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# Combination of biochar and PGPBs amendment suppresses soil-borne pathogens by modifying plant-associated microbiome

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## Abstract

Consecutive monoculture regimes lead to the emergence of soil-borne diseases, which in turn impair plant growth and soil health, and restrict sustainable agricultural production. Biochar and plant growth-promoting bacteria (PGPBs) amendment in soil is considered a potential strategy for reducing soil-borne diseases. However, the combined effects of biochar and PGPBs on plant microbiomes and the subsequent consequences on plant performance remain largely unexplored. Here, we investigated the mechanisms on how biochar and biochar combined with a *Bacillus* synthetic community (SynComm) alleviated replant disease in *Radix pseudostellariae* by modulating rhizosphere soil protistan and plant microbial communities based on lab and field experiment. First, we found that biochar with *Bacillus* SynComm treatment increased the abundance of beneficial *Bacillus* spp. and *Pseudomonas* spp. after exposure to *Fusarium oxysporum*. Field experiments showed that biochar alone and combined with *Bacillus* SynComm improved the physiological traits and main components of *Radix pseudostellariae*. Besides, we found both two treatments increased *Bacillus* abundances in the root and soil, and significantly decreased *Fusarium oxysporum* density in the three compartments. Last, we validated that the combined treatment significantly decreased the relative abundance of pathogenic *Ralstonia*, promoted the abundance of *Bacillus* in the root and *Paenibacillus* in the leaf, and increased the abundance of the predatory protists *Cercomonas* and *Paracercomonas* in the soil. In addition, the combined amendment significantly increased the relative abundance of parasitic protists and decreased the plant pathogens. Meanwhile, the abundance of soil parasitic protists exhibited a significantly negative relationship with *F. oxysporum* density, and positive relationship with *Bacillus* density. Especailly, soil pH and $NO_3^-$ -N had a significantly indirect and positive effect on the parasitic protists and biomass by influencing bacterial richness of *R. pseudostellariae* leaf and root. Overall, our results revealed that amendment with combined biochar and *Bacillus* SynComm efficiently improved soil-borne disease suppression and plant physiological parameters by remodeling the plant and rhizosphere microbiome. This study provides a practical basis for more sustainable agriculture by promoting plant health through the modulation of plant and soil microbiomes.

***Key words*:** Replant disease; Medicinal plant; Soil micro-food web; Allelopathy; Sustainable agriculture

## 1. Introduction

Consecutive monoculture practices have contributed to meeting the food and production demands of a rapidly growing population density (Wu et al., 2022a). However, such consecutive monoculture systems commonly suffer from severe soil sickness or replant diseases, leading to significant damage to crop quality and productivity of crops (Peruzzi et al., 2017; Wu et al., 2022c). Previous studies have suggested that consecutive monoculture cultivation of crops alters the physicochemical properties and microbial community composition in the rhizosphere soil, decreases the abundance of plant-beneficial microbes, accumulates soil-borne plant pathogens and viruses, and subsequently negatively affects soil health, eventually limiting crop growth and yield (Wu et al., 2016; Cesarano et al., 2017; Peruzzi et al., 2017; Wu et al., 2022b). Substantial evidence indicates that the application of biochar and plant growth-promoting bacteria (PGPBs) could provide a rhizosphere amendment solution to improve soil health and reduce soil-borne diseases with lower environmental impacts than chemical fertilizers and pesticides (Latini et al., 2019; Tesfaye et al., 2021; Wu et al., 2022a; Wu et al., 2022c).

In this context, biochar, with its high stable carbon content, high alkalinity, high porous structure and surface area, has been widely applied to improve soil fertility, contaminated soil remediation, soil carbon sequestration, and plant disease suppression (Dai et al., 2021). Previous studies have shown that biochar amendment enhances plant physiological parameters and suppresses plant diseases by altering soil physicochemical properties and influencing the soil biotic community (Jaiswal et al., 2014; Wu et al., 2020; Dai et al., 2021). Furthermore, PGPB application enhances disease suppression and increases the abundance of beneficial microbiota, with subsequent beneficial effects on soil and crop health (Tao et al., 2020; El-Saadony et al., 2022). Our previous results showed that biochar and the combination of biochar and PGPBs significantly alleviated soil sickness in *Radix pseudostellariae* plantations by decreasing the abundance of soil pathogens and promoting the proliferation of potentially beneficial microbiota (Wu et al., 2020; Wu et al., 2022a). To date, most studies have focused on examining the ability of biochar to decrease plant disease by altering soil physicochemical properties and microbial community composition (Dai et al., 2021; Zhao et al., 2022). However, the potential effects of biochar on soil protists and plant-associated microbial communities are poorly understood.

Plant health and growth are intimately modulated by the microbiome, which includes diverse assemblies of bacteria, fungi, viruses, protists, and several other microorganisms. The plant microbiota contributes to several plant functional traits, including nutrient absorption and plant development, and is important for preventing or fostering diseases (Sessitsch et al., 2019; Wu et al., 2021; Bai et al., 2022; Malacrinò et al., 2022). The complexity of the interactions within the microbial network (Berry and Widder, 2014; Corrochano-Monsalve et al., 2021) and microbial community assembly processes (Wen et al., 2022) have both been reported to influence the susceptibility of plants to soil-borne pathogens. Our previous results showed that leaf and root microbiomes play important roles in both the yield and quality of *R. pseudostellariae* (Wu et al., 2022c). In particular, soil protists mainly feed on bacteria and fungi, thereby influencing the structure and dynamics of the rhizosphere microbiome (Chandarana and Amaresan, 2022). Protistan communities have been proposed as bioindicators of soil quality, and are influenced by numerous soil properties and environmental factors (Dupont et al., 2016; Asiloglu et al., 2021a; Guo et al., 2021). For instance, soil protists can improve and increase plant health and productivity by directly feeding on plant pathogens and favoring the growth of beneficial plant microorganisms (Xiong et al., 2020; Chandarana and Amaresan, 2022). Different fertilizer applications can influence soil protistan communities more than soil fungal or bacterial communities (Zhao et al., 2019). However, we still do not know whether the application of biochar alone or in combination with PGPBs can alter the plant microbiota and soil protist community, and whether this has functional consequences on plants.

Considering the positive effects of biochar (both alone and in combination with PGPBs) on soil and plant health, we hypothesized that these beneficial effects would span different components of the soil and plant microbiomes, including bacteria, fungi, and protists. Consequently, this effect would influence plant-microorganism-soil interactions, with beneficial effects on plant physiological properties and the abundance of pathogens and beneficial microorganisms associated with plants. Thus, in this study, we aimed to evaluate the effects of biochar and a combination of biochar and *Bacillus* spp. on plants and soil microbiome under consecutive monocultures, and quantify the effects of both treatments on soil physicochemical properties, plant physiological characteristics, and the complex interactions within microbiomes in the rhizosphere soil, roots, and leaves of *R. pseudostellariae*.

## 2. Materials and Methods

### *2.1. The antagonistic activity of different Bacillus sp. communities against Fusarium oxysporum*

Two types of culture medium were prepared: 1/4 PDA and 3% biochar-1/4 PDA (w/v). In each Petri dish, the culture medium was divided into two parts: treatment with 1/4 PDA-biochar and control with 1/4 PDA. Ten *Bacillus* species (*B. megaterium*, *B. pumilus*, *B. aryabhattai*, *B. subtilis*, *B. pseudomycoides* and *B. toyonensis*) were cultivated in Luria broth (LB) until they reached an $OD_{600}$ of 0.7. Bacterial cultures were centrifuged, washed with sterile water, and suspended in an equal volume of sterile water. We then generated eight *Bacillus* synthetic communities (SynComms) by pooling equal amounts of 2, 4, 5, 6, 7, 8, or 9 randomly selected isolates, or all 10 strains (Tab. S1). Then, 4μL of each SynComm was streaked in the middle of each half of the Petri dish 2 cm from the center. *Fusarium oxysporum* was inoculated into the center of the Petri dish. The culture media were incubated at the 37°C for two days and then at 30°C for five days.

### *2.2. Microcosm experiment*

The microcosm experiment was conducted at the Anhui Agricultural University, Anhui Province, China (117°27′E, 31°85′N). The rhizosphere soil of *R. pseudostellariae* was selected in a field consecutively planted with *R. pseudostellariae* for two years. The pot experiments included three treatments: untreated soil (PTY), soil inoculated with biochar (PTYB), and soil inoculated with biochar and the selected *Bacillus* Syncomm (PTYBB). Each pot contained 650 g of soil and each group was replicated in four pots. The Biochar was generated via anoxic pyrolysis of rice hull at temperature of 500°C (Wu et al., 2022a).

Biochar was mixed with soil (3% w/w) in PTYB and PTYBB treatments. In the second week of the experiment, 10 ml *Bacillus* SynComm was added to the PTYBB treatment. *Bacillus* SynComm was prepared using all 10 isolates, as reported above. In the third week of the experiment, *Fusarium oxysporum* f. sp. *R. pseudostellariae* spores were added to the assigned pots at a concentration of $1\times10^6$ spores/g of soil. The pot incubated in a greenhouse at day/night temperatures 26°C/18°C. The soils of the three treatments were collected after 0, 1, 2, 3, 4, and 5 weeks from the start of the experiment. The soil samples were stored at −80 °C immediately after collection.

### *2.3. Field experiment and sampling*

Field experiments were conducted in Zherong City, Fujian Province, China (119°55′E, 27°17′N) in a field planted with *R. pseudostellariae* for two consecutive years. The field experiments included four treatments: a field never previously cultivated with *R. pseudostellariae* (CK), *R. pseudostellariae* consecutively planted in a two-year monoculture field (TY), *R. pseudostellariae* consecutively cultivated in a two-year monoculture field with biochar addition (TYB), and *R. pseudostellariae* consecutively cultivated in a two-year monoculture field with a combination of biochar and *Bacillus* SynComm (TYBB). We used *Radix pseudostellariae* variety "Zheshen 2" for this study.

The field experiment was conducted using a completely randomized block design, and contained five replicate plots for each treatment. Biochar and *Bacillus* SynComm were generated as described above. The TYB treatment was amended with biochar at a dose of 1 t $ha^{-1}$ in the two-year monoculture field. The TYBB field was amended with PGPB-loaded biochar (the biochar was blended with the PGPB culture at a ratio of 1:3 (w/v) and incubated for 2 h before application) at a dose of 1 t $ha^{-1}$ in a two-year monoculture field.

Rhizosphere soil samples were collected by gently uprooting *R. pseudostellariae* from the field, and the soil was collected by brushing off the soil closely attached to the rhizomes and roots. Samples from each replicate plot were collected from five random locations and then mixed together to form a pooled sample. The sampled soil was then immediately sieved through a 2-mm nylon mesh to remove stones and plant residues. All the rhizosphere soil samples were collected and divided into two groups: one batch was stored at -80°C for microbiome analyses, while the second batch was kept at room temperature before air-drying and used to analyze the soil physicochemical properties. Leaf and root samples were collected from the same plants which rhizosphere samples were collected. The loosely attached soil particles on the leaf and root samples were removed by rinsing with sterile water and by immersion and further cleaning in sterile phosphate buffered saline buffer three times in an oscillator for 20 min (Wu et al., 2022c). After completing the above washing process, the plant samples were further dried and stored at −80°C.

## *2.4. Plant traits and soil physicochemical analyses*

For each sampled plant, we measured the length and diameter of root tubers, leaves, and root biomass of *R. pseudostellariae*. The total polysaccharide content of *R. pseudostellariae* roots was extracted using water extraction and determined using the phenol-sulfuric acid method (Wu et al., 2012). Heterophyllin B was extracted and detected using HPLC-MS (Thermo LTQ XL) (Wang et al., 2010; Wu et al., 2022c). The detection parameters were as follows: mobile phase A, water; mobile phase B, acetonitrile; Ion Source, negative ion mode; APCI; Vaporizer temperature, 400°C; capillary temperature, 350°C; Capillary Voltage: -3 V. We also measured the soil pH and electrical conductivity (EC) using rhizosphere soil extracts [soil: $H_2O$ = 1:2 (w/v)]. Soil $NH_4^+$-N, $NO_3^-$-N, cellulase activity, and sucrase activity were determined as previously described (Wu et al., 2022a). Five technical replicates were used for each sample.

## *2.5. Quantification of specific microbial taxa*

The total DNA of pot soils, plants, and rhizosphere soils was extracted using the plant DNA Extraction Kit (Omega, United States) and soil DNA Extraction Kit (BioFast, China) according to the sample type. DNA concentration and quality were measured using a Nanodrop spectrophotometer (Thermo Scientific), and DNA integrity was assessed by agarose gel electrophoresis. The abundance of total bacteria (T Weedon et al., 2012), *Bacillus* spp. (Fierer et al., 2005), *F. oxysporum* (Lievens et al., 2005) and *Pseudomonas* spp. (Fierer et al., 2005) in plants and soils was determined and analyzed by qPCR using a CFX96 instrument (Bio-RAD, US). The primer sequences and reaction conditions are listed in Tab. S2 and S3. qPCR was performed as previously described (Wu et al., 2016).

### *2.6. DNA extraction, PCR, and sequencing*

The same DNA samples described above were used to prepare libraries for the 16S and 18S amplicon metagenomics. 16S libraries were prepared to characterize the bacterial community of leaves and roots by targeting the V5-V7 region of the 16S rRNA gene using the primers 799F and 1193R (Zhang et al., 2018b). The rhizosphere soil protist community was characterized by targeting the 18S rRNA gene using the primers V4_1f and TAReukREV3 (Xiong et al., 2020). The primer sequences and PCR conditions are listed in Tab. S2 and S3. Paired-end sequencing was performed on an Illumina NovaSeq PE250 sequencer. High-quality sequences were analyzed using QIIME2 (https://qiime2.org/). Taxonomy assignment was performed using the SILVA database (Quast et al., 2013) for 16S rRNA and PR2 databases (Guillou et al., 2012) for 18S rRNA. Within the 16S dataset, sequences identified as chloroplasts or mitochondria were discarded. Similarly, within the 18S dataset, ASVs matching Streptophyta, Rhodophyta, Fungi and Metazoa were removed. Protistan ASVs have been assigned to different functional groups according to their feeding modes (Dumack et al., 2020; Nguyen et al., 2020).

### *2.7. Data analysis*

Alpha diversity indices (richness and Shannon's index) of plant (leaf and root) bacterial and soil protist communities were estimated using the QIIME2 platform. Principal component analysis (PCA) was conducted using the Bray-Curtis distance matrix. Analyses below were performed using R 4.0.2, otherwise indicated. Random forest model analysis was conducted to estimate the mean predictor importance of *Fusarium oxysporum*, leaf bacterial, root bacterial and rhizoshpere soil protistan community diversity (richness index) and community structure (PCA1) for plant biomass by using the "randomForest" package (Liaw and Wiener, 2002). The potential importance of stochastic processes in bacterial and protistan community assemblies was evaluated by fitting our data to the Sloan-neutral community model (NCM) (Sloan et al., 2006). The beta-nearest taxon index (βNTI) was estimated to test whether stochastic and deterministic assembly processes drive the assembly of bacterial and protist communities (Stegen et al., 2013; Liu et al., 2022). The “niche breadth” approach was used to quantify habitat specialization and calculated as previous studies (Zhang et al., 2018a). Random forest regression model was analyzed with the “randomForest” package to identify the main plant (leaf and root) bacterial and soil protistan predictors of *R. pseudostellariae* biomass. Co-occurrence networks focused on correlations with absolute values of Spearman’s coefficient > 0.6 and $p < 0.01$ were generated using the “igraph” package (Csardi and Nepusz, 2006). The networks were visualized using the "Gephi" interactive platform. Spearman’s correlation tests were performed to test the relationship between the soil physicochemical characteristics and members of the microbiota. We constructed a structural equation model (SEM) to identify the indirect and direct effects of combined treatments (uncombined and combined amendments), soil properties (soil pH and $NO_3^-$-N), plant biomass, parasitic protists, and bacterial richness using Amos 21.0.0 software (IBM SPSS). The model fit was indicated by the root-mean-square error of approximation (RMSEA < 0.05), Bentler comparative fit index (CFI > 0.90), Tucker-Lewis coefficient (TLI > 0.95), and chi-square test $P > 0.05$ (Schermelleh-Engel et al., 2003). Significant differences among all treatments were identified using the least significant difference (LSD) multiple range test.

## 3. Results

### *3.1. In-vitro antagonistic activity*

The different *Bacillus* SynComms effectively suppressed the mycelial growth of *F. oxysporum* in 1/4 PDA and 3% biochar-1/4 PDA culture media (Fig. S1). SynComm combining all ten *Bacillus* spp. displayed the best antagonistic activity against *F. oxysporum*. The qPCR results showed that the abundance of soil bacteria, *Bacillus* spp., and *Pseudomonas* spp. increased within one week, after which the abundance sharply declined (Fig. 1). The biochar with PGPBs treatment increased the abundance of total bacteria, *Bacillus* spp., and *Pseudomonas* spp. after *F. oxysporum* addition (Fig. 1). The combined treatment maintained higher ratios of *Bacillus* spp. / *F. oxysporum* and *Pseudomonas* spp. / *F. oxysporum* after week 4.

### *3.2. Variations in soil physicochemical properties, plant biomass and microbial density under field experiment*

The combined treatment significantly increased the root diameter, root length, biomass, and total polysaccharide and heterophyllin B content compared to both the biochar and control treatments (Fig. 2). Biochar with PGPBs addition significantly improved the soil pH, EC and $NO_3^-$ -N content, but did not affect the cellulose and $NH_4^+$-N content (Tab. 1). The single biochar amendment significantly increased the abundance of total bacterial load in the leaf, root, and rhizosphere soils compared to the non-amended field (Fig. 3A). Biochar with PGPBs treatment also displayed higher bacterial densities in the roots and soil than third-year monocultures ($p < 0.05$). Biochar alone and the combined treatment both increased *Bacillus* densities in the roots and soil and decreased them in the leaves when compared to the control field (Fig. 3B). The abundance of *Fusarium oxysporum* sharply declined under the single biochar and combined treatments (Fig. 3C). The combined treatment had the most significant negative effect on the *F. oxysporum* load.

Random forest analysis showed that *F. oxysporum* abundance and microbial diversity in the three compartments were the strongest predictors of *R. pseudostellariae* biomass (*F. oxysporum* density: 30.6% increase in MSE; microbial diversity: 38.9% increase in MSE; Figure 3D). Leaf bacterial and rhizospheric soil protistan community diversity was the most important ($p < 0.05$) microbial parameter between the selected microbial indices with respect to explaining *R. pseudostellariae* biomass. In contrast, root bacterial and rhizospheric soil protistan structures were not correlated with the biomass.

### *3.3. Microbial diversity, community structure and composition*

Biochar addition alone significantly increased the leaf bacterial richness and root bacterial diversity (Fig. 4A). In contrast, the combined treatment decreased both bacterial richness and diversity compared with the third-year monocultures. In addition, the combined treatment significantly decreased soil protistan richness and diversity (Fig. 4B). PCA and Bray-Curtis distance results showed that the bacterial and protistan community compositions were generally separated when comparing different treatments and third-year monocultures in each of the three compartments (Fig. 4C, D, and E). The single biochar and combined treatments decreased the relative abundance of Proteobacteria in leaves (Fig. 4F). The results suggest a higher abundance of Actinobacteria in the roots than in the leaves, and the opposite trend was observed for Firmicutes. Meanwhile, the combined treatment increased the abundance of Firmicutes in leaves ($p < 0.05$). For protistan phyla, the single biochar and the combination of biochar and *Bacillus* spp. exhibited significant and positive effects on the abundance of Apicomplexa and decreased the abundance of Dinophyceae (Figure 4G).

### *3.4. Assembly processes and co-occurring network of bacterial and protistan communities*

The NCM results explained 55.4%, 65.5%, and 24.9% of the variability in the leaf and root bacterial communities and rhizospheric soil protistan communities across all treatments, respectively (Fig. 5A). Compared to third-year monocultures, the combined treatment decreased the explained fraction of variation and the mitigation rate of both the plant (leaf and root) bacterial and rhizospheric soil protistan communities (Figure S2). The explained fraction of variation and mitigation rate of leaf bacterial and rhizospheric soil protistan communities were lower under the TYB treatment than under the TY treatment. The βNTI values of the rhizospheric soil protistan and leaf bacterial communities were < 2 across all treatments, indicating that stochastic processes played a major role in the assembly of the leaf bacterial and rhizospheric soil protistan communities (Fig. 5B). Moreover, deterministic processes dominated the assembly of the root bacterial community. The root bacterial community had a lower niche breadth than that of the leaf bacterial community (Figure 5C). The single biochar amendment increased the average niche breadth of plant (leaf and root) bacteria and the rhizospheric soil protistan community compared with the control treatment. Moreover, the combined treatment showed the opposite trend.

Co-occurrence network results indicated that the network connections of the leaf and root bacterial communities were dominated by the phyla Proteobacteria, Actinobacteria, Firmicutes, Acidobacteria, and Bacteroidetes, and Proteobacteria and Actinobacteria accounted for more connections (Fig. 5D). The combined treatment increased the modularity values and clustering coefficient and decreased positive correlations proportion of plant (leaf and root) bacterial networks compared to the TY treatment (Tab. S4). The bacterial community co-occurrence suggested that the leaves had higher positive correlations, edge numbers, and clustering coefficients than the roots (Fig. 5E, Table. S4). Notably, the rhizospheric soil protistan community co-occurrence mainly consisted of omnivores, parasites, phototrophs, and bacterivores (Fig. 5E). The positive correlations and clustering coefficient of the rhizosphere soil protistan community were higher than those of the root bacterial community but lower than those for the leaf bacterial community.

### *3.5. Bacterial and protistan taxonomic and functional composition influenced the biomass of R. pseudostellariae*

The bacterial genera of the plants displaying the highest difference and variation belonged to the phyla Actinobacteria, Proteobacteria, and Firmicutes (Fig 6A). The results indicated that biochar treatment alone significantly increased the relative abundance of *Ralstonia*, *Bacillus*, *Burkholderia* and *Streptomyces* but decreased the abundance of *Paenibacillus* and *Pseudomonas* on the leaf compared to the TY treatment (Fig. S3). Furthermore, biochar treatment alone showed a significantly negative effect on the abundance of *Ralstonia*, and negative effect on the abundance of *Burkholderia* and *Streptomyces* in the root. The combined treatment decreased the relative abundance of *Ralstonia* and increased the abundance of *Bacillus* in the roots compared to the TYB and TY treatments ($p < 0.05$). *Paenibacillus* was more abundant in the leaves under

TYBB amendment than under TYB and TY amendment. Moreover, TYBB amendment significantly increased the abundance of *Platyophrya*, *Nassula*, *Cercomonas*, *Paracercomonas*, and *Viridiraptor* (Figure 6B, table S5). The single biochar addition and combined treatment had a negative effect on the abundance of bacterivores and increased the abundance of parasite protists compared to the TY treatment (Figure 6C, table S5). Moreover, the combined treatment significantly decreased the relative abundances of plant pathogens and phototrophs.

Random forest analysis indicated that *Paenibacillus*, *Pseudomonas*, *Bacillus*, *Streptomyces*, *Actinospica* and *Ralstonia* were important biomass predictors (Fig. 6D and 6E). Most of these beneficial and deleterious predictors were correlated with soil pH, EC, and $NO_3^-$-N. Biomass and pH were negatively correlated with *Ralstonia* and positively correlated with *Streptomyces* and *Bacillus*. In addition, most of the soil protistan biomarkers were negatively associated with biomass, pH, EC $NO_3^-$-N, and $NH_4^+$-N, whereas the majority of parasitic protists showed the opposite trend (Figure 6F).

The results also indicated that the relative abundance of soil parasitic protists was significantly negatively correlated with *F. oxysporum* abundance and positively correlated ($p$ <0.05) with *Bacillus* density (Fig. 6G). Moreover, soil parasitic protists were significantly positively associated with soil pH, EC, $NO_3^-$-N, and $NH_4^+$-N (Fig. S4). Structural equation models (SEMs) showed that soil pH and $NO_3^-$ -N were the most important factors that directly or indirectly drove plant biomass (Fig. S5). Soil pH showed the significantly positive and indirect effect on parasitic protists and biomass by influencing the microbial richness of leaves and roots (Fig S5A and S5B). Soil $NO_3^-$ -N had a significant and positive effect on parasitic protists and biomass and negative effects on leaf and root microbiota richness, respectively (Figure S5C and S5D).

EC, $NH_4^+$-N, and $NO_3^-$-N were negative for bacterial and protistan diversity and niche in the three compartments, whereas sucrase showed the opposite trend (Fig. 7A). Soil pH was a negative and strong predictor of leaf bacterial niche and soil protistan richness and was significantly positively correlated with protistan β diversity. Soil $NO_3^-$-N was significantly negatively correlated with leaf and root bacterial richness, protistan α-diversity, and protistan betaNTI. Subsequently, the combination of biochar and PGPBs practices affected plant biomass by altering soil properties, plant (leaf and root) bacterial diversity, and keystone taxa (Figure 7B). Biochar with PGPBs significantly directly and indirectly affects parasitic protists by influencing soil properties and plant (leaf and root) bacterial diversity. Specifically, we found that biochar with PGPBs treatment, soil parasite protists, and pH were the most positive factors directly driving plant biomass (Figure 7C). In contrast, leaf microbiota richness was negatively correlated with plant biomass under different treatments.

## 4. Discussion

### *4.1. Biochar and PGPBs regulated assembly of the plant microbiota*

Biochar has been reported to be a promising and effective soil amendment agent to enhance crop yields by modulating soil physicochemical properties and microbiome (Jiang et al., 2020; Zhao et al., 2022). Our field experiment indicated that the application of biochar alone and biochar with PGPBs increased the biomass and main components of *R. pseudostellariae* under the third-year monoculture field, which is similar to our previous study on second-year monoculture soil (Wu et al., 2022a). The biochar with PGPBs treatment significantly increased the bacterial densities in the root and soil, and decreased the leaf and root bacterial diversity compared to the third-year monocultures. Furthermore, combined biochar and *Bacillus* SynComm significantly decreased soil protist richness and diversity, which might be generated via selective filtering (Trivedi et al., 2020; Li et al., 2021). These results suggest that the combination of biochar and *Bacillus* SynComm increases the abundance of some specific microbes in the rhizosphere and endosphere of *R. pseudostellariae*.

The βNTI value analysis indicated that the stochastic process played an important role in the leaf bacterial assembly, and deterministic processes dominated the root bacterial community assembly, which agreed with previous results indicating that rhizosphere soil bacterial community assembly showed a deterministic process under biochar addition (Wu et al., 2022a). The addition of biochar alone increased the niche breadth of the plant (leaf and root) bacterial and rhizospheric soil protistan communities compared with the control treatment, due to biochar addition providing suitable abiotic environmental conditions. In addition, the biochar with *Bacillus* SynComm treatments shifted the stochastic assembly processes of the rhizospheric protistan community into deterministic assembly processes, decreasing the niche breadth of the plant (leaf and root) bacterial and rhizospheric soil protistan community, and decreasing the migration rate of the NCM in the plant (leaf and root) bacterial and rhizospheric soil protistan community. These effects might be generated by the combination of increased microbial densities and recruitment of specific microbial taxa, which influenced the protistan community by selecting taxa from specific feeding guilds, thus guiding a deterministic shift in the protist community of protists (Johnston et al., 2016; Amacker et al., 2022).

### *4.2. Biochar and PGPBs stimulated specific microbial taxa that enhanced suppression of Fusarium oxysporum*

Biochar and PGPBs amendments have been widely used to inhibit soil-borne pathogens and reduce plant diseases (Xiong et al., 2017; Zhao et al., 2022). In modern agricultural production, *F. oxysporum* is among the top 10 fungal pathogens in plant pathology and causes vascular wilt in more than 100 plants (Kamoun et al., 2015). Our previous studies have indicated that *F. oxysporum* is the main pathogen causing replant disease in *R. pseudostellariae* (Zhao et al., 2015; Chen et al., 2017; Wu et al., 2020). In this study, our results indicate that biochar alone and in combination with PGPBs decreased *F. oxysporum* density in the leaves, roots, and soil under the third-year monoculture treatment. In addition, the results showed that *F. oxysporum* density and microbial diversity in the three compartments were the strongest predictors of *R. pseudostellariae* root biomass. Previous findings have indicated that biochar may not play a direct role in suppressing *F. oxysporum* under field conditions (Zhao et al., 2022), and that their effects on plant health are mediated by alterations in the soil microbial community assembly (Tao et al., 2020; Chen et al., 2022). Our study also found that biochar treatment alone decreased the abundance of pathogenic *Ralstonia*, and enhanced the abundance of beneficial *Burkholderia* and *Streptomyces* in roots. The combined biochar and *Bacillus* SynComm treatment significantly decreased the abundance of potentially pathogenic *Ralstonia*, and increased the abundance of beneficial *Bacillus* in the roots and *Paenibacillus* in the leaves. Furthermore, biochar with *Bacillus* SynComm increased the abundance of potentially beneficial *Pseudomonas* spp. after *F. oxysporum* addition. Our results agree with those of a previous study indicating that biofertilizer contributes to alleviating replant disease by promoting the abundance of beneficial microorganisms in leaves and roots (Wu et al., 2022c). This indicated that biochar alone, and in combination with *Bacillus* SynComm, influenced the plant microbiome with beneficial effects on the susceptibility to plant disease.

In addition, our results suggest that the diversity of the rhizosphere soil protistan community has a significant influence on *R. pseudostellariae* biomass. The addition of biochar, alone and in combination with *Bacillus* SynComm, significantly increased the abundance of parasitic protists. Furthermore, the combined treatment significantly increased the abundance of predatory protists *Cercomonas* and *Paracercomonas*, and decreased the abundance of potentially pathogenic protists. Previous study had shown that Cercozoa were able to differentially modulate bacterial communities via grazing the genera *Cercomonas*, *Sandona* and *Paracercomonas* (Glücksman et al., 2010). *Cercomonas* spp. can increase plant yield by fostering the growth of plant-beneficial microorganisms (*Bacillus*, *Pseudomonas* and *Trichoderma*) and negatively influencing pathogenic *Fusarium* (Guo et al., 2021; Guo et al., 2022). Our results also suggest that the potentially beneficial genera *Paenibacillus*, *Pseudomonas*, *Bacillus*, *Streptomyces* and *Cercomonas* are the dominant predictors of plant biomass. These microbes have been widely reported to be biological control agents capable of suppressing plant pathogens (Viaene et al., 2016; Olishevska et al., 2019; Wu et al., 2019; Guo et al., 2022). Most of these beneficial biomarkers have been shown to have significant relationships with soil pH, EC, and $NO_3^-$-N. Further studies are needed to clarify the interactions between plants and protists that lead to selective recruitment of specific protists in the rhizosphere soil. These findings suggest that biochar, alone and in combination with *Bacillus* SynComm, stimulated and increased the abundance of beneficial and indigenous plant microbiota and soil protists to suppress host-specific pathogens.

### *4.3. Underlying driver factors of soil protistan composition and links with R. pseudostellariae biomass*

Plant-associated microbial community structure and composition are mediated by complex interactions between plant hosts, microorganisms, and the environment (Trivedi et al., 2020). The co-occurrence network suggested that the combined biochar and *Bacillus* SynComm increased the modularity values and clustering coefficient of the bacterial networks, which indicated that the combined treatment decreased niche overlap and interspecies competition of the leaf and root bacteria under third-year monoculture conditions. Previous studies have shown that soil physicochemical properties (pH, EC, exchangeable Ca, and Na) have a greater effect on protistan community composition than the rhizosphere effect in alkaline paddy-field soils (Asiloglu et al., 2021b). Our results showed that in acidic soils, the concentrations of EC, $NH_4^+$-N, and $NO_3^-$-N were negatively correlated with protistan diversity and niche. Soil pH was a negative and strong predictor of soil protist richness and positively correlated with protist β diversity, indicating the importance of soil properties in regulating soil protist communities. This is in line with the results that the pH may be a key factor altering the protistan diversity in alkaline paddy soil (Asiloglu et al., 2021b).

Predatory (or consumers) protists have been shown to function as omnivores and bacterivores, and prey on a wide range of fungi and bacteria, as well as other eukaryotes (Geisen et al., 2018), and affect ecosystem-level processes such as pathogen control and nutrient cycling (Sun et al., 2021). Our results indicate that the rhizospheric soil protistan community co-occurrence network consists mainly of omnivores, parasites, phototrophs, and bacterivores. Among the top 30 most important genera of soil protists as key drivers of plant biomass, omnivorous taxa were the most abundant and negatively correlated with soil physicochemical characteristics. Our results are consistent with those of a previous study indicating that omnivores and bacterivores are significantly influenced by fertilization treatment in soil (Sun et al., 2021). In addition, we found that parasitic protists were more abundant under biochar amendment, alone or in combination with *Bacillus* SynComm, which is consistent with results showing that fertilization enriches the functional groups of protist parasites and consumers (Li et al., 2021; Sun et al., 2021). Protistan parasites were able to parasitize on various hosts (plants, animals, or other organisms), and the amended soils influenced the main hosts (Schulz et al., 2019) and provided the most abundant sources of hosts for them (Sun et al., 2021), which may explain their presence in soil habitats.

Furthermore, the majority of the parasite protist biomarkers were positively associated with plant biomass. The parasite protists were significantly negatively correlated with the abundance of *F. oxysporum* and positively correlated with the abundance of *Bacillus*, suggesting that parasite protists might differentially modulate the abundance of beneficial and pathogenic microbiota in rhizosphere soil. Previous studies have shown that predatory protists improve crop yields by recruiting plant-beneficial microorganisms that negatively affect plant pathogens (Guo et al., 2021). The parasitic protists significantly and negatively impact the abundance of *F. oxysporum*, suggesting *F. oxysporum* can be important food sources impacting the growth of parasitic protists via bottom-up regulations. Although we cannot provide direct evidence for predation of *F. oxysporum* by parasitic protists, the ecological function of parasite protists and their interactions with plant pathogens or beneficial bacteria in soils should be explored in future studies. Nitrogen and pH are important factors strongly influencing the protist communities (Zhao et al., 2019; Asiloglu et al., 2021b). In this study, the SEMs results showed that soil pH and $NO_3^-$–N exhibited significant positive and indirect effects on parasitic protists and biomass by influencing the microbiota richness of leaves and roots. Soil protists can significantly shape a wider rhizosphere microbial community through top-down control effects (Flues et al., 2017; Li et al., 2021). Specifically, we found that biochar with PGPBs directly and indirectly affected parasitic protists by influencing soil properties and plant (leaf and root) bacterial diversity, indicating that soil sources and plant microbiota were able to modulate protistan community assembly through bottom-up regulations.

## 5. Conclusions

In conclusion, our results indicated that biochar and biochar with *Bacillus* SynComm could have beneficial effects on plant health through their influence on the plant microbiota and specific predatory and parasitic protists. Soil parasites can control the abundance of plant pathogens through top-down mechanisms. This study also highlighted the potential use of this strategy to suppress soil-borne pathogens in agroecosystems. Nevertheless, considering the effect of biochar on the structure and function of the soil micro-food web, further studies are necessary to explore the effects of plant-microbiome-environment interactions on biochar-induced amelioration.

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## Declaration of competing interest

The authors declare that they have no conflict of interest.

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**Table 1** Physicochemical characteristics of rhizosphere soils across all the treatments.

| **Treatment** | **EC (μs/cm)** | **pH** | **Cellulose (mg glucose·g$^{-1}$ soil·d$^{-1}$)** | **Sucrase (mg glucose·g$^{-1}$ soil·d$^{-1}$)** | **$NO_3^-$ -N ($mg \cdot kg^{-1}$)** | **$NH_4^+$-N ($mg \cdot kg^{-1}$)** |
|---|---|---|---|---|---|---|
| CK | 75.64±2.26d | 5.51±0.05a | 5.28±1.37a | 1.48±0.45c | 34.23±9.87c | 16.23±0.94a |
| TYS | 91.36±0.91c | 5.36±0.04c | 4.67±0.92ab | 3.52±0.34a | 107.30±19.02b | 15.44±1.21a |
| TYBS | 97.18±8.24b | 5.43±0.07b | 3.67±0.18b | 3.69±0.58a | 110.52±8.24b | 15.78±0.76a |
| TYBBS | 106.84±1.78a | 5.45±0.03ab | 3.60±0.32b | 2.54±0.31b | 152.66±7.63a | 16.92±1.33a |

TYS, TYBS, and TYBBS represent rhizosphere soils under the TY, TYB, and TYBB treatments, respectively. CK represents soil that had never been previously cultured with *R. pseudostellariae*. Different letters in each column represent significant differences ($p < 0.05$, n=5).

## Figure Legends

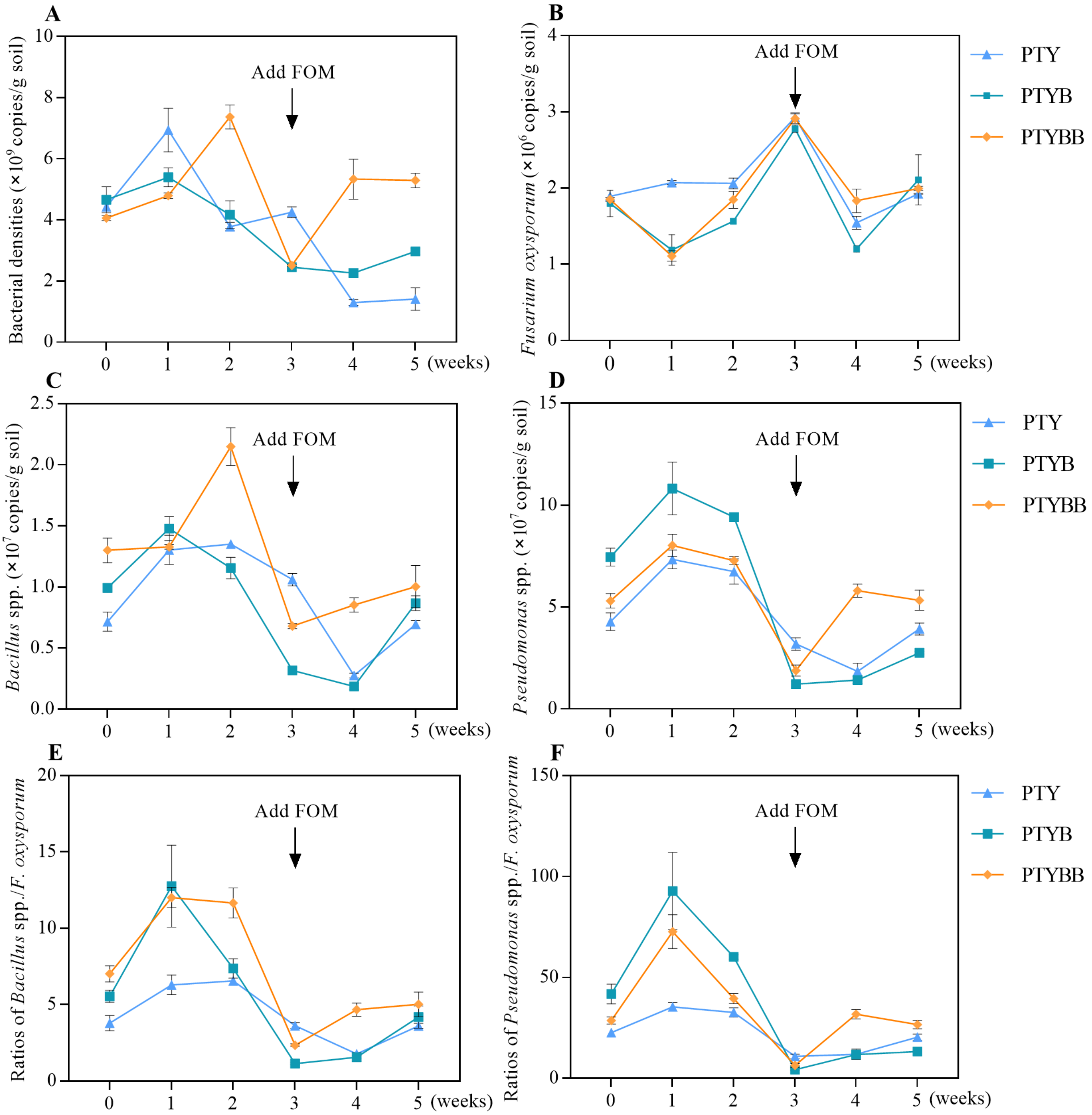

**Figure 1** Abundance of microorganisms in the soils under pot experiments detected by qRT-PCR. Add FOM: the *F. oxysporum* f.sp. *R. pseudostellariae* spores added the three treatments in the third week. PTY: two-year monoculture soil of *R. pseudostellariae*; PTYB: two-year monoculture soil with biochar addition; PTYBB: two-year monoculture soil with the biochar and then *Bacillus* sp. addition in the second week.

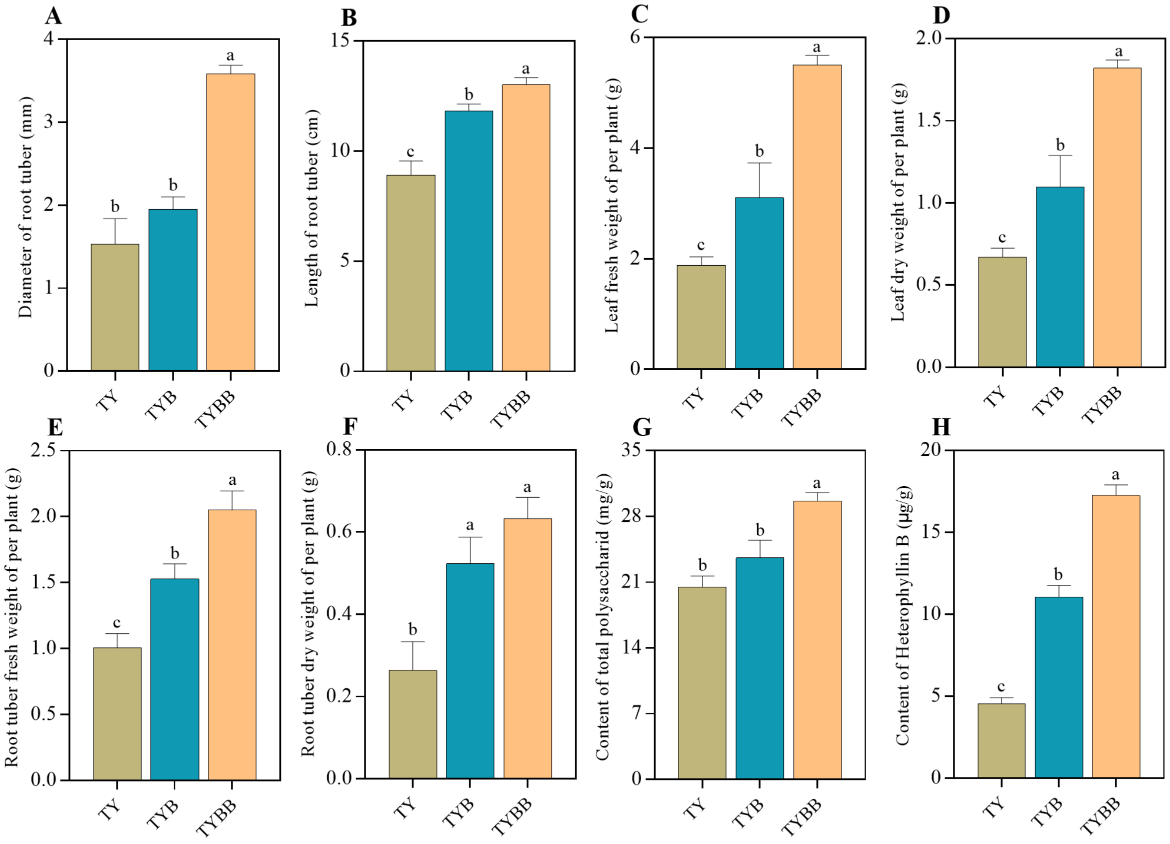

**Figure 2** Effect of different treatments on plant characteristics and components of *R. pseudostellariae* in the field. TY: *R. pseudostellariae* consecutively cultivated in two-year monoculture field; TYB: *R. pseudostellariae* consecutively cultivated in two-year monoculture field with biochar addition; TYBB: *R. pseudostellariae* consecutively cultivated in two-year monoculture field with the combination of biochar and *Bacillus* SynComm addition. The different letters in each column display significant differences ($p < 0.05$, n = 5).

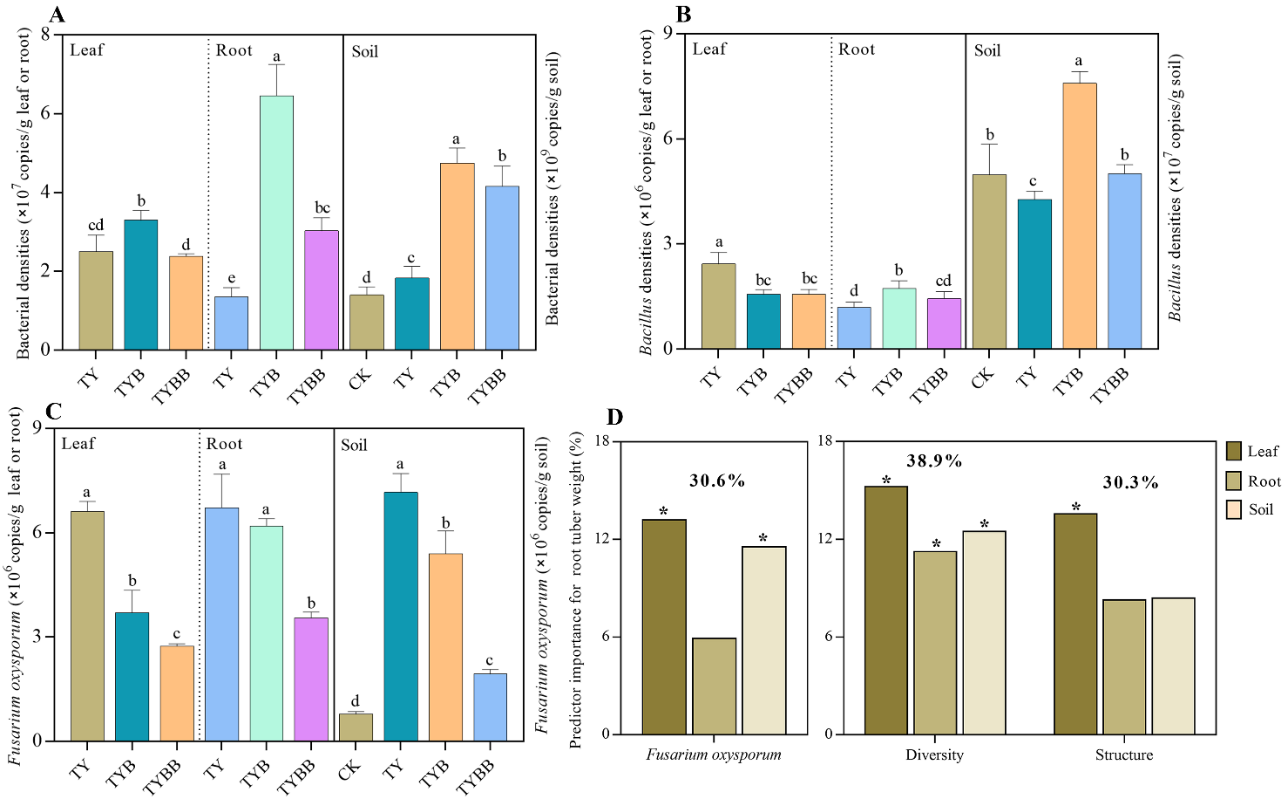

**Figure 3** Abundance of total bacteria **(A)**, *Bacillus* spp. **(B)** and *F. oxysporum* **(C)** under different treatments detected by qRT-PCR. Random forest mean predictor importance (% increase of the MSE) of *Fusarium oxysporum*, leaf bacterial, root bacterial and rhizoshpere soil protistan community diversity (richness index) and community structure (principal component analysis) for plant biomass **(D)**. The different letters in each column display significant differences ($p < 0.05$, n = 5).

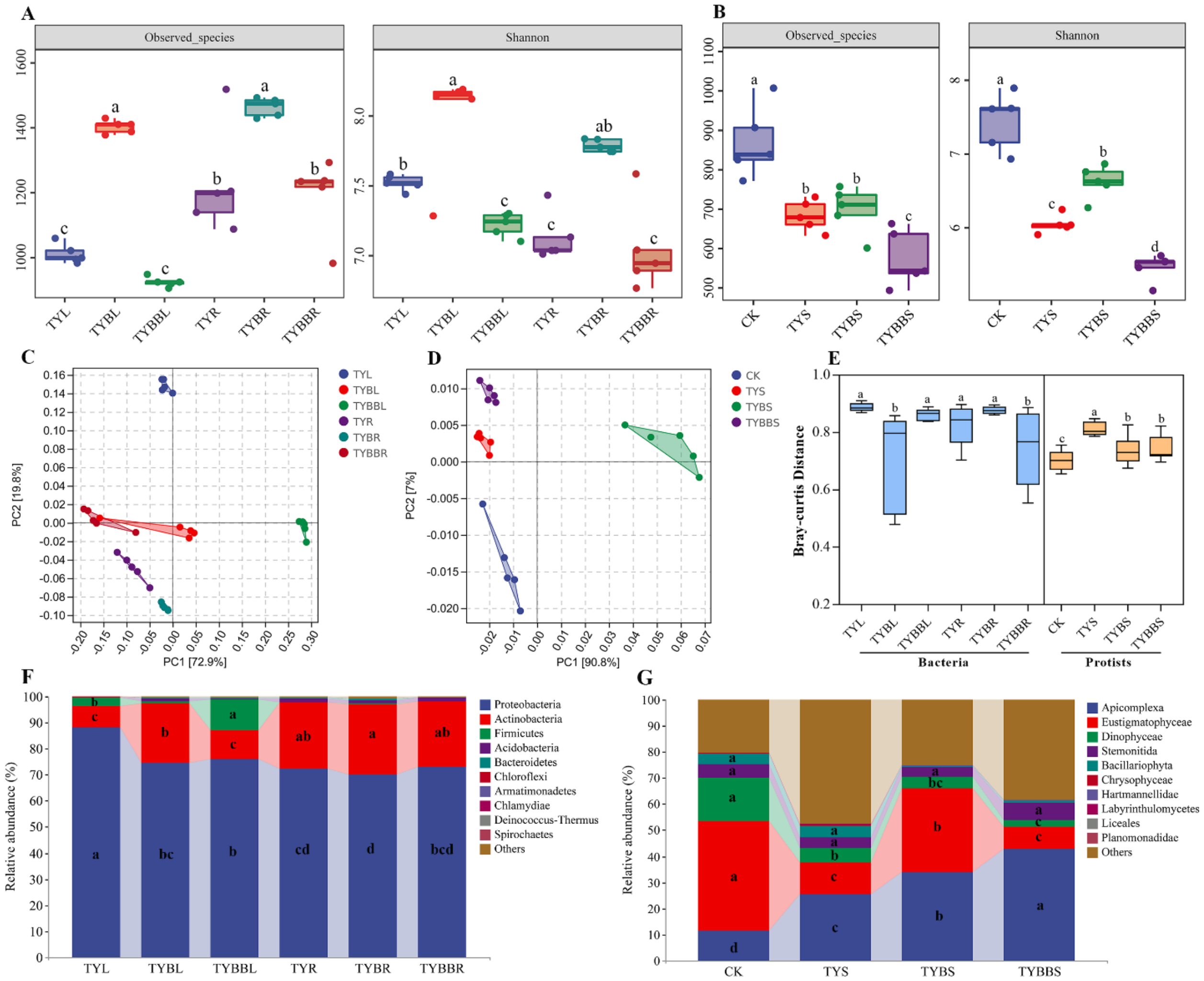

**Figure 4** Richness and Shannon diversity index for **(A)** bacteria (leaf and root) and **(B)** protists (rhizosphere) across all treatments. Principal component analysis (PCA) for **(C)** bacteria (leaf and root) and **(D)** protists (rhizosphere). **(E)** Bray-Curtis distance of bacteria (leaf and root) and protists (rhizosphere) for each treatment. Relative abundance of **(F)** bacterial (leaf and root) and **(G)** protistan (rhizosphere) phyla across all treatments. TYL, TYBL, and TYBBL represent the *R. pseudostellariae* leaves under the TY, TYB and TYBB treatments, respectively; TYR, TYBR, and TYBBR represent the *R. pseudostellariae* roots under the TY, TYB and TYBB treatments, respectively; TYS, TYBS, and TYBBS represent the rhizosphere soils under the TY, TYB and TYBB treatments, respectively; CK represents the soil of never previously cultured with *R. pseudostellariae*.

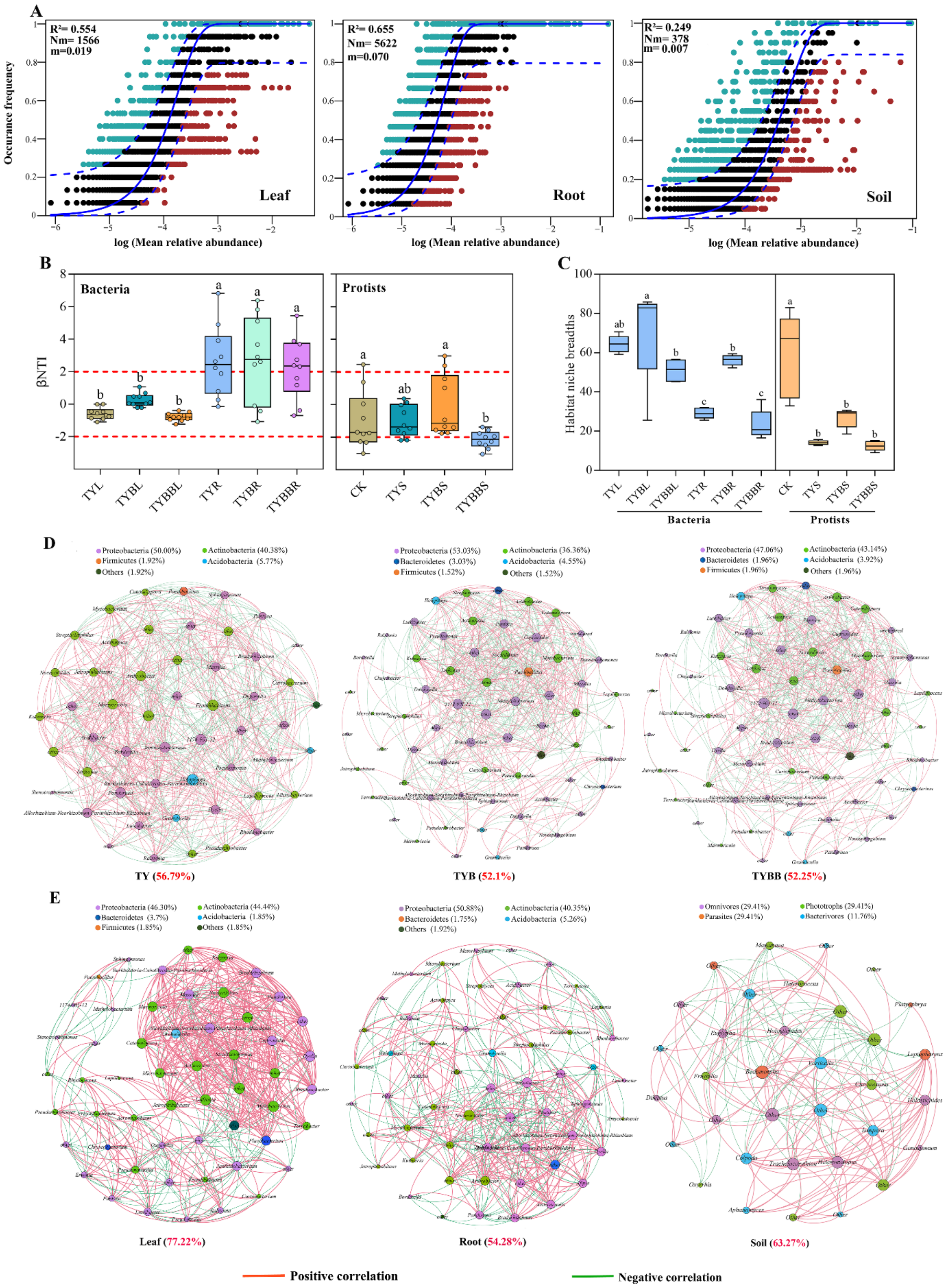

**Figure 5** Sloan neutral community model (NCM) analysis for the leaf bacterial, root bacterial and rhizoshpere soil protistan community, respectively **(A)**. The βNTI values of assembly processes of plant (leaf and root) bacteria and soil protists **(B)**. Average niche breadth from all average niche breadth under different treatments **(C)**. Co-

occurring network of plant (leaf and root) bacterial **(D)** and soil protistan **(E)** genera based on Spearman's correlation analysis.

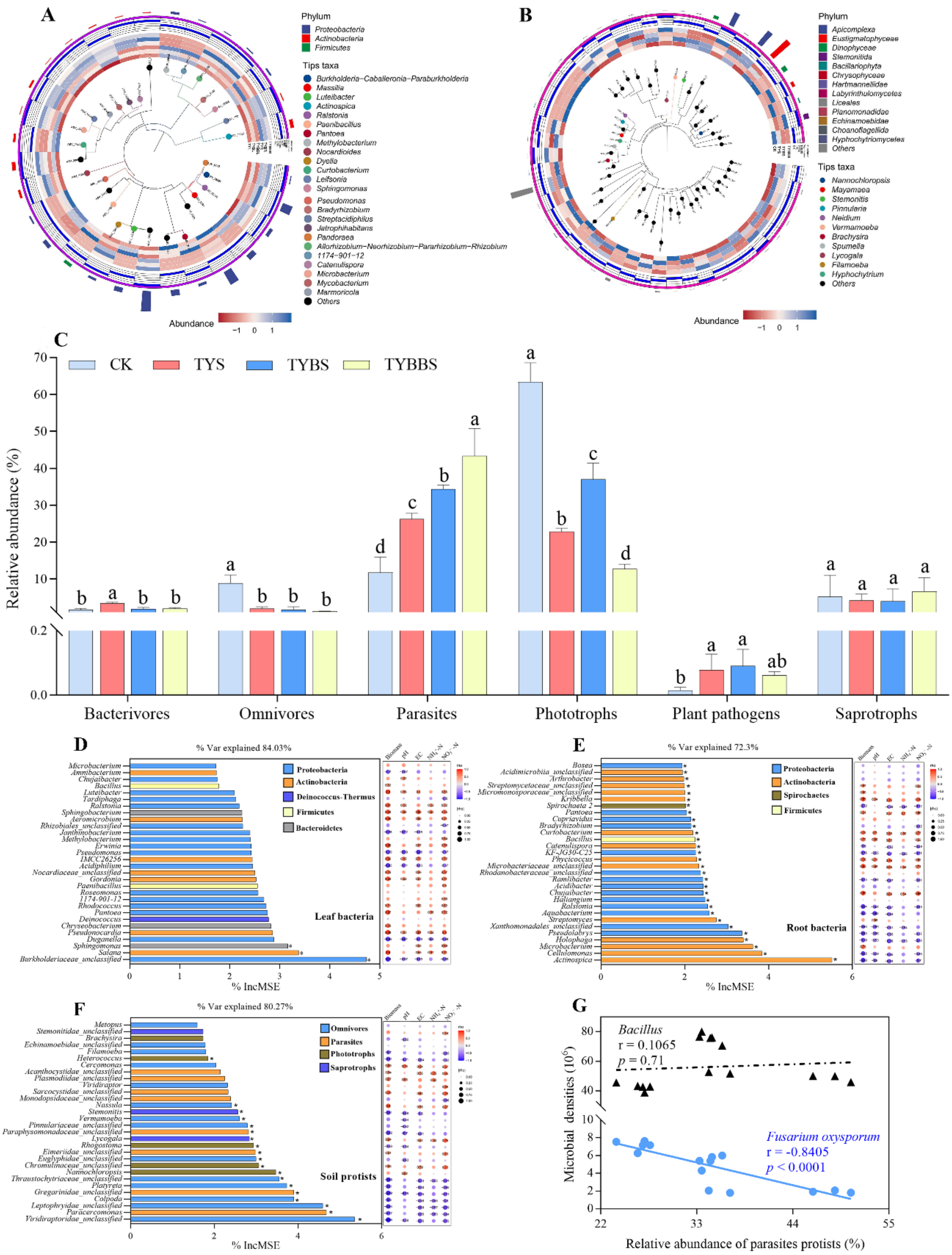

**Figure 6** Phylogenetic tree plot showing the top 30 abundant plant (leaf and root) bacterial **(A)** and soil protistan **(B)** genera across the different treatment samples. Distribution of the relative abundance of soil protistan functional groups **(C)**. Random forest model shows the top 30 most important genus of leaf bacteria **(D)**, root bacteria

**(E)** and soil protists **(F)** as key drivers of plant biomass, respectively. Bubble chart shows the correlation between the important taxa and plant biomass, soil pH, EC, $NO_3^-$-N and $NH_4^+$-N, respectively. The numbers represent the significant ($p$<0.05) correlation coefficients. **$p$ < 0.01, *$p$ < 0.05. Correlation between the relative abundances of parasitic protists with the densities of *Bacillus* and *F. oxysporum*, respectively **(G)**.

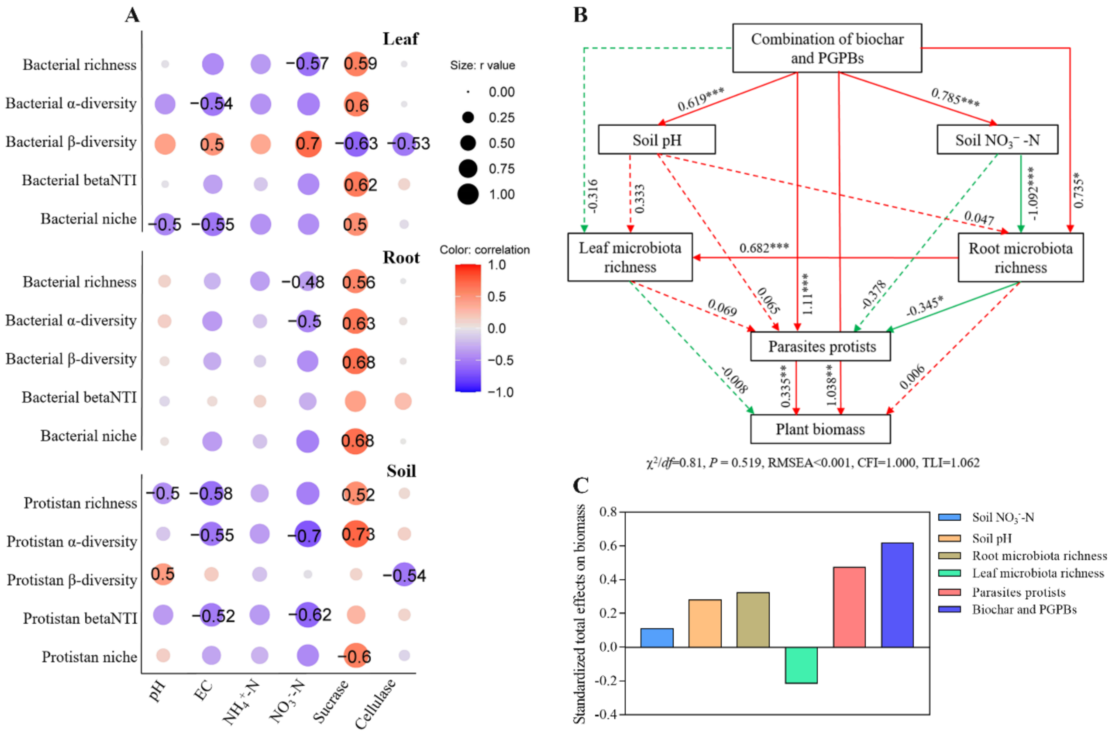

**Figure 7** Bubble chart showing the correlation between the bacterial and protistan diversity as indicated by soil physicochemical characteristics **(A)**. The numbers represent significant ($p$<0.05) correlation coefficients. SEMs analyzing the effect of the combined biochar and *Bacillus* sp. on soil properties and microbiota **(B)**. Standardized total effects of soil properties and microbial diversity on plant biomass derived from the SEMs used above **(C)**.