Pseudostellaria heterophylla cultivar mixtures driven changes in rhizosphere metabolites to suppress soil-borne Fusarium disease

# *Pseudostellaria heterophylla* cultivar mixtures driven changes in rhizosphere metabolites to suppress soil-borne *Fusarium* disease

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**Abstract**

Crop diversification contributes to a decrease in soil-borne crop diseases, as well as an increase in agricultural productivity. However, few studies have investigated the changes in the composition of the rhizosphere microbial communities and rhizosphere metabolites, as well as their suppressive effect on soil-borne diseases under different

crop cultivar mixture regimes. We carried out a series of experiments to assess changes in the rhizosphere microbial community and metabolites profile under different *Pseudostellaria heterophylla* cultivar mixture cultivation in consecutive monoculture fields by employing amplicon metagenomics (16S rRNA, ITS, and 18S rRNA) and non-targeted metabolomics. The impact of key metabolites on pathogenic *Fusarium oxysporum*, crop growth, and soil microorganisms was assessed under controlled conditions. Our study indicated that the cultivar mixtures improved the *P. heterophylla* performance, increased the fresh root biomass by 81.9% to 115.4% and the heterophyllin B content by 35% compared to the consecutive monoculture, respectively. Cultivar mixtures increased the abundance of beneficial bacteria (*Lactobacillus*, *Pseudomonas*, *Nitrosospira*) and consumer protists, and decreased the abundance of pathogenic fungal genera (*Fusarium*, *Alternaria*, *Curvularia*, *Stemphylium*, *Gibberella*). The qPCR results indicated that the cultivar mixtures significantly decreased the abundance of pathogenic *F. oxysporum* by 64.0% to 84.3% compared to the consecutive monoculture treatment. Non-targeted metabolomics analysis showed that the cultivar mixtures significantly altered the soil metabolite profiles, and increased the contents of d-galactose, galactinol, d-sorbitol, glycerol, melibiose, D-fructose and D-tagatose. Subsequently, the key upregulated metabolites (glycerol, d-fructose, gluconic acid, quinic acid, and l-valine), identified through the random forest analysis, significantly inhibited the growth of *F. oxysporum*. The crucial metabolites in the presence of a pathogen (*F. oxysporum*) and single metabolite treatment significantly increased the biomass, SOD and CAT activity and decreased the POD and MAD activity of *P. heterophylla* compared to FOP (*F. oxysporum* treatment). Furthermore, the crucial metabolites under pathogen treatment significantly lowered the abundance of total fungi and *F. oxysporum* and increased the abundance of *Pseudomonas* spp. compared to FOP. Therefore, our study was able to emphasize the efficacy of using cultivar mixtures to combat soil-borne *Fusarium* disease through the modulation of rhizosphere metabolites.

**Keywords:** Soil-borne disease; Crop diversification; Rhizosphere microbiome;

## 1. Introduction

The increasing incidence and severity of soil-borne disease outbreaks pose significant and growing risks to crop productivity and global food security worldwide (Singh et al., 2023; Batista et al., 2024). In particular, in consecutive monoculture practices, *Fusarium* disease causes vascular wilt in more than 100 different host plants, posing an enormous constraint on cucumber (Zhou et al., 2023a), banana (Ren et al., 2024), peanut (Zhou et al., 2023b) and Chinese medicinal plants (Liao and Xia, 2024) yields. Soil-borne *Fusarium* disease is managed by applying chemical pesticides (Chen et al., 2020), implementing biological control agents (Tao et al., 2020), cultivating disease-resistant varieties (Rahman et al., 2021), or diversifying crops (Zhu et al., 2000; Zhou et al., 2023a). The diversification of cultivar mixtures contributed to a decrease in plant and soil-borne diseases and increases crop yield (Mundt, 2002; Hiddink et al., 2005; Jia et al., 2024). Crop cultivar mixtures have significantly contributed to the reduction of fungal disease spread (Vidal et al., 2017), while also decreasing the fungicide input and mitigating the development of fungicide resistance (Kristoffersen et al., 2020). As a result, crop cultivar mixtures provide a more environmentally friendly and sustainable strategy for suppressing crop diseases.

Crop cultivar mixtures were shown to have significant positive effects on agricultural productivity by improving yield stability, increasing soil microbial diversity, and reducing diseases (Yang et al., 2019). For example, rice cultivar mixtures decreased rice blast disease (caused by *Magnaporthe oryzae*) and increased grain yield compared with monocultures (Zhu et al., 2000). Mixed cropping of maize cultivars enhanced the resistance of maize to *Puccinia polysora*, agent of the southern corn rust disease (Liu et al., 2013). Cultivar mixtures of maize varieties had a pronounced impact on shaping and defining the rhizosphere bacterial community, thereby increasing nutrient accumulation and improving plant growth (Jia et al., 2024). Our previous studies also showed that intraspecific intercropping changed microbial community diversity and alleviated the serious soil-borne disease of *Pseudostellaria heterophylla* by increasing

the abundance of potentially beneficial microorganisms and decreasing the number of pathogens (Wu et al., 2020). Furthermore, crop cultivar mixtures effectively mitigate the harmful effects of pathogens on crop production by disrupting the growth of pathogens, thereby providing a cost-effective alternative for controlling soil-borne diseases. However, how crop cultivar mixtures regulate the effect of rhizosphere microbial communities on crop soil-borne diseases are still largely unknown.

Rhizosphere soil microbial communities and root-derived metabolites have been shown to be affected by consecutive monocultures, leading to the accumulation of soil-borne pathogens in soil (Wu et al., 2019; Chen et al., 2022). Compared to consecutive monocultures, diversification cultivation strengthened the capacity of soils to prevent the entry of pathogens into the rhizosphere and inhibited the development of soil-borne diseases (Zhou et al., 2023b). Previous research has demonstrated that diversification cultivation systems are able to help recruiting beneficial indigenous soil microbes via root exudates, thereby contributing to improved host resistance against pathogens (Zhalnina et al., 2018; Zhu and Morel, 2018). For example, tomato-potatoonion intercropping was able to enhance tomato resistance to *Verticillium* wilt disease by altering the rhizosphere microbiome and recruiting beneficial bacteria via root exudation of taxifolin (Zhou et al., 2023a). Changes in rotation-driven rhizosphere metabolites increased the soil microbial functional capacity and diversity, further improving rice production (Wang et al., 2023). The rhizosphere microbiome and its metabolites established a key bridge connecting soil functionalities and the health of plants (Bi et al., 2022). Thus, crop communities that fluctuate among different agricultural practice regimes were able to produce varied rhizosphere metabolites and further alter the stability of agricultural ecosystems (Zhou et al., 2023b). However, only a few studies have revealed the composition of crop rhizosphere metabolites and their suppressive effect on soil-borne diseases under crop cultivar mixture cultivation.

Here, we focused on *Pseudostellaria heterophylla* (Caryophyllaceae), a prized medicinal herb originating from China. It is frequently utilized as a substitute for ginseng among children due to its gentle effects, which invigorate the spleen, replenish qi, nourish the lungs, and promote overall blood health (Hu et al., 2019). However, it

suffers from serious soil-borne *Fusarium* disease under consecutive monoculture regimes (Wu et al., 2019; Wu et al., 2020). We conducted a series of field experiments to assess the impact of different *P. heterophylla* cultivar mixture cultivation on the soil-borne *Fusarium* disease in consecutive monoculture field, by comprehensively analyzing the rhizosphere microbial community and metabolite profile. Furthermore, we thoroughly analyzed the influence of key metabolites on pathogenic *F. oxysporum*, *P. heterophylla* growth, and soil microorganisms based on laboratory experiments. We hypothesized that when exposed to different crop cultivar mixtures, *P. heterophylla* would recruit beneficial microorganisms and change the metabolite compositions in rhizosphere soil, and that the interaction between different metabolites and microorganisms would promote crop growth and the suppression of soil-borne *Fusarium* disease.

## 2. Materials and Methods

### 2.1. Field experiment and sampling

Field experiments were carried out in Zherong City, Fujian Province, China, located at 119°55′E, 27°17′N, featuring an altitude of 660 m, an annual average precipitation ranging from 2000 mm, and an annual mean temperature of 16.2°C. The study was conducted in a field specifically cultivated with *P. heterophylla* for two consecutive years. The field soil had the following characteristics: red-yellow soil, pH 5.36, total nitrogen 1.853 g $kg^{-1}$, total potassium 2.788 g $kg^{-1}$, and total phosphorus 0.358 g $kg^{-1}$. We used the *P. heterophylla* varieties Zheshen 2 (ZSII), Qiantaizishen 1 (QT), Shitai 1 (ST), Xuanshen 1 (XS), and Kangdu 1 (KD), which are predominantly cultivated at scale in their respective geo-authentic production zones across Fujian, Guizhou, Anhui, and Shandong provinces. These varieties exhibited significant variations in plant height, root length, root circumference, and overall yield (Lin et al., 2022). The field experiments consisted of four different treatments, including a control plot that had never been cultivated with *P. heterophylla* before (CK), a single *P. heterophylla* cultivar (ZSII) planted in the consecutively two-year monoculture soil (TY), a mixed *P. heterophylla* cultivar (ZSII: QT: ST: XS: KD=2:1:1:1:1, w/w) planted

in the consecutively two-year monoculture soil (MixW), and a mixed *P. heterophylla* cultivar (ZSII: QT: ST: XS: KD=3:1:1:1:1, w/w) planted in the consecutively two-year monoculture soil (MixH). The planting density of *P. heterophylla* was 450 kg per hectare in the field. The *P. heterophylla* was sown to the ridges by ditch cultivation. The field experiment used a randomized block design, assigning five replicate plots to each treatment group. Each treatment comprised an area of 667 $m^2$. The application concentrations of fertilizers during the cultivation phases of *P. heterophylla* were as stipulated: a fused calcium-magnesium phosphate (fertilizer) concentration of 1500 kg $hm^{-2}$, a urea concentration of 640 kg $hm^{-2}$ and a potassium sulphate concentration of 288 kg $hm^{-2}$. Additionally, the fertilizer application during the growth periods of *P. heterophylla* was as follows: a urea concentration of 160 kg $hm^{-2}$ and a potassium sulphate concentration of 72 kg $hm^{-2}$. Throughout the experiment, the weed and pest management of the *P. heterophylla* cultivation adhered strictly to the Good Agricultural Practice (GAP) standards. At the harvest growth stage, we collected samples of leaves, roots, and rhizosphere soil of *P. heterophylla* for analysis on June 15, 2023. Each experimental treatment was performed with five replicates. Rhizosphere soil samples were randomly gathered from five spots within each replicate, and subsequently combined to create composite samples for analysis. A set of soil samples were preserved at -80°C to ensure their integrity for DNA and metabolite analysis, while the remaining samples were maintained at room temperature and subsequently air-dried to facilitate the examination of their physicochemical properties.

### 2.2. Plant traits and soil physicochemical analyses

The diameter and length of the root tubers, leaf area, stem length, and root biomass of *P. heterophylla* were measured for each sampled plant. We used water extraction and phenol-sulfuric acid approach to analyze the overall polysaccharide content of *P. heterophylla* roots (Wu et al., 2022b). The heterophyllin B content of *P. heterophylla* roots was determined using HPLC-MS (Wang et al., 2010; Wu et al., 2022b). Soil pH, electrical conductivity (EC), $NO_3^-$-N, $NH_4^+$-N, sucrase activity, and cellulase activity were assessed using methods described in previous studies (Wu et al., 2022a).

### 2.3. Quantification of specific microbial taxa

Total DNA from both rhizosphere and pot soils was isolated using a soil DNA Extraction Kit (BioFast, China) following the specific protocols for each sample type. The concentration and quality of the extracted DNA were assessed using a Nanodrop spectrophotometer and the integrity of the DNA was evaluated by agarose gel electrophoresis. The abundance of *Fusarium oxysporum* (Lievens et al., 2005) and *Pseudomonas* spp. (Fierer et al., 2005) in soils was assessed and quantified using quantitative polymerase chain reaction (qPCR) with a CFX96 instrument from Bio-RAD, US (see Tab. S2 and S3 for details). The qPCR procedure followed the methodology outlined in a previous study (Wu et al., 2016).

### 2.4. Soil bacterial, fungal and protist community analysis

The soil DNA samples were used to create libraries for metagenomic analysis of 16S, ITS, and 18S amplicons. The bacterial community was characterized by preparing 16S libraries, focusing on the V3-V4 region of the 16S rRNA gene, using primers 341F and 806R (Wang et al., 2019). The fungal community was enhanced through amplification using the primers ITS1F and ITS2R (Adams et al., 2013). The protist community within the rhizosphere soil was profiled by focusing on the 18S rRNA gene using primers V4_1f and TAReukREV3 for specific characterization (Xiong et al., 2020). The primer sequences utilized for the PCR reactions are comprehensively outlined in Table S2, while the specific conditions required for conducting these reactions are detailed in Table S3. Paired-end sequencing was conducted using an Illumina NovaSeq 6000 instrument with the SP 250PE chemistry.

### 2.5. Extraction and profiling of rhizosphere soil metabolites

Five replicates of rhizosphere soil were extracted and analyzed from the TY, MixW, and MixH treatments. Briefly, we added 1 g of each soil sample to 1 mL methanol-water solution (v:v=1:1) containing 20 μL 2-Chloro-L-phenylalanine (0.3 mg/mL), precooled the mixture for 5 min at -20°C, and ground the mixture for 20 min in the miller at 0°C. After centrifugation at 10,000 rpm for 10 min, the liquid

supernatant was collected into a new tube. The resultant extracts were subjected to freeze-drying, followed by redissolution in a 400 μL mixture of methanol and water (v:v=1:1). Subsequently, the solution was vigorously swirled for a duration of 2 minutes, and then ultrasonically processed in an ice-water bath for another 2 minutes. Finally, the mixture was centrifuged at a speed of 12,000 rpm for a total of 10 minutes. To continue, a volume of 300 μL of the supernatant was transferred to the injection bottle and subjected to freeze-drying. Subsequently, 80 μL of methoxyamine hydrochloride pyridine solution (15 mg/mL) was incorporated, followed by shaking for 2 minutes to initiate the oximation reaction. This reaction was allowed to proceed for 90 minutes at 37°C within a shock incubator. Then, the 80 μL BSTFA (contain 1%TMCS), 20 μL n-Hexane, and 10 μL internal standard (C8/C9/C10/C12/C14/C16, 0.16 mg/mL; C18/C20/C22/C24/C26, 0.08 mg/mL) were added to the injection bottle. The above extracts were shaken for 2 min, reacted for 60 min at 70°C, and then subsequently analyzed using a Gas Chromatography-Mass Spectrometer (Agilent, USA).

Extracts were separated using a capillary column (30 m × 0.25mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA, USA) under the following conditions: initial oven temperature 60°C, initial hold 0.5 min; ramp at 8°C $min^{-1}$ to 125°C; ramp at 5°C $min^{-1}$ to 210°C; ramp at 10°C $min^{-1}$ to 270°C; ramp at 20°C $min^{-1}$ to 305°C, hold for 5 min. The MS ion source was 230°C, four stage rod temperature was 150 °C and electron energy was 70 eV. Metabolites were identified using the untargeted database of GC-MS from Lumingbio (Oebiotech, Shanghai, China).

We conducted a comparative analysis of the relative abundance of rhizosphere metabolites across all treatments in order to ascertain the specific compounds that exhibited greater abundances in the cultivar mixture treatments. Using the random forest model, we identified 14 metabolites (quinic acid, glycolic acid, lactobionic acid, talose, L-proline, gluconic acid, D-arabinose, maltotriose, D-galactose, glycerol, D-tagatose, D-fructose, L-valine, and melibiose) with high relative abundances in cultivar mixture samples (see Results). Upregulated metabolites were selected for further investigation.

## 2.6. Impacts of the up-regulated metabolites on the growth of pathogenic *F. oxysporum*

The culture medium used in the experiment was prepared by diluting potato dextrose agar (PDA) four times and then adding a range of concentrations of specifically chosen artificial metabolites. The concentration gradient series of single and mixed metabolites was prepared at concentrations of 0, 1, 50, and 100 mM. Mixed metabolites were prepared from 14 metabolites at equal volume ratios. After the culture medium plates were prepared, *F. oxysporum* obtained from the actively growing colony margins was carefully placed in the center of the plates. Subsequently, the plates were transferred to a constant temperature incubator set at 30°C for a period of 7 days, allowing the mycelia to grow. The diameter of the mycelia was measured after incubation. The entire process was independently conducted thrice for each treatment.

## 2.7. Impacts of the up-regulated metabolites on the *P. heterophylla* and soil microorganism

A mixture of metabolites was formulated using an equal molar concentration of all 14 metabolites described above. The experimental design encompassed the following four treatments: (1) CK: water, (2) UM: mixture metabolite, (3) FOP: *F. oxysporum*, and (4) FOP-UM: mixture metabolite and *F. oxysporum*. Each treatment was conducted in five replicates (4 plants per replicate). *P. heterophylla* variety Zheshen 2 (ZSII) was selected for this experiment.

Following the initial growth phase of *P. heterophylla* lasting one month, the soil was treated with a mixture metabolite (consisting of 14 upregulated metabolites, each mixed in equal volumes with a 10mM solution) or water for a duration of four weeks. The weekly concentration was 1 μmol $g^{-1}$ soil. *F. oxysporum* ($5 \times 10^5$ spores $g^{-1}$ soil) was inoculated into the soil after the first application of metabolites. The plants were irrigated daily to sustain soil moisture throughout the entire duration of the greenhouse experiment. We then collected samples of the leaf and rhizosphere soil of *P. heterophylla* for analysis. The superoxide dismutase (SOD), peroxidase (POD), catalase (CAT), and malonaldehyde (MAD) activities in the leaves were analyzed using

the plant enzyme activity detection kit (Suzhou Comin, China). We also determined the abundance of total bacteria (T Weedon et al., 2012), total fungi, *F. oxysporum* (Lievens et al., 2005), *Pseudomonas* spp. (Fierer et al., 2005) and *Bacillus* spp. (Fierer et al., 2005) in the rhizosphere soils by qPCR.

**2.8. Data analysis**

The QIIME2 platform was used to estimate the alpha diversity indices (including shannon and chao1 index) of the soil bacterial, fungal, and protist communities. The FUNGuild database was used to identify the potential fungal pathogens (Nguyen et al., 2016). Potential beneficial bacteria were identified using the global plant-beneficial bacteria database (Li et al., 2023). ASVs matching Streptophyta, Rhodophyta, Fungi, and Metazoa were excluded in the analysis of the 18S dataset. Additionally, protistan ASVs have been categorized into various functional groups based on their feeding behavior (Dumack et al., 2020; Nguyen et al., 2020).

Principal Coordinate Analysis (PCoA) was conducted using the Bray-Curtis distance matrix as the basis for comparison. The Bray-Curtis distance matrix served as the foundation for conducting Principal Coordinate Analysis (PCoA). To assess the relative importance of soil bacterial, fungal, and protistan diversity (measured by Shannon index) and community structure (captured by PCoA1) in predicting *P. heterophylla* heterophyllin B content and biomass, a random forest model analysis was conducted using the "randomForest" package (Liaw and Wiener, 2002). The beta-nearest taxon index (βNTI) was calculated to investigate whether the bacterial, fungal, and protist communities are shaped primarily by stochastic or deterministic assembly processes (Stegen et al., 2013; Liu et al., 2022). The stability of the soil microbial communities was evaluated using the Average Variation Degree (AVD), which quantifies the deviation from the mean relative abundance of normally distributed ASVs (Xun et al., 2021). A decrease in the AVD value indicated greater microbial stability. The "niche breadth" approach was utilized to measure the degree of habitat specialization, following the methodology of previous research studies (Zhang et al., 2018). We utilized the "igraph" package to construct co-occurrence networks that

specifically targeted correlations with an absolute value of Spearman's coefficient greater than 0.6 and a p-value less than 0.01 (Csardi and Nepusz, 2006). The network was visualized using Cytoscape. To investigate the linkage between soil metabolites and microorganisms, Spearman's correlation tests were carried out. Our study involved the development of a structural equation model (SEM) using Amos 21.0.0 software (IBM SPSS) to analyze the indirect and direct effects of cultivar mixtures, soil $NO_3^-$-N, functional microbial abundances, and crop biomass. The goodness of fit of the model was assessed using a combination of fit indices, including the root-mean-square error of approximation (RMSEA < 0.05), Tucker-Lewis coefficient (TLI > 0.95), Bentler comparative fit index (CFI > 0.90), and the chi-square test ($p$ > 0.05) (Schermelleh-Engel et al., 2003). Statistical significance in variations across all treatments was ascertained using the LSD multiple range test ($p$ < 0.05).

## 3. Results

### 3.1. Field-based variations in soil physicochemical parameters, plant phenotype, and microbial predictors

Cultivar mixtures significantly increased soil cellulose and sucrase activities compared to the TY treatment (Table S3). The MixH treatment significantly improved both soil pH and $NO_3^-$ -N content. The cultivar mixtures displayed positive effects on the performance of *P. heterophylla* compared to the monoculture. Compared to the control treatment, the MixW treatment significantly augmented various growth parameters such as root diameter, length, leaf area, stem length, biomass, and heterophyllin B content (Figure 1A-H). The MixH treatment also displayed higher biomass and heterophyllin B content in *P. heterophylla* than the control treatment. Random forest analysis showed that the bacterial and protistan community structure in the rhizosphere soil was the strongest predictor of heterophyllin B content and *P. heterophylla* biomass (Figure 1I-H). Furthermore, bacterial and protistan community diversity had a high level of explanatory power with respect to the heterophyllin B content. Fungal community diversity was a better microbial parameter for explaining *P. heterophylla* biomass.

### 3.2. Variations in the microbial diversity and composition

The MixW treatment significantly increased the Shannon diversity indices of bacterial, fungal, and protistan communities compared to the monoculture treatment (Figure 2A). MixH had a significant positive effect on the Shannon index of the bacterial community (Figure 2A). The cultivar mixtures had no significant effects on the chao 1 indices of the microbial community compared to the monoculture treatment (Figure 2B). Our results also showed that the shannon and chao 1 index of the bacterial community and protistan Shannon indices significantly declined after *P. heterophylla* cultivation. Cultivar mixtures affected the community structure of the bacterial, fungal, and protist groups (Figure S1).

The rhizosphere soils exhibited a bacterial community primarily consisting of the Acidobacteria, Proteobacteria, Actinobacteria, and Chloroflexi phyla (Figure 2C). Cultivar mixtures significantly decreased the relative abundance of Acidobacteria and Actinobacteria compared to the monoculture treatment. The dominant phyla in the fungal communities were Ascomycota, Basidiomycota, and Mortierellomycota (Figure 2C). The MixW treatment significantly decreased the relative abundance of Basidiomycota compared to all treatments. The protistan communities were taxonomically dominated by Chlorophyta, Cercozoa, and Intramacronucleata (Figure 2C). The MixW treatment significantly increased the abundance of Cercozoa across all treatments.

The ternary plot analysis indicated that the relative abundances of potentially beneficial bacterial genera (*Lactobacillus*, *Pseudomonas*, *Nitrosospira*, *Lechevalieria*, *Burkholderia-Caballeronia-Paraburkholderia*, *Mesorhizobium*), fungal genera (*Sarocladium*, *Purpureocillium*, *Penicillium*), and protistan genera (*Eocercomonas*, *Paracercomonas*, *Cercomonas*, *Fisculla*, *Platyophrya*, *Rhogostoma*) in MixW were significantly enriched (Figure 3A). The MixH treatment significantly increased the abundances of bacteria (*Mucilaginibacter*, *Rhizobacter*), fungi (*Clonostachys*, *Trichoderma*, *Didymella*) and protists (*Protoderma*, *Platynematum*, *Phascolodon*, *Kraken*). The abundance of pathogenic fungal genera (*Fusarium*, *Alternaria*, *Curvularia*, *Stemphylium*, *Gibberella*) was significantly higher in TY. The qPCR results

indicated that the cultivar mixtures significantly decreased the abundance of pathogenic *F. oxysporum* and increased the *Pseudomonas* spp. compared to the monoculture treatment (Figure 3B). The MixW treatment maintained lower *F. oxysporum* abundance and ratios of *F. oxysporum* /*Pseudomonas* spp.

Our results showed that the cultivar mixtures affected the community structure of potentially beneficial bacterial and fungal pathogen groups compared to the control treatment (Figure 3C). The consumer protist community structures of MixW and MixH were similar and significantly different between the CK and TY treatments. The dominant genera of potentially beneficial bacteria included *Arthrobacter*, *Rhodanobacter*, *Sphingomonas*, *Bryobacter*, *Gemmatimonas*, *Bradyrhizobium* and *Terrabacter* across all the treatments. The relative abundance of beneficial bacteria in the cultivar mixture treatments was significantly increased. The dominant genera of fungal pathogens include *Didymella*, *Verticillium*, *Rickenella*, *Alternaria*, *Botrytis* and *Clonostachys*. The relative abundance of fungal plant pathogens in the MixW treatment was significantly lower than that in the MixH and TY treatments (Figure 3D). Compared to the TY treatment, the cultivar mixtures exhibited a beneficial influence on the population of consumer protists and a suppressive effect on the abundance of parasites and phototroph protists (Figure 3E).

### 3.3. Assembly processes and co-occurring network of microbial communities

The βNTI values of the bacterial community exceeded 2 in both the TY and MixW treatments, suggestive of deterministic processes primarily driving community assembly (Figure 4A). MixH, instead, drove the assembly of bacterial communities towards stochastic processes. Regarding the fungal community, all treatments exhibited a βNTI value below 2, suggesting that stochastic factors were major drivers of the assembly of fungal communities. Stochastic processes dominated the assembly of the protistan community under the TY and MixH treatments. MixW drove the assembly of protistan communities towards deterministic processes. The MixW treatments significantly decreased the average variation degree (AVD) of the bacterial community compared to the TY and MixH treatments (Figure 4B). However, the AVD of fungal

and protistan communities was not significantly different under *P. heterophylla* cultivation. The MixH treatments significantly decreased the niche breadth of the bacterial and protistan communities compared to TY and MixW. MixH displayed the lowest niche breadth in the fungal community (Figure 4C). We developed multi-bipartite co-occurrence networks to untangle the interconnections between beneficial bacteria, fungal pathogens, and consumer protist genera (Figure 4D). Notably, beneficial bacteria- consumer protist interactions were dominated by positive correlations, including Proteobacteria, Actinobacteria, and Cercozoa. The interaction between beneficial bacteria and consumer protists and fungal pathogens was predominantly characterized by negative correlations.

### 3.4. The changes of rhizosphere soil metabolites

A total of 256 metabolites were obtained by GC-MS/MS non-targeted metabolomic analysis, which mainly included organonitrogen compounds, carboxylic acids and derivatives, and fatty acids (Figure 5A). MixW significantly increased metabolomic diversity compared to other treatments (Figure 5B). PCA revealed significant differences in metabolite profiles between the MixW and MixH treatments compared to those observed under TY treatment (Figure 5C). Specifically, the abundance of 71 and 42 metabolites increased under MixH and MixW treatments, respectively, compared to TY treatment (Figure 5D). These differential metabolites were mainly associated with ABC transporters, mineral absorption, protein digestion and absorption, galactose metabolism, and biosynthesis of amino acids (Figure S2). Furthermore, the cultivar mixture treatments significantly increased the contents of d-galactose, galactinol, d-sorbitol, glycerol, melibiose, D-fructose and D-tagatose compared to TY (Figure 6).

### 3.5. Correlation between microbial community, metabolites and soil properties

The results revealed that both the abundance and structure of fungal pathogens were strongly correlated with $NO_3^-$-N, $NH_4^+$-N and EC (Figure 7A). Fungal diversity and pathogen abundance were strongly correlated with metabolite diversity. The

structures of fungal pathogens and protists showed strong correlations with the pH. Both the abundance and structure of beneficial bacteria showed strong correlations with cellulose and sucrase activity. SEM results showed that the cultivar mixtures had a positive effect on the abundance of beneficial bacteria and consumer protists (Figure 7B). Consumer protist abundance was negatively correlated with fungal pathogens. In addition, the cultivar mixture practices significantly and indirectly improved crop biomass by altering the soil properties, metabolites, and microbial abundance.

Subsequently, we used random forest analysis to pinpoint the crucial metabolites among the differential metabolites across all the samples (Figure 7C). Based on the top 24 metabolites, 14 were selected. The selected metabolites were significantly enriched in the cultivar mixture treatments, including quinic acid, glycolic acid, lactobionic acid, talose, L-proline, gluconic acid, D-arabinose, maltotriose, D-galactose, glycerol, D-tagatose, D-fructose, L-valine, and melibiose (Figure 7D). Furthermore, the selected metabolites were significantly positively correlated with the abundance of beneficial bacteria (*Arthrobacter*, *Rhodanobacter*, *Sphingomonas*, *Bradyrhizobium*, *Pseudomonas* and *Pandoraea*) and consumer protists (*Protaphorura*, *Oppiella*, *Sandona*, *Isotomurus*, *Cercomonas*, *Paracercomonas*), and negatively correlated with fungal pathogens (*Verticillium*, *Alternaria*, *Botrytis*, *Fusarium*, *Stemphylium*, *Curvularia*) (Figure 7E).

### 3.6. The mixture metabolite suppressed soil-borne *Fusarium* disease

Our results showed that glycerol, d-fructose, gluconic acid, quinic acid, and l-valine significantly inhibited the growth of *F. oxysporum* at concentrations ranging from 50 mM to 100 mM (Figure 8 and S3). Melibiose, l-proline, glycolic acid, maltotriose, and lactobionic acid significantly inhibited the growth of *F. oxysporum* at a concentration of 100 mM. Moreover, the mixture of metabolites had a significantly negative effect on *F. oxysporum* at all test concentrations (Figure 8D). Metabolites with pathogen (*F. oxysporum*) and single metabolite treatment significantly increased the *P. heterophylla* biomass, SOD and CAT activity and decreased the POD and MAD activity of *P. heterophylla* compared to FOP (*F. oxysporum* treatment) (Figure S4 and 8E-H).

Metabolite addition significantly increased the abundance of total bacteria, total fungi, *Pseudomonas* spp., and *Bacillus* spp. in the rhizosphere soil compared with CK (Figure 8I-P). Furthermore, the metabolites with pathogen treatment notably lowered the abundance of total fungi and *F. oxysporum* and enhanced the presence of the *Pseudomonas* spp. compared to FOP.

## 4. Discussion

### 4.1. Cultivar mixtures alter rhizosphere microbial community to alleviate the soil-borne disease

The severity of soil-borne diseases has been increasing owing to the escalation of agricultural intensification. This can be attributed to inappropriate field management practices and consecutive monoculture practices that contribute to the prevalence of crop diseases. As a result, these diseases have had detrimental impacts on crop yield, yield stability, and overall crop quality (Wu et al., 2019; Zhou et al., 2023a). Previous studies have demonstrated that cultivar mixtures globally enhanced crop yields and temporal yield stability (Huang et al., 2024). In continuous monoculture agricultural systems, the co-evolution of plants and pathogens resulted in the emergence of numerous physiologically diverse pathogen strains, ultimately exacerbating their ability to cause diseases in the native plant host (Zhu, 2013; Wu et al., 2020). In this study, we cultivated several non-native plant varieties in consecutive monoculture fields. Our result showed that higher diversified varieties mixture treatment (MixW) displayed a lower abundance of fungal pathogens and had a positive effect on plant characteristics. This phenomenon has been attributed to the fact that non-host plant varieties often develop root systems that act as physical barriers, inhibiting the spread of pathogens from native host plants to another (Zhu and Morel, 2018).

Soil microbial community composition and diversity have been widely recognized as crucial indicators of plant health (Zhou et al., 2023b). Crop diversification cultivation has been shown to have the potential to decrease plant diseases by influencing the composition of the soil microbial communities (Zhou et al., 2019; Ren et al., 2024). Our study showed that the bacterial and protistan community structures were the

strongest predictors of heterophyllin B content and *P. heterophylla* biomass. This finding aligns with previous research indicating that protist communities are able to serve as strong indicators of disease suppression abilities (Ren et al., 2024). Cultivar mixtures significantly affected the structure of the bacterial and protistan communities and significantly increased the diversity of the bacterial community. The cultivar mixture system decreased the pathogenic *F. oxysporum* density and the ratios of *F. oxysporum* /*Pseudomonas* spp., and also increased the abundance of potentially beneficial bacteria compared to the monoculture. Furthermore, the potentially beneficial *Pseudomonas*, *Lactobacillus*, *Nitrosospira*, *Lechevalieria Sarocladium*, *Purpureocillium* and *Penicillium* were significantly enriched in MixW. These microorganisms have been confirmed to effectively suppress *Fusarium* disease (Wu et al., 2022a; Yan et al., 2024). Our results showed that the density of the host crop tended to decrease as the number of crop varieties increased, which resulted in a dilution effect on pathogens within highly diverse crop communities and further mitigated the adverse impacts of soil-borne pathogens on crop yield. This finding is consistent with earlier research showing that a greater distance between rice varieties diluted the concentration of a specific disease-causing agent as it spread among susceptible host varieties (Zhu et al., 2000). Moreover, MixW increased the stability of the bacterial community and decreased the niche breadth of the fungal community, and MixH treatments significantly decreased the niche breadth of bacterial and protistan communities. This indicated that the cultivar mixtures led to a greater overlap in ecological niches and increased competition between different species within the soil fungal community and decreased them in the bacterial and protistan communities.

Consumer protists have the ability to suppress diseases through various mechanisms, such as direct predation on fungal pathogens (Geisen et al., 2016), selective enrichment of beneficial microbes with antagonistic functions, and activation of bacterial functions that are responsible for suppressing plant pathogens (Guo et al., 2022; Ren et al., 2024). Our results showed that cultivar mixtures had a positive effect on the abundance of consumer protists, especially those of *Paracercomonas* and *Cercomonas*. In vitro experiments validated the predatory effect of *Cercomonas* on

*Fusarium* pathogens (Ren et al., 2024). *Paracercomonas* and *Cercomonas* were shown to play essential roles in improving plant productivity by promoting beneficial microorganisms and suppressing the growth of pathogenic *Fusarium* (Glücksman et al., 2010; Guo et al., 2022). These findings suggested that the presence of potential prey in the cultivar mixture system was able to indirectly lead to an increase in consumer protists, potentially enhancing disease suppression, supporting previous results that diversification cultivation improved crop disease suppression by reshaping consumer protists (Guo et al., 2022; Ren et al., 2024). Notably, interactions between beneficial bacteria and consumer protist were dominated by positive correlations, and those between consumer protists and fungal pathogens were predominantly characterized by negative correlations. Therefore, the disease-suppressive effects of cultivar mixtures may be partially attributed to the increased abundance of specific consumer protistan taxa, such as *Cercomonas*, which aid in pathogen suppression through direct predation.

### 4.2. Changes in rhizosphere soil metabolites compositions in response to cultivar mixtures

The release of root exudates into the soil can exert a direct influence on target organisms, while also undergoing degradation or transformation by soil microorganisms (Scavo et al., 2019). Additionally, these exudates may trigger a third species to produce a compound that interferes with donor plants and subsequently leads to alterations in soil abiotic factors, thereby affecting target plants (Scavo et al., 2019). Root-secreted metabolites have been demonstrated to play a pivotal role in shaping rhizosphere microbial communities and their functionalities, thereby exerting a profound influence on soil fertility and plant development (Bakker et al., 2018). Under different abiotic stresses, variations in rhizosphere metabolites were able to potentially have significant effects on plants, soil properties, and microbial communities (Tiziani et al., 2022; Yuan et al., 2024). Our results suggested that MixW significantly increased metabolomic diversity. The cultivar mixtures changed the structure of metabolite profiles. Furthermore, the cultivar mixture treatments altered the galactose metabolism, which significantly increased the contents of d-galactose, galactinol, d-sorbitol,

glycerol, melibiose, D-fructose and D-tagatose. Previous studies have indicated that galactose metabolism is essential for biofilm formation by *Bacillus subtilis*, which increased its ability to colonize plants (Chai et al., 2013). Metabolites produced from galactose metabolism exhibited greater antimicrobial activity against pathogens (Mizzi et al., 2020). Our findings indicate that genotype-specific metabolic processes in *P. heterophylla* played a key role in shaping the soil metabolite composition in response to cultivar mixtures. Soil microbes and plant roots were shown to be crucial performers driving metabolic activities in the soil (Wang et al., 2023). In the rhizosphere, the interaction between metabolites and microorganisms played a vital role in mediating the soil microenvironment, which influenced the activity and function of microbial communities (Cotton et al., 2019). Our studies showed that the important differential metabolites were significantly positively correlated with the abundance of beneficial bacteria (*Sphingomonas*, *Bradyrhizobium*, *Pseudomonas*) and consumer protists (*Cercomonas*, *Paracercomonas*) and negatively correlated with fungal pathogens (*Verticillium*, *Alternaria*, *Botrytis*, *Fusarium*). This finding is consistent with our previous studies, which showed a significant negative correlation between pathogen abundance and the metabolites that increased the most under intercropping treatment (Wu et al., 2020). Our results showed that the use of cultivar mixtures significantly increased crop biomass directly and indirectly by changing metabolite composition and microbial abundance in consecutive monoculture systems.

### 4.3. Cultivar mixtures mediate the interaction between metabolites and microorganisms to improve the suppression of soil-borne *Fusarium* disease

Increasing evidence has suggested that root exudates of non-host plants are able to directly inhibit plant pathogens or indirectly activate the plant immune system in diverse agricultural systems (Zhu and Morel, 2018; Zhang et al., 2024). In this study, we identified a specific group of important rhizosphere metabolites (amino acids, organic acids, and carbohydrates) that were directly linked to the treatment of cultivar mixtures. Importantly, these metabolites significantly inhibited *F. oxysporum* and increased the *P. heterophylla* biomass. Previous studies have shown that low-molecular-

weight amino acids, organic acids, and carbohydrates can recruit beneficial microbial communities and trigger plant defense against pathogens (Bacilio-Jiménez et al., 2003; Sasse et al., 2018; Luo et al., 2022). Our results also indicated that the metabolite blend mitigated the detrimental effects of *F. oxysporum* infection on crop growth, reduced the abundance of total fungi and *F. oxysporum*, and increased beneficial *Pseudomonas* spp. The plant metabolites were able to recruit beneficial microorganisms, including *Bacillus*, *Pseudomonas* and *Streptomyces* in the soil (de Weert et al., 2002; Ling et al., 2011). Our findings support the idea that rhizosphere metabolites produced by healthy plants contribute to reducing pathogen invasion by stimulating rhizosphere microbes (Wen et al., 2023). This finding implies that rhizosphere metabolites present in cultivar mixtures are able to bolster the growth and enrich the diversity of beneficial microbes within the plant rhizosphere, leading to the effective suppression of soil-borne *Fusarium*. This may be because the microbes stimulated by metabolites outcompeted pathogens in occupying niches, thereby reducing the occurrence of crop diseases (Wen et al., 2023). Previous studies have primarily focused on the role of rhizosphere microbiomes in supporting the suppression of soil pathogens against significant soil diseases in diverse cropping systems (Zhou et al., 2023b). Our study identifies a novel mechanism for suppressing pathogens through metabolite production in diversified cropping systems, which differs from previous reports that have focused on plants actively recruiting beneficial microbes to target specific pathogens (Carrión et al., 2019).

## 5. Conclusions

Taken together, our results indicate that crop cultivar mixtures significantly alleviated soil-borne *Fusarium* disease in consecutive monoculture systems. Our work showed that cultivar mixtures significantly affected the community structure of bacterial and protistan communities, significantly decreased pathogenic *F. oxysporum* density, and increased the abundance of consumer protists and potentially beneficial bacteria (Figure 9). Furthermore, the key metabolites in the cultivar mixtures reduced the negative effect of *F. oxysporum* infection on crop growth, decreased the abundance of *F. oxysporum*, and increased the number of beneficial microbes. Therefore, our study

highlights crop cultivar mixture-driven changes in rhizosphere metabolites to suppress soil-borne *Fusarium* disease. These findings suggest a potential strategy for utilizing rhizosphere metabolites to control soil-borne diseases in modern agricultural crops.

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**Declaration of competing interest**

The authors declare that they have no conflict of interest.

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**Figure Legends**

**Figure 1** Impact of varying treatments on field-grown *P. heterophylla*'s vegetative traits **(A-H)**. Importance of bacterial, fungal, and protistan community diversity and structure in predicting heterophyllin B (**I**) and plant biomass (**J**) using Random Forest (% increase in MSE). The distinct letters within each column denote statistically significant variations (LSD-test, $p < 0.05$). **$p < 0.01$, *$p < 0.05$.

**Figure 2** Variation in rhizosphere soil microbial alpha diversity across bacterial, fungal, and protistan communities **(A-B)**. Distribution of bacterial, fungal and protistan phyla among all sampled specimens **(C)**. The distinct letters within each column denote statistically significant variations (LSD-test, $p < 0.05$). The distinct letters within same microorganism denote statistically significant variations in the stacked column chart ($p < 0.05$).

**Figure 3** Ternary plot showing the enriched and depleted genera for bacterial, fungal and protistan community composition **(A)**. Abundance of *F. oxysporum* and *Pseudomonas* spp. across all the treatments detected by quantitative polymerase chain reaction (qPCR) **(B)**. Changes in community compositions of potentially beneficial bacteria, fungal pathogens and consumer protists across all the treatments as revealed by principal Coordinate Analysis (PCoA) **(C)**. Relative abundances of potentially beneficial bacteria and of fungal pathogens at the genus level **(D)**. Distribution of the relative abundance of functional groups of soil protists **(E)**.

**Figure 4** The βNTI values of assembly processes of soil bacteria, fungi and protists **(A)**. Average variation degree **(B)** and habitat niche breadth **(C)**. Co-occurring network analysis of beneficial bacteria, fungal pathogens and consumer protists genera utilizing Spearman's correlation analysis **(D)**.

**Figure 5** Metabolites changes of rhizosphere soil across TY, MixW and MixH

treatments **(A)**. Shannon index **(B)** and principal component analysis **(C)** of metabolome profiles across treatments. Volcano plot showing significantly differentially occurring metabolites **(D)**. The volcano plot illustrates that the red upregulated metabolites signify a significant increase in metabolite abundance in the treatment group in comparison to the control.

**Figure 6** Pathway mapping analysis revealing the metabolites changes in galactose metabolism. On the pathway diagram, the metabolite emphasized in red corresponds to the metabolite that has been upregulated.

**Figure 7** Mantel tests depict the association of microbial diversity, structure and abundance with soil variables **(A)**. Structural equation models (SEM) assessing the effects of cultivar mixtures on soil properties, metabolites and microbiota **(B)**. The red line signifies a positive impact, whereas the green line denotes a negative one. The identification of 24 most significant marker metabolites were analyzed by utilizing random forest classification **(C)**. Heatmap revealed differential abundance of selected differential metabolites across all the samples **(D)**. Correlation analysis showed the correlation between differential metabolites and specific microbes **(E)**. $^{**}p < 0.01$, $^{*}p < 0.05$.

**Figure 8** The impact of varying concentrations (0, 1, 50, and 100 mM) of single and mixed metabolites on the growth of *F. oxysporum* (A-D). Impacts of the mixed up-regulated metabolites and *F. oxysporum* treatment on the SOD, POD, CAT and MAD activity of *P. heterophylla* (E-H). Impacts of the mixed up-regulated metabolites and *F. oxysporum* treatment on the microbial abundance in the rhizosphere soil of *P. heterophylla* (I-P). CK: water addition; UM: mixture metabolite addition; FOP: *F. oxysporum* addition; FOP-UM: mixture metabolite and *F. oxysporum* addition. The distinct letters within each column denote statistically significant variations (LSD-test, $p < 0.05$).

**Figure 9** Schematic representation of crop cultivar mixtures on suppressing soil-borne pathogens. “+” and red line represent the positive effect; “—”and green line represent the negative effect.