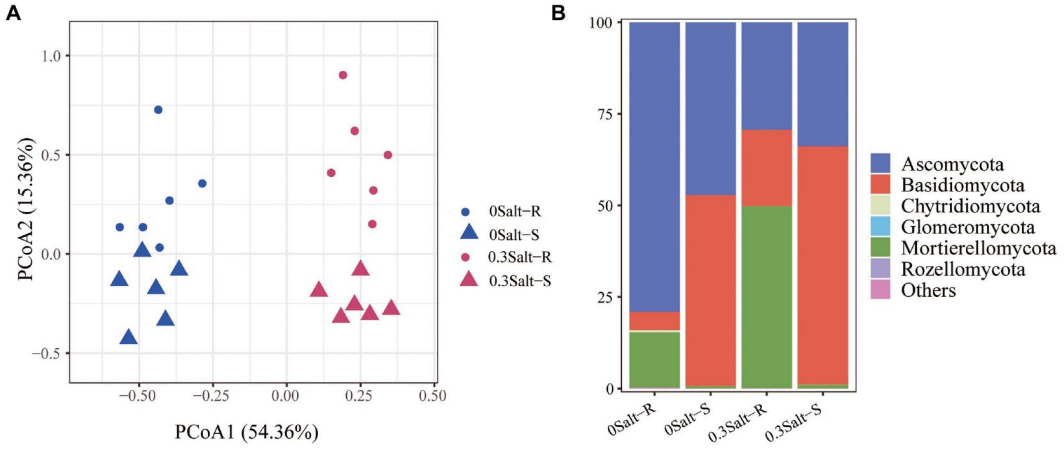

FIGURE 3
Principal co-ordinates analysis (PCoA) reveals the significant differences in the rhizospheric fungal community structures with and without salt stress **(A)**. The PERMANOVA F-ratios and *p*-values (n=6) for the factor genotype are provided in the corner of the plots. Bar chart for calculating relative abundance of soybean rhizospheric fungal community on dominant phyla **(B)**. Salt-R: salt-resistant soybean genotype; Salt-S: salt susceptible soybean genotype.

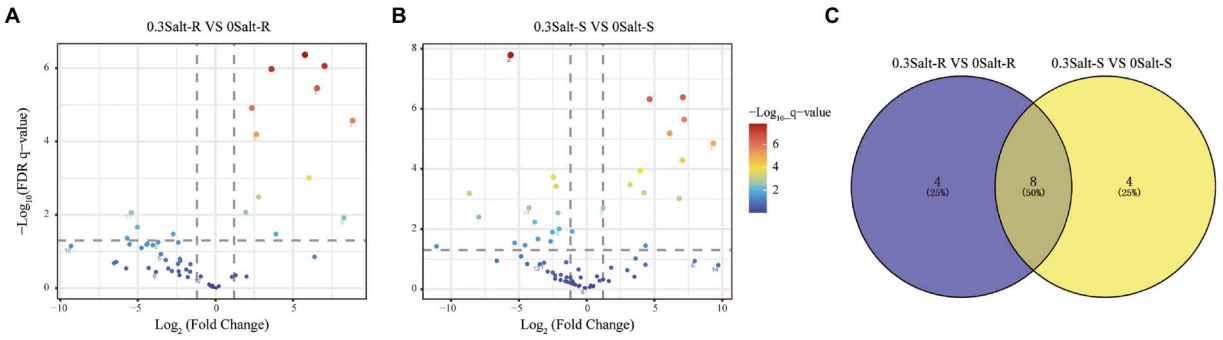

FIGURE 4
Enrichment and depletion of ASVs of Salt-R **(A)** and Salt-S **(B)**. Each point represents an independent ASV. Venn analysis was used to count the number of shared and unique enriched ASVs in the two comparison groups mentioned above **(C)**. Salt-R: salt-resistant soybean genotype; Salt-S: salt susceptible soybean genotype.

increased the shoot and root biomass of Salt-S soybeans by 21.9 and 19.7%, respectively, and *Cladosporium* increased the shoot and root biomass of Salt-S soybeans by 26.9 and 29.5%, respectively (Table 4).

Discussion

This study was conducted to reveal how salt stress affects the structure of the rhizospheric fungal community of salt-tolerant (Salt-R) and susceptible (Salt-S) soybean genotypes. In comparison to the Salt-S genotype, the fungal communities of the Salt-R genotype were higher in abundance and significantly different in structure, but not in diversity (Figures 1–3). The amount and type of root exudates secreted by different soybean genotypes was variable, which led to a diverse response to salt stress (Li et al., 2021). Therefore, Salt-R genotypes, except for secreting abundant organic acids directly to dilute NaCl, might also enrich some fungi with ability to secrete organic acids around the rhizosphere, thus increasing their resistance to salinity (Lian et al., 2020). Notably, salt stress usually amplified the segregating trend in fungal community structure between resistant and susceptible genotypes, which was in accordance with the observations of Lian et al. (2020) and Lian et al. (2020).

The composition of the rhizospheric fungal community was greatly altered by salt stress in both genotypes (Figure 3A). This was in line with previous studies that fungal community structure was influenced by the complicated effects of saline alkaline soil environments (Yao et al., 2021). There was also, however, genotype-dependance in the fungal community structure with non-salt stress (Figure 3A), which was in contrast with previous study (Wang et al., 2008). Wang et al. (2008) reported that fungal communities were not found to be significantly different among the three genotypes at the same growth stage (Wang et al., 2008). This phenomenon might be explained by the lower methodological resolution to test fungal communities (Gomes et al., 2003). It was possible that differences in soil physicochemical properties directly contributed to changes in the structure of fungal communities under salt stress (Figure 5). Moreover,

Na^+, Olsen-P, NH_4^+, NO_3^- and pH were the most important factors that shaping the rhizosphere microbial community.

A number of studies have demonstrated that salt resistant genotypes had the ability of secreting some special root exudates to make plants more adapted to salt stress (Innes et al., 2004; Lian et al., 2020). We have previously shown that the resistant genotype can recruit beneficial bacteria and hypothesize that the same is true for fungi (Lian et al., 2019a). The relative abundance of several fungal taxa in the rhizosphere of Salt-R was higher compared to Salt-S under salt stress like *Talaromyces*, *Saitozyma* and *Cladosporium*. Moreover, *Talaromyces* and *Cladosporium* isolated from the rhizosphere soil were verified that significantly increased the shoot and root biomass of soybean (Table 4). It has been previously revealed that *Talaromyces* was able to solubilize phosphate at salinity and thus showed high tolerance to salt stress (López et al., 2020). Thus, *Talaromyces* may be a key species for improving salt tolerance in soybean. However, the other five genera have not yet been found to be associated with soil salt stress and their role needs to be investigated in more detail.

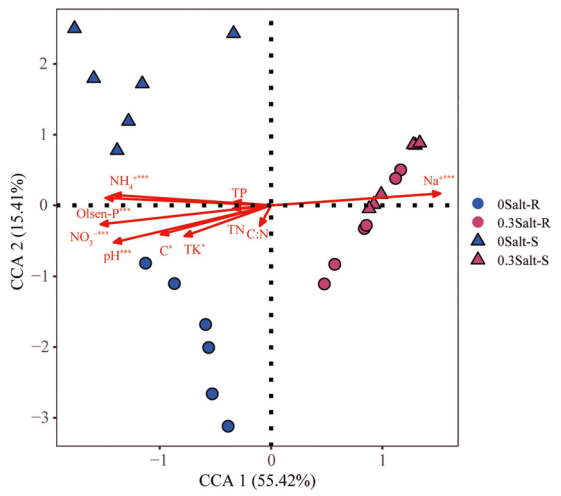

FIGURE 5
Canonical correspondence analysis (CCA) showed the relationship between rhizospheric fungal community structure and soil physicochemical factors.

The two genotypes also showed differences in the relative abundance of specific trophic groups (Table 1). Saprotrophs were the dominant trophic mode in the present study. Saprotrophs, as the dominant guild, had the highest relative abundance in the Salt-R genotype under salt stress. It has reported that fungi belonging to saprotrophs might have an essential function in promoting nutrient conversion and controlling plant pathogens (Lian et al., 2019b). The increased abundance of saprotrophs is again directly linked to the presence of different *Talaromyces* species. Previous studies have well established that *Talaromyces* could facilitate plant growth through better utilization of nutrients by plants (Shi et al., 2022).

Co-occurrence network analysis revealed that network properties were inherently different among salt-resistant and susceptible genotypes (Figure 6; Table 2). Compared to Salt-R genotypes, there was fewer negative correlations and higher modularity in fungal networks of Salt-S genotypes under salt condition, according to network theory, probably because of weaker competitive relationships between microbial species within the rhizosphere (Saavedra et al., 2011; Fan et al., 2018). Additionally, Salt-R genotype exhibited a higher number of positive correlations than Salt-S genotype under salt stress, suggesting that most fungal members were connected through a series of cooperative relationships (Coyte et al., 2015; de Vries et al., 2018). However, this network structure was considered unstable because fungal members might be strongly influenced by environmental fluctuations, thus increasing unstable coupling (Coyte et al., 2015; de Vries et al., 2018). In addition, core species served as a critical pointcut to analyze how to alleviate salt stress (Table 3). For example, ASV23, ASV8, and ASV14 were identified as *Aspergillus*, which alleviated salt stress by producing organic acids to form organic acid-salt complexes (Ali et al., 2021). However, rhizosphere microbes also include bacteria, which can help soybeans resist salt stress by releasing hormones and promoting plant nutrient uptake, among other things, which cannot be ignored (Li et al., 2021). Bacteria should be explored in

TABLE 1 Functional potentials of rhizospheric fungal community based on different salt stress and genotypes.

	0Salt-R	0Salt-S	0.3Salt-R	0.3Salt-S	*p* value
Wood Saprotroph	32.550 ± 4.795 c	36.687 ± 7.506 bc	51.808 ± 10.079 a	45.132 ± 13.642 ab	0.0107*
Undefined Saprotroph	33.873 ± 7.145 a	38.698 ± 10.415 a	17.040 ± 7.846 b	28.675 ± 7.037 a	0.0013**
Plant Pathogen	6.841 ± 4.219 b	4.289 ± 1.536 b	15.189 ± 10.558 a	6.4263 ± 3.828 b	0.0268*
Endophyte	1.418 ± 1.141 b	0.914 ± 0.312 b	8.679 ± 5.411 a	7.621 ± 5.495 a	0.003**
Ectomycorrhizal	0.244 ± 0.191 a	0.311 ± 0.218 a	0.371 ± 0.323 a	0.721 ± 0.645 a	0.1824
Fungal Parasite	0.379 ± 0.230 a	0.542 ± 0.188 a	0.952 ± 0.761 a	1.067 ± 0.850 a	0.1708
Soil Saprotroph	0.382 ± 0.637 b	2.279 ± 2.813 ab	3.591 ± 1.753 a	4.341 ± 1.519 a	0.0077**
Animal Pathogen	0.371 ± 0.287 ab	0.180 ± 0.081 ab	0.091 ± 0.0590 b	0.437 ± 0.414 a	0.1006
Arbuscular Mycorrhizal	0.007 ± 0.009 b	0.034 ± 0.021 a	0.003 ± 0.004 b	0.013 ± 0.018 b	0.0094**
Dung Saprotroph	0.128 ± 0.141 ab	0.213 ± 0.146 a	0.023 ± 0.017 b	0.027 ± 0.027 b	0.0125*
Unknown	23.800 ± 8.307 a	15.847 ± 3.914 b	2.248 ± 1.260 c	5.536 ± 2.435 c	0.0001***

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

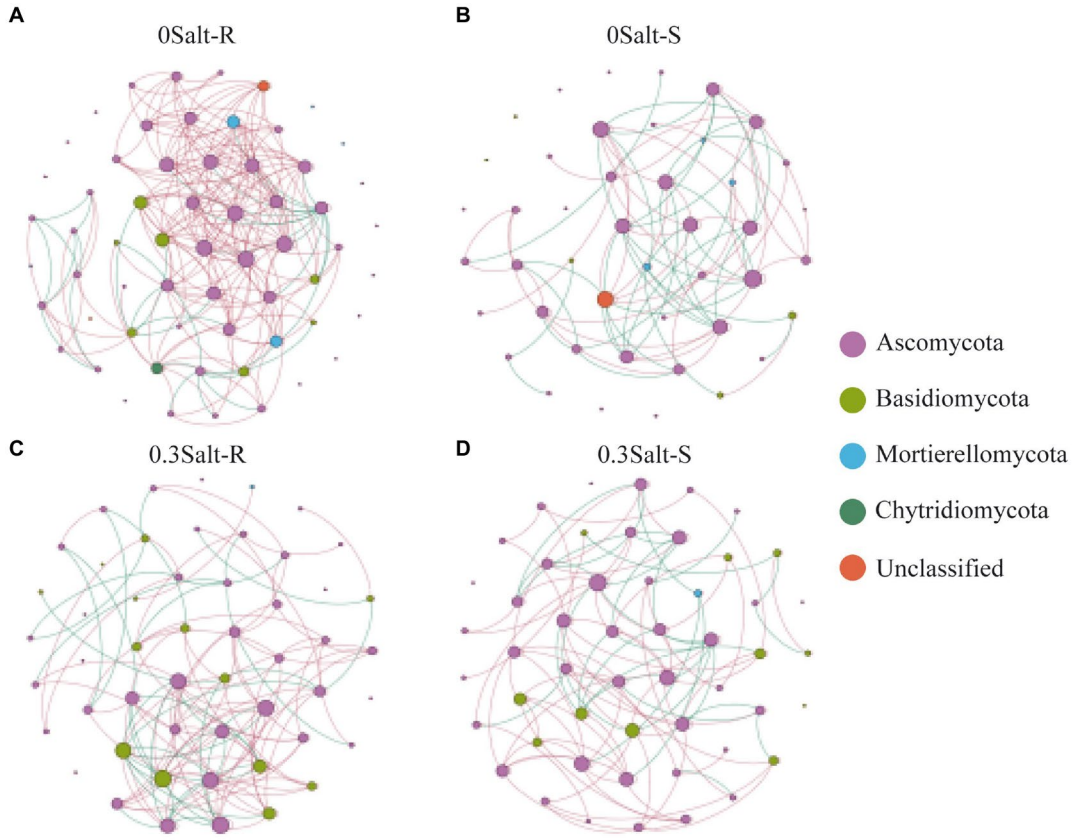

FIGURE 6
Co-occurrence network analysis of rhizospheric fungal communities. 0 Salt-R **(A)**, 0 Salt-S **(B)**, 0.3 Salt-R **(C)**, and 0.3 Salt-S **(D)**. OTUs are indicated by nodes, coloring-coded in phyla, and degree is indicated by node size. Lines shown in red indicate positive correlation (r >0.8), lines shown in green indicate negative correlation (r <−0.8), and p <0.05. Salt-R: salt-resistant soybean genotype; Salt-S: salt susceptible soybean genotype.

TABLE 2 Network characteristics of rhizospheric microbial networks among different treatments.

Network characteristics	0Salt-R	0Salt-S	0.3Salt-R	0.3Salt-S
Number of nodes	61	44	51	51
Number of edges	276	119	170	153
Number of positive correlations	242	82	130	118
Number of negative correlations	34	37	40	35
Average path length	2.68	3.34	3.19	3.26
Graph density	0.15	0.13	0.13	0.12
Network Diameter	7	7	7	6
Average clustering coefficient	0.59	0.66	0.63	0.58
Average weighted degree	10.57	2.04	9.34	4.24
Connecting components	15	12	9	6
Modularity	0.40	14.85	1.29	1.46

future studies and analyses in conjunction with fungi to explore the synergistic role of different microbial communities in helping the host to resist stress.

In conclusion, the rhizospheric fungal community of the two genotypes differed under salt stress. The Salt-R genotype recruited salt-resistant fungal species to the root zone to help alleviating salt stress. Different co-occurrence structure of the fungal community associated with the resistant genotype indicate more complex along with environmental changes. Taken together, the study provides new evidence for the important role of the soybean rhizosphere microbiome in conveying resistance to salt stress. In the future, the rhizospheric fungal community could serve as a promising breeding strategy to select for plants that are more resistant towards different stresses.

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TABLE 3 Keystones of the networks of different treatments.

Genotype	ASV	Degree	Closness centrality	Betweeness centrality	Phylum	Class	Order	Family	Genus	Species
0Salt-R	ASV12	19	0.505	74.348	Ascomycota	Sordariomycetes	Sordariales	Chaetomiaceae	*Humicola*	*Humicola_olivacea*
	ASV26	19	0.517	70.091	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*	*Talaromyces_neofusisporus*
0Salt-S	ASV20	12	0.444	70.288	Ascomycota	Saccharomycetes	Saccharomycetales	Phaffomycetaceae	*Barnettozyma*	*Barnettozyma_californica*
	ASV23	11	0.475	103.343	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus_pseudodeflectus*
0.3Salt-R	ASV45	14	0.423	74.713	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Talaromyces*	*Talaromyces_helicus*
	ASV8	13	0.410	115.182	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus_pseudodeflectus*
0.3Salt-S	ASV114	11	0.366	116.255	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus_sepultus*
	ASV23	8	0.353	155.681	Ascomycota	Eurotiomycetes	Eurotiales	Aspergillaceae	*Aspergillus*	*Aspergillus_pseudodeflectus*

TABLE 4 Effect of *Talaromyces* and *Cladosporium* on the soybean biomass under salt stress.

Treatment	Shoot biomass	Root biomass
Control	30.10 ± 2.5	10.23 ± 1.51
Talaromyces	36.7 ± 2.18	12.25 ± 0.4
Cladosporium	38.2 ± 1.79	13.25 ± 1.02
p value	0.0025**	0.021*

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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

DWH, MY, and LW: conceptualization, investigation, writing – review and editing, visualization, and supervision. MY, DZ, ZW, and ZZ: methodology. MY, HS, WW, and DZH: software. ZQ and BM: validation. JW: formal analysis. MY and DZ: data curation. MY: writing – original draft preparation, project administration, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1142780/full#supplementary-material

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