The combination of biochar and PGPBs stimulates the differentiation in rhizosphere soil microbiome and metabolites to suppress soil-borne pathogens under consecutive monoculture regimes

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Abstract
The application of biochar and plant-growth-promoting bacteria (PGPBs) in biocontrol soil-borne pathogens has garnered worldwide interest recently. However, how agricultural replanting disease is alleviated by a combination of biochar and PGPBs treatment (SYBB) remains largely unexplored. In this study, we investigated the beneficial effects of single biochar addition and the combination of biochar and PGPBs on alleviating replanting disease by altering the rhizosphere microbiome and metabolites. Our field experiment showed that the SYBB treatment had a better alleviating effect on replanting disease than the single biochar addition. The study indicated the dominant effect of deterministic processes on the bacterial community and of stochastic processes on the fungal community under biochar and PGPBs treatment. The combination of biochar and PGPBs tended to convert the stochastic processes of fungal community assembly into deterministic processes. We found SYBB treatment increased the abundance of potentially beneficial *Pseudomonas*, *Lysobacter*, *Gemmatimonadetes* and *Nitrospira*, and decreasing the abundance of potentially pathogenic *Fusarium*, *Talaromyces* and *Fusarium oxysporum*. Moreover, the SYBB treatment increased the abundances of carbohydrates, fatty acids and plant hormones, and decreased the abundances of amino acids in the rhizosphere soil. Co-occurrence network analysis indicated that SYBB treatment increased the connections within the microbial communities and drove the alteration of co-occurrence network among the microbial communities and metabolites, which increased positive correlations in bacteria-metabolite networks and decreased positive correlations in fungus-metabolite networks. Spearman correlation analysis showed the abundances of beneficial *Streptomyces*, *Pseudomonas* and *Lysobacter* were significantly and positively correlated to the metabolites most increased under SYBB treatment. The combination of biochar and PGPBs alleviated replanting disease by mediating the
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1 | INTRODUCTION

Consecutive monoculture is a global trend, contributing in helping to meet the food needs of an increasing world population density and subsequent global changes. However, the same crop being repeatedly planted in the same field will result in severe replanting disease or soil sickness, which causes the accumulation of soil-borne pathogens and serious declines in the productivity and the quality of crops (Wu, Lin, et al., 2020; Wu, Qin, et al., 2019). Replanting disease is common in crops grown under intensive consecutive monoculture and is especially severe in the cultivation of fruits and medicinal herbs (Wu, Qin, et al., 2020). The factors underlying replanting disease may be associated with soil nutrient imbalance (Huang et al., 2013), accumulation of root exudate autotoxicity (Zhang et al., 2019) and rhizosphere microbial community change (Li et al., 2014; Wu, Qin, et al., 2019). Increasing evidence showed that the accumulation of soil-borne pathogens at the expense of plant-beneficial microbes has been proven to be the major driving factor of replanting disease (Lareen et al., 2016; Wu, Qin, et al., 2020; Xiong et al., 2017).

Replanting disease is considered to be a type of biological pollution that causes the accumulation of soil-borne pathogens, excessive pesticide residues, soil acidification and environmental pollution. As a logical extension of such findings, the principal strategy for the remediation of replanting disease is to decrease the abundance of soil-borne pathogens from the soil, that is, bio-fertilizers application and soil sterilization. The introduction of plant-growth-promoting bacteria (PGPBs) contributing to disease suppression holds promise to control of replanting disease. In addition, biochar amendment has been shown to reduce plant disease and increase crop yields, thus minimizing the health and ecological risks of soil-borne pathogens in the soil (Dai et al., 2020; Wang et al., 2020; Wu, Qin, et al., 2020). In our previous pot experiment, we found that biochar was able to alleviate replanting disease by decreasing the abundance of pathogenic Fusarium oxyporum and Talaromyces helicus, and significantly influence metabolic processes of pathogenic F. oxyporum while abating the virulence on plants (Wu, Qin, et al., 2020). The biochar amendment contributed to enriching the beneficial Lysobacter and Bacillus and suppressing an elevated abundance of pathogenic Ilyonectria and Fusarium in consecutive monoculture regimes of Panax notoginseng (Wang et al., 2020). However, most of the biochar effects on the replanting disease were mainly focused on pot experiments in the greenhouse (Wang et al., 2020; Wang, Ma, et al., 2019; Yang et al., 2019). In this sense, the effect of biochar on crop growth and composition of the microbial community of consecutive monocultures in field trials need to be taken into account, especially at the rhizosphere.

Recently, the combination of biochar and PGPBs has been shown to be effective to remediate soil (Chen et al., 2019; Tu et al., 2020; Wu, Wang, et al., 2019). Biochar has been shown to be a good carrier of PGPBs, thereby increasing the biomass of plant, enhancing soil catalase and urease activities, and improving the soil microbial community in heavy metal-polluted soil (Tu et al., 2020; Wu, Wang, et al., 2019). Previous studies have shown biochar acting as shelter in addition to providing nutrients to indigenous microbes; biochar was shown to adsorb pathogenic bacteria and reduce rhizosphere colonization by pathogens, thereby changing the microbial community and soil properties; biochar was also able to reduce pathogen growth via the sorption of root exudates (Gu et al., 2017; Tu et al., 2020; Wu, Qin, et al., 2020). However, the combinatory mechanism and respective contribution of biochar and exogenous PGPBs on the remediation of replanting disease is not well understood. Since both biochar and PGPBs were shown to shape soil microbial communities (Li et al., 2020; Xiong et al., 2017), we hypothesized that the combination of biochar and PGPBs could result in the selection of disease-resistant and pro-growth beneficial microbiome, consequently leading to a tighter microbial cooperation.
network and increasing the antagonistic activity of beneficial microbes against pathogens.

Previous studies had reported that plants released about 10% of the net carbon fixed by photosynthesis to the rhizosphere soil (Mohanram & Kumar, 2019). These root exudates are able to shape the rhizosphere microbial communities (Lareen et al., 2016). Meanwhile, the metabolism of these root exudates by soil microbes also, in turn, affects plant growth and root functions (Petriacq et al., 2017). In the process of root-microbe interaction, soil metabolomics is deemed as a window into microbial behaviors (Li et al., 2020; Swenson et al., 2018). Correlating soil metabolism composition to the microbial community composition enables deeper insights into the complex rhizosphere process in soil (Li et al., 2020). However, an integrative study of soil microbial communities and soil metabolomics under a combined treatment of biochar and PGPBs is still lacking.

Hence, we focused on a valuable medicinal plant from China, Ratex pseudostellariae, mainly cultivated in Fujian Province in southeastern China and suffers from serious replanting disease (Wu et al., 2016; Wu, Qin, et al., 2019). *R. pseudostellariae* was used as a model plant to better understand the effect of rice hull biochar and the combined treatment of biochar and PGPBs amendment on replanting disease in the field. Our aims were (1) to evaluate the effects of biochar amendment on plant functional traits, soil physicochemical and biological characteristics, and soil microbial community assembly processes and composition to investigate the effectiveness of biochar application to alleviate replanting disease under consecutive monoculture regimes and (2) to investigate rhizosphere processes by analyzing the rhizosphere soil microbiome and metabolites under both biochar and a combined biochar and PGPBs treatment. Our study intends to decipher the underlying mechanism of the combined biochar and PGPBs treatment and in remediating replanting disease.

2 | MATERIALS AND METHODS

2.1 | Field experiments and soil sampling

The experiments were conducted in Youxi, Fujian Province, China (118°21'E, 26°10'N) at 200 m altitude, a mean annual temperature of 19.2°C and an annual precipitation averaging 1600 mm. Two experimental fields were selected: never-planted soil (never previously planted with *R. pseudostellariae*) and 1-year monoculture soil (planted with *R. pseudostellariae* in the previous year). Five treatments were designed as follows: *R. pseudostellariae* planted in never-planted soil (FY), planted in never-planted soil with biochar treatment (FYP), planted in 1-year monoculture soil (SY), planted in 1-year monoculture soil with biochar (SYB) and planted in 1-year monoculture soil with biochar and PGPBs (SYBB), respectively (Table S1, Figure S1). All the treatments had three replicate plots. The rice hull biochar was generated at 500°C using the rice hull, which has been confirmed to alleviate replanting disease as shown in our previous pot study (Wu, Qin, et al., 2020). The PGPBs used in this study were a mixture of effective and antagonistic *Bacillus* spp. (including *Bacillus pumilus*, *Bacillus subtilis*, *Bacillus megaterium*, *Bacillus aryabhattai* and *Bacillus toyonensis*), isolated and subsequently stored in our laboratory. Each treatment had collected five rhizosphere soil samples. On April 22, 2019, 25 soil samples were collected from the five treatments. The field experiments and soil sampling are described in detail in the Supplementary Information (Text S1).

Planting of all *R. pseudostellariae* plots was conducted in December 21, 2018, and sampled on April 22, 2019. The rhizosphere soil samples were collected and divided into two groups. Thereafter, one batch of fresh soil samples was selected to store at −80°C for extracting the soil microbes and metabolites, whereas another group of soil samples was kept at normal room temperature before air drying for soil physicochemical property analysis. Rhizosphere soil samples were collected from five random locations within each replicate and mixed together to form a composite sample. For each treatment, we performed five replicates. Meanwhile, we harvested 10 whole plants and analyzed the *R. pseudostellariae* functional traits in each treatment.

2.2 | Plant functional traits and soil physicochemical and biological characteristics

The leaf areas, root diameters, stem and root lengths, and fresh biomass of every *R. pseudostellariae* were analyzed. The rhizosphere soil extracts (soil:H2O = 1:2 [w/v]) were used to measure the soil pH and electrical conductivity (EC). We determined the soil cellulase activity, sucrase activity, chitinase activity, NH4+-N and NO3−-N using the Soil Kit (Comin). Five independent replicate assays were extracted and measured for each sample.

2.3 | High-throughput sequencing

Total DNA extraction was done using a soil total Genomic DNA Extraction kit (BioFlux). The bacterial and fungal communities were characterized by amplifying the
V3–V4 regions and internal transcribed spacer (ITS1), respectively. The polymerase chain reaction (PCR) primers and reaction conditions are listed in Tables S2 and S3 (Roggenbuck et al., 2014; Wang, Wang et al., 2019). The library was then sequenced on an Illumina HiSeq 2500 platform. Five independent sequencing were performed for each treatment. The detailed procedures of PCR amplification and miseq sequencing are shown in Text S2. The raw data were submitted to the NCBI SRA database with the submission accession PRJNA769257.

2.4 Effect of biochar treatment on the antagonistic activity of PGPBs against the pathogenic fungi

We used an amended agar disk diffusion method to evaluate the antagonistic activity. Briefly, the culture medium consisted of 1/4 potato dextrose agar (PDA) and different concentration gradients (1%, 2% and 3%, w/v) of biochar. In each culture dish, we set up half of the culture medium as the control with 1/4 PDA, the other half consisted of 1/4 PDA-biochar. Three strains of PGPBs (Burkholderia contaminans, Pseudomonas tolaasii and Burkholderia contaminans) were streaked onto the medium and dispersed over the control and biochar treatments with the same distance to the center of the plate, respectively. The pathogenic fungi were then removed from the margins of actively growing colonies of F. oxysporum FJ, T. helicus and F. oxysporum GZ, and placed into the center of the plate. The plates were then incubated for 2 days at 37°C, followed by an additional 5 days at 30°C.

2.5 Quantitative Real-time PCR (qRT-PCR) analysis of microbial communities

Total soil DNA was extracted using the soil Genomic DNA Extraction Kit (BioFast). Five replicates of each treatment were extracted. The abundances of total bacteria (Eub338/Eub518) (Kielak et al., 2008; Weedon et al., 2012), total fungi (ITS1F/ITS4) (Gao et al., 2008; Okubo & Sugiyama, 2009), F. oxysporum (ITS1F/AF308R) (Lievens et al., 2005), T. helicus (TH1F/TH1R) (Wu et al., 2016), Pseudomonas spp. (Ps-for/Ps-rev) (Fierer et al., 2005) and Bacillus spp. (BacF/1378) (Fierer et al., 2005) were determined using a CFX96 Real-Time system (Bioretd. The primers and PCR amplification conditions are listed in Table S1 and S2. qRT-PCR was performed as previously described (Wu et al., 2016). Five independent quantitative PCR assays were performed for each treatment.

2.6 Extraction and detection of soil metabolites

In this study, we extracted and detected the rhizosphere soil metabolites under SY, SYB and SYBB treatments. All the rhizosphere soils were extracted and analyzed in five replicates, respectively. In short, we added 15 g of each soil sample into 35 ml of methanolic acetonitrile:H2O = 2:2:1 (v/v/v). In addition, 20 μl of 2-chloro-L-phenylalanine (3.5 mg ml⁻¹ stock in methanol) was added as internal standard and mixed by vortexing for 30 s. Homogenization of the sample then proceeded in a ball mill for 4 min at 45 Hz, followed by ultrasound treatment for 5 min (incubated in ice water). After homogenization for three times, the sample was incubated for 1 h at −20°C to precipitate proteins. The samples were centrifuged for 15 min at 4°C and 13,800 g, and the supernatants were transferred into 50 ml EP tubes. Thereafter, 35 ml of methanol:acetonitrile:H2O = 2:2:1 (v/v/v) was added to the soil sample. The ball milling and centrifugation steps were repeated. All the resultant extracts were pooled and evaporated completely in a vacuum concentrator at 4°C and then dissolved in 400 μl of acetonitrile:H2O = 1:1 (v/v) followed by vortexing for 30 s and ultrasound for 10 min (incubated in ice water). Next, the suspension was centrifuged at 16,200 g for 15 min at 4°C. Thereafter, the liquid supernatant was collected. The supernatant (75 μl) was transferred into a fresh 2 ml liquid chromatography/mass spectrometry (LC/MS) glass vial for the ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry analysis, and 20 μl was taken from each sample and pooled as quality control samples.

The extracts were subsequently analyzed using an ExionLC Infinity series UHPLC System (AB Sciex), equipped with a UPLC BEH Amide column (2.1 x 100 mm, 1.7 μm; Waters). To acquire MS/MS spectra on an information-dependent basis during an LC/MS experiment, TripleTOF 5600 mass spectrometry (AB Sciex) was utilized. The detailed detection condition of metabolites is shown in the Supplementary Information (Text S3).

2.7 Statistical analysis

Alpha diversity indices (Shannon, Simpson, Chao1 and ACE) were calculated at the operational taxonomic units (OTU) level using QIIME platform on the basis of cumulative sum scaling normalization and sub-sampling. Principal coordinates analysis (PCoA) was used to analyze the discrepancies among samples at the level of OTUs. The data were visualized using Circos (Version
The Sloan neutral community model (NCM) was used to evaluate the potential importance of stochastic processes to soil microbial community assembly (Sloan et al., 2006). We further used the checkerboard score (C-score) metric to indicate the microbial co-occurrence patterns, and standardized effect size (SES) to estimate the relative importance of stochastic and deterministic processes (Mo et al., 2021; Stone & Roberts, 1990). The beta Nearest Taxon Index ($\beta$NTI) was used to assess the relative importance of stochastic and deterministic processes in bacterial and fungal assembly, respectively (Stegen et al., 2013). For the microbial community, $|\beta$NTI$| < 2$ and $>2$ indicated the dominance of stochastic and deterministic processes, respectively. $\beta$NTI > +2 indicated variable selection while $\beta$NTI < −2 indicated homogeneous selection. $|\beta$NTI$| < 2$ and RCbray > 0.95 indicated dispersal limitation; $|\beta$NTI$| < 2$ and RCbray < −0.95 indicated homogenizing dispersal. Furthermore, the undominated fraction was quantified as the percentage of pairwise comparisons with $|\beta$NTI$| < 2$ and $|\text{RC}_{\text{bray}}| < 0.95$.

The differential metabolites were screened out using analysis of variance and the least significant difference test (significance level of $p$ value < 0.05), and using orthogonal partial least squares discriminant analysis with a variable importance (VIP) values > 1.0. The diversity index of the soil metabolites was calculated as the Shannon index using R 4.0.2. The co-occurrence networks were generated using “igraph” package (Csardi & Nepusz, 2006) in R 4.0.2 and focused on the correlation with the absolute value of Spearman coefficient >0.6 and $p < 0.01$, and visualized by the “Gephi” interactive platform (Ver 0.9.2, https://gephi.org). The correlations among the soil physical-chemical properties, microbial properties and metabolites were then evaluated using Spearman’s rank correlation test. Structural equation models (SEMs) were constructed using IBM SPSS Amos 21.0.0 software package. Microbial activity (cellulase activity, sucrase activity, chitinase activity) and soil nutrients ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) represent the principal components analysis (PCA) data of the first axis of PCA of the corresponding indicators.

## 3 | RESULTS

### 3.1 | Variations in *R. pseudostellariae* functional traits and soil physicochemical characteristics

Biochar addition increased the leaf area and diameter of root tuber of *R. pseudostellariae* compared to the newly planted and second-year monocultures, respectively (Figure S2). The combined effect of biochar and PGPBs
significantly increased the leaf area and biomass of *R. pseudostellariae* as compared to the single biochar treatment (SYB) and second-year monocultures, respectively. The biochar addition significantly increased the EC and NO₃⁻-N in the rhizosphere soils of newly planted and second-year monocultures, respectively. In addition, the combined effect of biochar and PGPBs led to a significant increase in EC, pH, chitinase and sucrase activities when compared to the single biochar treatment and second-year monocultures, respectively (Table 1).

### 3.2 Microbial community structures

After the quality control, the bacterial 16S rDNA V3–V4 and fungal ITS high-throughput sequencing yielded a total of 1,511,131 and 1,598,459 effective tags across all soil samples, respectively. The bacterial and fungal sequencing yielded the number of OTUs per sample varying within the ranges of 3107–3534 and 527–910, respectively (Figure S3). When compared to newly planted (FY) and second-year monocultures (SY), a slight but insignificant decrease in the ACE, Chao1 and Shannon indices of the bacteria was observed in FYB and SYB treatments, respectively (Figure S4). The biochar addition increased (*p* > 0.05) the ACE, Chao1 and Shannon indices of the fungi in the second-year monocultures, while a significant contrasting tendency was shown in the newly planted soil (Figure S4). In addition, the combined effect of biochar and PGPBs displayed a positive effect on the bacterial alpha diversity indices as compared to the single biochar treatment (SYB) and the second-year monocultures (SY), respectively. Meanwhile, the combined effect of this treatment decreased the fungal diversity when compared to the single biochar treatment. The PCoA was conducted to elucidate shifts in the soil bacterial and fungal communities. The result showed treatment under biochar amendment and without biochar separated the bacterial communities along the second coordinate axis and separated the fungal communities along the first coordinate axis in the soil, respectively (Figure S5). The bacterial communities under combined amendment treatment were distributed between the newly planted and second-year monocultures. In addition, the fungal communities under the combined amendment treatment were distinctly different from the other samples.

The bacterial community was classified into a total of 38 different phyla, with the most predominant phyla being Proteobacteria (30.82%), Chloroflexi (17.14%), Actinobacteria (12.53%) and Acidobacteria (11.88%) (Figure 1a). Fungal sequences were associated with 14 phyla and the predominant phyla were Ascomycota, Basidiomycota and Mortierellomycota (Figure 1b). The biochar addition significantly decreased the relative abundance of the Zixibacteria, Nitrospirae, Zoopagomycota in the newly planted and second-year monocultures, respectively, while a positive effect was observed regarding the relative abundance of Actinobacteria (Figure 1c). Furthermore, the combined effect of biochar and PGPBs had a positive effect on the relative abundance of Nitrospirae, Gemmatimonadetes, Zoopagomycota, Glomeromycota, Chytridiomycota and Olpidiomycota as compared to the single biochar treatment (SYB) and second-year monocultures, respectively.

Based on the 60 most abundant bacterial and fungal genera, we used fold change to calculate the variations in the relative abundance of the genera in the SYB and SYBB treatments compared to SY treatment and in the FYB treatment compared to FY treatment, respectively. As shown in Figure 2, the microbial genera displaying the highest variation belonged to the phylum Proteobacteria, Chloroflexi, Actinobacteria, Ascomycota and Basidiomycota. Compared to the FY and SY treatments, the biochar addition significantly increased the abundance of *Bacillus*, *Streptomyces* and *Gibberella*, and significantly decreased *Gemmatimonadetes*, *Nitrospira* and *Talaromyces*. Moreover, *Pseudomonas*, *Lysobacter*, *Gemmatimonadetes*, *Nitrospira* and *Gibberella* displayed a higher relative abundance under SYBB treatment in comparison to SY and SYB treatments. In addition, the abundance of *Fusarium* and *Talaromyces* was decreased under SYBB treatment when compared to SY and SYB treatments, respectively. The relative abundance of *Bacillus* did not show significant differences between the SYBB and SY treatments.

### 3.3 The assembly processes of bacterial and fungal communities

The contribution of stochastic processes on bacterial and fungal community assembly was investigated by the Sloan NCM. The NCM explained 75.4% variability of the bacterial community across all treatments (Figure 3a), and 57.5%–63.6% variability of each treatment (Figure S6). The mitigation rate of bacteria was lower in a single biochar addition than under without-biochar treatment (Figure 3a). The explained fraction of variation and mitigation rate were lower in SYBB than in SY and SYB, respectively. For the fungal community, the biochar treatment increased the mitigation rate as compared to without-biochar addition (Figure 3b). However, the combined effect of biochar and PGPBs (SYBB) decreased the
mitigation rate as compared to the single biochar treatment (SYB) and second-year monocultures, respectively (Figure S7).

The $\beta$NTI value of the bacterial community across all treatments was higher than 2, indicating the deterministic process played a major role in the bacterial assembly (Figure 3c). The $\beta$NTI value of bacteria in the single biochar addition was higher than that under without-biochar treatment. Homogenizing dispersal and variable selection influenced the single biochar treatment more than the without-biochar treatment, while dispersal limitation was prevalent under the without-biochar treatment. Meanwhile, the SYBB had significantly lower $\beta$NTI value than SY and SYB.

For the fungal community, the $\beta$NTI value of all treatments was lower than 2 (Figure 3d). In addition,
FIGURE 2 Changes in the relative abundances of microbial genera under different treatments. (a, b) The fold change of bacterial and fungal genera under FYB, SYB and SYBB treatments relative to the FY and SY treatment. Comparisons were performed based on the 60 most different microbial genera, using variance and \( p < 0.05 \) as the threshold of significance. Error bars represent the standard deviation of the five replicates. (c) The relative abundances of potential beneficial and pathogenic microbial genera. The different letters in each column display significant differences (LSD-test, \( p < 0.05, n = 5 \)). LSD, least significant difference.
Figure 3  Fit of the Sloan neutral community model for the bacterial (a) and fungal (b) community of different treatments, respectively. Solid blue line represents the best fit to the Sloan neutral community model, and dashed blue line represents 95% confidence intervals around the neutral community model prediction. Operational taxonomic units that occur more or less frequently than predicted by the neutral community model are shown in green and red, respectively. $R^2$ indicates the fit to this model; $m$ indicates migration rate. The \( \beta \text{NTI} \) values and relative contributions of ecological processes of bacteria (c) and fungi (d) in different treatments, respectively. \( \beta \text{NTI} \), beta nearest taxon index. The different letters in each column display significant differences (LSD-test, \( p < 0.05 \)). LSD, least significant difference.
the single biochar treatment had a lower $\beta$NTI value than the without-biochar treatment. Furthermore, a higher $\beta$NTI value and prevalent variable selection were observed under the SYBB treatment than that under the SY and SYB treatments, respectively. We further employed the co-occurrence patterns by the checkerboard score (C-score) and SES metric. The result shown that the SES of bacteria and fungi under FY treatment was higher than under SY treatment (Figure 4a,b). In addition, the SYBB treatment decreased bacterial SES and increased fungal SES when compared to the SY treatment. The average niche breadth across all treatments was significantly higher for bacterial community than for the fungal community (Figure 4c). The SYBB treatment had a slight but insignificant increase in bacterial niche breadth and decrease in fungal niche breadth as compared to the SYB and SY treatments, respectively (Figure 4c). More importantly, the niche breadth of fungal community was lower in SY than in FY, respectively.

### 3.4 The effects of biochar on the antagonistic activity and populations of specific microorganism

The qRT-PCR analysis showed biochar addition significantly decreased the bacterial and fungal populations in the newly planted soil, and significantly increased the fungal populations in the second-year monocultures (Figure 5). Compared to the FY and SY, the biochar treatments also significantly decreased the amount of pathogenic *F. oxysporum* and *T. helicus*, and increased the abundance of *Bacillus* spp. In addition, the combined effect of treatment with both biochar and PGPBs significantly increased the amounts of total bacteria and of *Pseudomonas* spp., and had a negative effect on *F. oxysporum* and the ratio of *F. oxysporum*/*Pseudomonas* spp. when compared to SY and SYB treatment, respectively. The results displayed antagonistic activity by showing that PGPBs (*B. aryabhattai*, *P. tolaasii*,

![Figure 4](image-url)  
**Figure 4** Checkerboard score (C-score) metric showing random co-occurrence patterns of bacterial (a) and fungal (b) communities, respectively. The value of simulated C-score (C-score sim) > observed C-score (C-score obs) represents random co-occurrence patterns. The value of standardized effect size (SES) >2 and <−2 indicates segregation and aggregation, respectively. Comparison of mean habitat niche breadth (c) from all taxa for each treatment. The different letters in each column display significant differences (LSD-test, $p < 0.05$, $n = 5$). LSD, least significant difference
B. contaminans) were able to suppress the mycelial growth of the pathogenic fungi, *T. helicus* and *F. oxysporum*, when they were co-cultured with or without addition of biochar (Figure 5).

### 3.5 The changes of rhizosphere soil metabolites under biochar treatments

A total of 246 metabolites, including carbohydrates, amino acids, fatty acids, steroids, organic acids, alcohols, phenolic acids, etc., were detected and identified across SY, SYB and SYBB treatments. We observed a significant decrease in soil metabolite diversity under SYBB treatment (Figure S8). The PCA analysis showed that the metabolite profiles under SYB and SYBB treatments were significantly different from those under SY treatment, and they could be separated along the first and third coordinate axes (Figure 6a). Overall, 126 different metabolites were identified across the three groups. Specifically, the abundance of 54 metabolites increased and that of 28 metabolites decreased under SYB treatment compared to SY treatment; the abundance of 33 metabolites increased and those of 36 metabolites decreased under SYBB treatment compared to SY treatment; the abundance of 19 metabolites increased and those of 38 metabolites decreased under SYBB treatment compared to SYB treatment (Figure 6c). Out of the 126 different metabolites, 12 were overlapped among the three groups (Figure 6b).

Among the 126 differentially abundant metabolites, 73 comparably abundant metabolites (*p* < 0.5, VIP > 1) in relation to the biochar and PGPBs treatments are shown in Figure 7. Generally, gibberellin A53, abscisic acid,
ginsenoside Ia, maltohexaose, stachyose, cyclolinopeptide I, N-acetylglutamic acid, bovinic acid, azelaic acid, arachidonic acid, acetylatederonic acid, lipoic acid, hepxilin B3, hexadecanedioic acid and medicagenic acid significantly increased, while pyroglutamic acid, L-phenylalanine, L-leucine and L-lactic acid significantly decreased under SYBB treatment compared to SY treatment (Figure 7a). The most increased metabolites were carbohydrates and fatty acids, and most decreased metabolites were amino acids under SYBB treatment. Moreover, the plant hormones (gibberellin A53 and abscisic acid) significantly increased under SYBB treatment compared to SYB treatment.

3.6 Correlation analysis of soil properties, microbial communities and metabolites

Co-occurrence network analysis was used to compare the complexity of microbiome associations in different treatments. The bacterial empirical co-occurrence networks differed significantly with the biochar amendment into the systems as revealed by the network parameters (Figure S9, Table S4). The FYB and SYB treatments significantly decreased the number of edges but increased the modularity and positive correlations of the bacterial networks compared to FY and SY treatments, respectively (Table S4). Notably, the SYBB treatment increased the density and the number of edges compared to the SY and SYB treatments. The fungal community co-occurrence under FYB treatment showed higher edge numbers and positive correlations compared to FY treatment, while the opposite trend was observed under SYB treatment in comparison to SY treatment (Figure S10, Table S4). The SYBB treatment occupied higher edge and node numbers but with less positive correlations compared to SY treatment.

To further elucidate the correlation between the most differently abundant microbial genera and differentially occurring metabolites, six interactive networks were constructed (Figure 8). Generally, the SYB and SYBB...
treatments increased the node numbers and positive correlations in the bacteria-metabolite networks compared to the SY treatment (Table S5). While the opposite trends were displayed in fungi-metabolite networks. Agglomeration of co-occurrence networks was observed in bacteria-metabolite networks (Figure 8). The beneficial Gemmatimonadetes and Nitrospira were strongly co-occurring with other nodes in network of SYBB treatment, which occupied a higher abundance under SYBB treatment. The fungi and metabolites were uniformly distributed in networks without intensive clusters (Figure 8).

Spearman correlation analysis was used to evaluate the correlation between soil physical–chemical properties, microbial properties and metabolites. The metabolite diversity exhibited negative correlation to pH, EC, microbial diversity and richness, respectively (Table S6). Soil pH was positively and significantly correlated to sucrose and chitinase activities. The abundances of T. helicus, Talaromyces, F. oxysporum and Fusarium were significantly negatively correlated to metabolites most increased under SYBB treatment, while significantly positively correlated to decreasing metabolites (Figure 9). Furthermore, opposite trends were observed in the abundances of Gibberella, Streptomyces, Pseudomonas and Lysobacter. The Ginsenoside Ia and cyclolinopeptide I had positive correlation ($p < 0.05$) to the abundances of Penicillium, Bacillus and Trichoderma. Meanwhile, the Bacillus was significantly negatively correlated to decreasing metabolites under SYBB treatment. The gibberellin A53, abscisic acid, maltohexaose and stachyose contents were significantly positively correlated to the presence of Pseudomonas and Lysobacter, respectively. Gemmatimonadetes was significantly positively correlated with ginsenoside Rg3 and galactose, and negatively correlated with cyclolinopeptide I.

We analyzed the contributions of soil properties to the dissimilarities of microbial phyla and microbial communities. The results showed the contributions of soil properties were significant to the differences in relative abundances of most phyla (Figure 10a). And the contents of pH, $\text{NH}_4^+$-$\text{N}$ and chitinase were positive and strong predictors for differences in relative abundances of most phyla, and dissimilarities of microbial communities. The contents of $\text{NO}_3^-$-$\text{N}$ and moisture were negative predictors for the differentially microbial phyla. SEMs indicated that the combination of
biochar and PGPBs treatment had a significantly positive and direct effect on the soil pH, microbial activity (soil enzyme activities) and plant biomass under consecutive monoculture regimes (Figure 10b). Furthermore, the combination of biochar and PGPBs had a more direct or indirect influence on bacterial richness than on fungal richness. The soil nutrient (NH$_4^+$-N and NO$_3^-$-N) had not significantly positive effect on the soil microbiota.

**4 | DISCUSSION**

**4.1 | Biochar altered the microbial diversity, composition and assembly processes under continuous 2 years monoculture regimes**

Biochar has promise as a soil amendment improving crop growth and increase crop yields by modulating soil conditions (Jiang et al., 2020; Liu et al., 2017). However, there is documentation showing a large variation in plant productivity responses to biochar application in soil in previous studies due to biochar properties, experimental types (pot or field), soil conditions, types of target plants and conditions of fertilizer utilization (Dai et al., 2020; Hussain et al., 2017). Hussain et al. (2017) had summarized the responses in plant productivity in biochar-amended soils ranging from −35.8% to +294%. In this study, our field experiment indicated the biochar application was able to increase root biomass and alleviate serious replanting disease of *R. pseudostellariae*, which is consistent with the results of previous pot experiment (Wu, Qin, et al., 2020).

There is general consensus that the process of replanting disease was mainly due to the changes in soil microbial community and structure. In this study, biochar amendment had an insignificant negative effect on the bacterial community diversity in newly planted and...
second-year monocultures, but increased the fungal diversity and abundance in second-year monocultures (Figure 5j; Figure S4). Our findings are consistent with previous studies showing biochar addition increased the fungal diversity in replant disease soil of apple (Wang, Ma, et al., 2019), and had no significant effect on bacterial community diversity at a concentration of 0.5% (w/w) in replant disease soil of *P. notoginseng* (Wang et al., 2020). The biochar effect on microbial diversity might be influenced by biochar types, biochar application rate and soil conditions (Han et al., 2019; Li et al., 2020; Wang et al., 2020; Wang, Ma, et al., 2019). Recent laboratory studies have revealed that biochar could influence the metabolic processes of pathogenic *F. oxysporum* while inhibiting the mycelial growth and abating the virulence on crops (Wu, Qin, et al., 2020). When looking at bacterial wilt, biochar addition was able to adsorb pathogenic *Ralstonia solanacearum* and inhibit the swarming motility of pathogens and subsequent...
rhizosphere colonization (Gu et al., 2017). In this study, biochar amendment was shown to significantly increase the abundance of potentially beneficial *Trichoderma*, *Mortierella* and *Acremonium*, while decreasing the abundance of pathogenic *Fusarium*, *Talaromyces*, *F. oxysporum* and *T. helicus* in second-year monocultures (Figures 2c and 5j). In addition, the abundance of beneficial *Bacillus*, *Streptomyces* and *Pseudomonas* spp. was also significantly increased under biochar treatment in second-year monocultures. Our results indicated that biochar amendment enriched fungal diversity and perhaps increased the ratio of beneficial microbes to pathogens, thereby alleviating serious replanting disease. More studies are needed to elucidate the mechanisms of how biochar decreased pathogens and increased beneficial microbes in rhizosphere soils of crops in long-term field trials.

Our study indicated that continuous monoculture and biochar addition had an important influence on the assembly of bacterial and fungal communities in the rhizosphere soils, primarily by affecting the balance between stochastic and deterministic processes. Previous study had shown that the environmental changes and agricultural practices were able to mediate the balance between deterministic and stochastic processes to govern the microbial community assembly (Zhou & Wu, 2021). Our study was supported by the null model analysis, which demonstrated the dominant effect of deterministic processes on the bacterial community and stochastic processes on the fungal community under consecutive monoculture regimes. In addition, the single biochar addition increased the βNTI value and decreased the migration rate of Sloan neutral community model in the bacterial community, but a reverse pattern was observed in the fungal community. This indicated the biochar addition promoted the deterministic processes of bacterial community assembly, which was consistent with previous experiments (Lu et al., 2021). This might be due to biochar creating a favorable habitat by providing suitable abiotic environmental conditions and excluding specific communities of certain taxa. Meanwhile, the SYBB treatment resulted in narrower niche breadth and lower migration rate, and contained higher βNTI value and SES than in second-year monocultures soil, indicating that the fungal community assembly was more strongly converting stochastic processes into deterministic processes under the combination of biochar and PGPBs treatment, likely because SYBB treatment altered the fungal community structure and the deterministic processes tended to have higher SES and have a stronger effect on a narrow community-level niche breadth than on the wide one (Mo et al., 2021; Wu et al., 2018). Interestingly, the opposite effect was observed in the bacterial community under the combination of biochar and PGPBs treatment. The potential mechanism might be that the combination of biochar and PGPBs was able to act as a shelter and provide nutrients for the soil microbiota, leading to a wider niche breadth and higher diversity of the bacterial community, which again increased stochastic processes (Mo et al., 2021). Therefore, more studies are needed to explore the mechanism of ecological processes governing soil microbiota under different agricultural environment.

### 4.2 A combination of biochar and PGPBs stimulated the indigenous and beneficial soil microbes to suppress host-specific pathogens

Biochar and PGPBs have often been used in soil remediation due to their suppressive effects on soil-borne disease (Wang et al., 2020; Xiong et al., 2017). However, not many field trials have been conducted to evaluate biocontrol by a combination of biochar and PGPBs on replanting disease of Chinese medicinal plants. In the present study, a combination of biochar and PGPBs treatment exhibited a more pronounced alleviation effect on replanting disease compared to single biochar treatment (Figure S2). Previous studies indicated that replanting disease could have serious negative effects on biological activities of soil microbes, which significantly altered the soil chitinase, cellulase and sucrase activities (Wu, Lin, et al., 2020). The chitinase and cellulase activities are involved in the antagonistic activity against soil-borne pathogens (Budi et al., 2000; Prasad et al., 2013). Sucrease is an extracellular enzymes involved in soil C and N cycles, and is widely used to assess the soil maturity and soil fertility (Shen et al., 2019). Our study suggested that a combination of biochar and PGPBs significantly increased chitinase and sucrase activities when compared to the single biochar and without biochar treatments (Table 1). Soil enzymes are mainly secreted by soil microorganisms and the enzymes activities would be influenced by a change in the soil microbial community (Shen et al., 2019; Wang et al., 2016). Furthermore, we also found the combined effect of biochar and PGPBs significantly increased bacterial abundance and decreased fungal abundance, but had not significant effect on microbial diversity as comparing to the signal biochar treatment. PCoA results showed significant differences in microbial community composition between SYBB and other treatments. These findings indicated that the combined effect of biochar and PGPBs significantly influenced variations in microbial community composition and structure.

Co-occurrence network analysis indicated that a combination of biochar and PGPBs treatment was responsible for the alteration and increasing the connections in microbial communities. A highly connected
microbiome surrounding the root has been verified to decrease the success of pathogen invasion (Mendes et al., 2018; Wei et al., 2015). A significant increase in plant-beneficial bacteria (e.g. *Pseudomonas, Lysobacter, Gemmatimonadetes*) and a decrease in pathogens (e.g. *Fusarium, Talaromyces* and *F. oxysporum*) were observed under SYBB treatment compared to SY and SYB treatment. We also found PGPBs did suppress the mycelial growth of pathogenic fungi when they were co-cultured with the addition to biochar. The increased abundance of beneficial bacterial members appeared to be caused by *R. pseudostellariae* root exudates recruiting these bacteria. Under SYBB treatment, PGPBs were a mixture of *Pseudomonas* spp. However, the relative abundance of *Bacillus* spp. was significantly lower in SYBB treatment compared to single biochar treatment (Figure 2c). These introduced PGPBs might compete for resources in their niche with the indigenous microbes in soil, which would reduce their level of colonization and adaptive abilities (Haas & Defago, 2005; Ye et al., 2020). This indicated the artificially introduced PGPBs conferred little direct antagonistic effects on soil-borne pathogens. Our results are in line with the results previously reported Xiong et al. (2017), who observed the introduced *Bacillus* spp. and *Trichoderma* spp. had only a limited capacity of survival in soil while increasing the abundance of indigenous beneficial microbes. These results indicated the combination treatment of biochar and PGPBs stimulated indigenous and beneficial soil microbes to suppress host-specific pathogens.

Our previous study showed plant-beneficial microbes were able to increase the pH value of the respective environment over time, while pathogenic fungi decreased the pH and promoted the $H^+$ efflux of the plant root (Wu, Qin, et al., 2019). Therefore, the combined effects of biochar and PGPBs significantly increasing the soil pH might due to the accumulation of beneficial microbes, but this hypothesis needs further verification. Moreover, our results showed SYBB treatment significantly increased the relative abundance of *Gibberella* and the content of gibberellin A53 in comparison to SY treatment. *Gibberella* is a well-known pathogen of rice and wheat plants, causing plants to excessively grow by secreting gibberellins. Gibberellins are widely used to promote root hair abundance, root growth, seeds germination, regulation of vegetative and reproductive bud dormancy and involvement in yield increases of crops (Bottini et al., 2004). Nevertheless, the changes in *Gibberella* and gibberellin under the combination treatment of biochar and PGPBs were interesting phenomena, which are worthy of further investigation of their ecologic niche and function in the rhizosphere of *R. pseudostellariae*.

### 4.3 A combination of biochar and PGPBs regulated rhizosphere soil metabolites to mediate the microbial communities

Soil metabolite analysis was used to provide hints as to the reasons causing changes in the microbial community and functional properties in replanting disease soils mediated by introducing biochar and PGPBs. We observed a significant decrease in soil metabolite diversity under treatment by a combination of biochar and PGPBs, and negative correlation to soil microbial diversity and richness. SEMs indicated that combination of biochar and PGPBs had an indirect effect on the soil microbiota and plant biomass by influencing metabolite diversity. This suggests that a microbial community displaying a higher abundance could rapidly consume metabolites, thereby decreasing accumulation and diversity of soil metabolites (Li et al., 2020). We found the combination of biochar and PGPBs significantly increased the content of phytohormones (gibberellin A53 and abscisic acid). Abscisic acid not only regulates the response of plants to drought, salt and cold stress, but also has a primary function in modulating disease resistance and plant pathogen defense (Asselbergh et al., 2008). This indicated that rhizosphere interactions had a positive effect on the growth of *R. pseudostellariae* under the combination treatment. Interestingly, the cardinal components (ginsenoside Ia and cyclolinopeptide I) of *R. pseudostellariae* also increased under SYBB treatment (Figure 7). These two components were negatively correlated to the presence of pathogens and positively correlated to the presence of most beneficial microbes (Figure 9). This might confirm that the increase in beneficial microbes contributed to the accumulation of pharmacodynamic components and vice versa.

Moreover, the relative abundances of fatty acids and sugars (maltohexaose and stachyose) were higher under combination treatment by biochar and PGPBs. Previous studies have shown the observed high levels of fatty acids conferred an increase in resistance to pathogens (Abu-Nada et al., 2007). Our study also showed that fatty acids were negatively associated with the presence of pathogens and positively associated with the presence of beneficial microbes. The sugars provided energy for microbial growth in the rhizosphere soil (Chaparro et al., 2013) and had been identified as the most efficient chemoattractant for beneficial myxobacteria (Ye et al., 2020). Our results indicated that sugars (maltohexaose and stachyose) were significantly positively correlated to the presence of beneficial *Pseudomonas* and *Lysobacter*. The maltohexaose and stachyose may play important roles in chemotaxis and colonization of *Pseudomonas* and *Lysobacter*. The ecological function of sugars in interactions between...
the root and beneficial microbes deserves further investigation in future studies. Moreover, the combination of biochar and PGPBs enhanced alteration of co-occurrence networks among the microbial communities and metabolites, which increased positive correlations in the bacteria metabolite networks and decreased positive correlations in the fungi-metabolite networks. The observed positive correlation between microbes and metabolites might contribute to the proliferation of some specific microbes and protection against pathogens infection. Therefore, the relationship between the microbial communities and soil metabolites will guide regulation of rhizosphere processes through biochar and PGPBs amendments to reduce plant disease and increase plant yields.

5 | CONCLUSIONS

The positive effect of amendment with PGPBs and biochar on increasing crop yields and alleviating soil-borne pathogens has been widely recognized (Dai et al., 2020; Wang et al., 2020; Xiong et al., 2017), while the combination of biochar and PGPBs for the biocontrol of replanting disease has received less attention. Our study indicated the dominant effect of deterministic processes on bacterial community and of stochastic processes on fungal community under consecutive monoculture regimes. The combination of biochar and PGPBs tended to convert the stochastic processes of fungal community assembly into deterministic processes. The SYBB treatment was able to extend bacterial niche breadth and decrease fungal niche breadth as compared to the SYB and SY treatments, respectively. The combination of biochar and PGPBs stimulated the indigenous and beneficial soil microbes to suppress host-specific pathogens by mediating an increase production of rhizosphere soil metabolites, further alleviating the serious replanting disease. This study can provide a starting point for a better understanding of a combined application of biochar and PGPBs that could be an effective and promising strategy for the sustainable and green remediation of replanting disease in future.

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CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available in the Supporting Information of this article.

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