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Effect of *Purpureocillium lilacinum* on inter-root soil microbial community and metabolism of tobacco

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Abstract

Background Numerous chemical pesticides have been used in agricultural production to combat crop diseases and pests. Despite ensuring certain economic advantages, they have also resulted in issues like environmental contamination, declining soil quality, and pesticide residues. Because biological control is environmentally friendly and difficult to acquire resistance to, it has been used in practice recently.

Methods In this study, we isolated the endophytic fungus *Purpureocillium lilacinum* from *Portulaca oleracea* L., which was previously found to have inhibitory effects on soil pathogens in tobacco fields. To find out how the biocontrol agent *P. lilacinum* affects soil microorganisms and plant metabolism in tobacco cultivation, we used amplicon sequencing technology and gas chromatography-mass spectrometry to look at the structure of soil microbial communities and the networks of interactions between microorganisms and metabolites in the inter-rhizosphere soil of tobacco fields treated with different amounts of *P. lilacinum*.

Results The findings showed that there was a trend toward less microbial diversity among inter-root microorganisms as solid-state fermentation (SSF) products of *P. lilacinum* increased; however, submerged fermentation (SmF) had no discernible impact on microbial diversity when compared to the direct use of SSF. Additionally, the relationship between inter-root fungi and volatile compounds in tobacco leaves was dominated by a negative correlation.

Conclusions The result demonstrated that *P. lilacinum*'s antagonistic interaction in the inter-rhizosphere microbial community was dominant and valuable for biopesticide application. *P. lilacinum* can work more effectively on tobacco roots by using SSF products. *P. lilacinum*'s opposition to fungal colonies may enhance the volatile chemicals in tobacco leaves. These provide some implications for the biocontrol application of *P. lilacinum*.

Keywords *Purpureocillium lilacinum*, Rhizosphere microbiome, Plant-microbe interactions, Nicotiana tabacum, Solid-state fermentation

Introduction

Soil-borne illnesses pose a serious threat to agriculture's productivity. Increased soil deterioration, monocropping with limited crop rotations, and agricultural intensification all contribute to more frequent outbreaks of soil-borne illnesses and correspondingly higher output losses (Zhang et al. 2022). The same issue affects both tobacco production and cultivation. The primary limiting factors in tobacco production have been viral, bacterial, fungal, and insect-borne illnesses

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as well as environmental dangers (such as weather spots, drought, and temperature) (Kakar et al. 2020). Tobacco brucellosis, tobacco black tibia, and tobacco root rot are typical soil-borne diseases for tobacco cultivation and maintenance. These diseases make tobacco a huge challenge to cultivate and manage. To sanitize the soil before planting tobacco, the majority of producers now use soil fumigants. This can somewhat lower the prevalence of diseases that are transmitted through the soil. However, the use of these products is known to cause various health problems and environmental damage (Arcury and Quandt 2006; Yokoyama 2007).

Purpureocillium lilacinum is in the genus Penicillium. It can be grown on PDA medium and has ellipsoidal to fusiform conidia on the surface of its colonies. *P. lilacinum* has been shown to have potential for biopesticide applications (Xue et al. 2006; Gao et al. 2023). Numerous studies have demonstrated that adding *P. lilacinum* to nematode infestation situations, particularly Meloidogyne spp. infestations, can enhance the plant's reaction to the pathogen's presence and even encourage plant development (Singh et al. 2013). According to Lopez and Sword's evaluation of *P. lilacinum*'s endophytic colonization of cotton, *P. lilacinum* was able to improve plant dry mass and flower count, proving that inoculation with *P. lilacinum* in the absence of pathogens can encourage plant development (Lopez and Sword 2015). Similarly, the strain of *P. lilacinum* QLP12, which was found in the soil of Qinling, China, could also live inside the eggplant. Research on the biological activity of *P. lilacinum* showed that the pretreated QLP83 fermentation solution reduced the number of cases of yellow wilt disease in eggplant soil by 82.12%. Additionally, the fungus had good growth-promoting effects on the germination of eggplant seeds, the development of shoots, the amount of chlorophyll, and root activity (Lan et al. 2017).

As the in-depth study of microbial physiological activities progresses, the commercial production of bacteria and fungi is also gaining more attention. Both submerged fermentation (SmF) and solid-state fermentation (SSF) are now used to prepare biocontrol fungal agents. Solid-state fermentation has been studied a lot (Domínguez et al. 2000; Robinson et al. 2001; Machado et al. 2004) to find ways to make more enzymes, hormones, organic acids, antibiotics, aflatoxins, and other fungal metabolites. SSF, when compared to SmF, has several advantages, including the capacity to boost fermentation product yields and production speeds while utilizing little water and not necessitating rigorous aseptic conditions (Hölker et al. 2004). The growth capacity of the maize plant is enhanced by the development of Guizhou *Xylella vulgaris NJAU 4742* through SSF of stevia residues supplemented with amino acids from animal carcass hydrolysis (Liu et al. 2021).

Microorganisms play a very important role in plant growth and development and are highly associated with plant metabolism and changes in volatiles within the plant. The addition of numerous beneficial bacteria altered or had an impact on altering plant volatiles and metabolites. It has been shown that some soil microorganisms can improve crop nutrition and promote plant growth by mobilizing nutrients that are not readily available to plants and that they produce microbial volatile organic compounds (mVOCs), which contain alcohols, benzenes, aldehydes, olefins, acids, esters, terpenoids, and ketones (Morath, Hung, and Bennett 2012; Lemfack et al. 2018; Misztal et al. 2018; Guo et al. 2020), which can have a positive effect on plants, either directly or indirectly. For instance, by using *Bacillus licheniformis* and *Pseudomonas fluorescens*, *Vitis vinifera* was able to produce more terpenoids and alkenes, as indicated by GC-MS (Salomon et al. 2014; Salomon et al. 2017). *Glomus mosseae* or *Glomus intraradices* applied to *Sorghum bicolor* resulted in an increase in alcohols, olefins, and certain ethers and acid volatiles in plants (Sun and Tang 2013). Sesquiterpene volatiles decreased in *Vicia faba* plants as a result of intercropping between *Glomus* ssp. and *Vicia faba* (Babikova et al. 2014). MVOCs produced by some fungi, like *Trichoderma* spp., *Alternaria alternata*, *Penicillium charlesi*, and *P. aurantiogriseum*, have also shown plant growth promotion activity. (Ezquer et al. 2010; Li et al. 2011; Hung, Lee, and Bennett 2013; Sánchez-López et al. 2016). Plant roots have a large role in ecological function in soil ecosystems by detecting foreign chemical signals and releasing VOCs involved in below-ground biological interactions (Delory et al. 2016). However, the role of root-emitted VOCs in signaling between and within plants remains poorly documented.

An essential step in the manufacturing of tobacco is the spontaneous fermentation of tobacco leaves. The economic worth of tobacco is directly correlated with its odor quality (Zappe et al. 2020). The scent is a product of the volatile molecules that tobacco leaves can produce, and the fermentation of tobacco is a crucial step in producing aroma (Huang et al. 2010). In the realm of plant metabolomics, gas chromatography is incredibly sensitive for the detection of volatile and semi-volatile compounds. Ibraimov et al. provide a comprehensive description of the chromatographic methods used to examine cigarette products. The gas chromatography is extremely sensitive for the detection of volatile and semi-volatile chemicals in the field of plant metabolomics. Ibraimov et al. (Kapar et al. 2018) provided a thorough overview of chromatographic techniques for the examination of cigarette products.

Even though the understanding and knowledge of plant-soil microbial interactions have increased considerably in recent years, the underlying mechanisms appear to be increasingly complex, and more remains to be learned. When utilized in the cultivation of tobacco, *P. lilacinum*'s effects on soil microorganisms and plant metabolism have not been studied. It is yet unclear how *P. lilacinum* functions in tobacco fields. In this work, we used amplicon sequencing and gas chromatography-mass spectrometry (GC-MS) detection to look at how the structure of the soil microbial community changed in the inter-rhizosphere of tobacco fields treated with *P. lilacinum*. In addition to metabolites, a microbial-metabolite network was made to provide a theoretical framework for the future development of tobacco biocontrol agents based on multi-omics.

Materials and methods

Isolation and identification of *P. lilacinum*

The endophytic fungus *P. lilacinum* was isolated from *P.oleracea* L. Briefly, the leaves, stems, and roots were first chopped into small pieces and thoroughly cleaned with fresh water. Then, the surfaces were successively immersed in 70% ethanol for 2 minutes, 0.1% $HgCl_2$ for 1 min, and rinsed with sterile water six times to sterilize them. They were then inoculated onto potato dextrose agar (PDA), cultured at 28°C for 7–10 days, and checked daily to see whether any fungal colonies were growing. For each fungal colony, single hypha was carefully subcultured onto fresh PDA plates to obtain pure isolates. Spores and mycelia of each fungal strain were preserved in 50% (v/v) glycerol at −80℃.

For species identification, morphological features of fungal cells were examined using light microscopy, and fungal DNA was extracted for amplification and sequencing of the ITS coding region to identify the species.

Preparation of strain *P. lilacinum*

A 5-mm-diameter cake was cut from the colony's edge and injected into a 2-l flask containing 1 l of PDB medium for *P. lilacinum* SmF culture. *P. lilacinum* was first inoculated on potato dextrose agar (PDA) plates at 26°C for 7 days to prepare conidia. SSF culture: 200g of an SSF medium, like rice or corn, was put into bags for edible fungal culture and sterilized at 121°C for 20 min. Pour 50 mL of *P. lilacinum* fermentation SmF into each bag after it has cooled. Stir thoroughly, then place the bags in an incubator or other similar equipment for 5–7 days at 26°C so that mycelium can disperse throughout the SSF medium. To prevent clumps from forming during this process, the bags are required to be rubbed every day. The SSF medium had been entirely covered by mycelium on the third day when the fungus culture bag was opened for open fermentation. Each gram of SSF produced 7 days later contains roughly 10^{10} strains, as determined by a hemocytometer plate. The precipitate was obtained by centrifuging the filtrate after the SSF medium had been suspended and filtered, and it was then freeze-dried to produce spore powder.

Field experiment

From February 3 to July 30, 2021, a field experiment was carried out in Guo Lao village, Baise City, Guangxi Province, China (23°02′ N, 106°35′ E). According to the Baise climate survey, the annual precipitation was 1114.9 mm, and the average temperature ranged from 19.0 to 22.1°C. After transplanting the tobacco seedlings, the fermentation products were buried in the topsoil close to the roots using the SSF method of *P. lilacinum*. With 0 g, 30 g, and 50 g of SSF products per tobacco plant, three gradients were built up. Additionally, root irrigation treatment employed 30 mL of *P. lilacinum* spore suspension (9×10^{10} /mL). Soil samples were collected from the 10~15 cm surface layer of the sample plots using the five-point sampling method. After removing weeds, debris, and other impurities, the collected soil samples were placed in sealed bags, placed in an ice box, and stored at 4°C for use in the laboratory (storage time not exceeding 7 days) for soil microbial diversity determination.

DNA extraction and high-throughput Miseq sequencing

To obtain soil microbial DNA, parallel soil samples were mixed well and taken at 1g for extraction with the Omega Bio-tek Soil DNA Kit D5625. All operations were performed in accordance with the manufacturer's protocols. To assess the DNA quality, OD 260/230 nm and OD 260/280 nm values were measured by the NanoDrop2000 Spectrophotometer (Thermo Fisher Scientific). For each sample, 260/230 ratios must be larger than 1.7, and 260/280 ratios must be larger than 1.8.

Each sample took 10 ng of DNA for PCR-amplification (20 μl reaction, 30 cycles) in triplicate. In detail, primer sets 338F (5'-ACTCCTACGGGAGGCAGCAG-3')/806R (5'-GGACTACHVGGGTWTCTAAT-3') (Mori et al. 2014) and ITS1F (5'-CTTGGTCATTTAGAGGAA GTAA-3')/ ITS2R (5'-GCTGCGTTCTTCATCGAT GC-3') were used to amplify the 16S rDNA V3-V4 region gene and the ITS1 region gene. After that, the amplified products were sequenced with the 2×300 bp kit using the Illumina MiSeq platform of Majorbio Corporation (Shanghai, China).

Determination of volatile compounds in tobacco

The tobacco leaves at the sampling location were roasted by nearby tobacco farmers, graded in accordance with the standards of the national standard GB 2635–1992

"Roasted Tobacco." These plant materials were frozen in the −80 °C refrigerator before use. During pretreatment, 50 mg of each sample were taken for immersing with liquid nitrogen and powdering using a grinding machine (LUKYM24, Guangzhou, China). Mixture of methanol and dichloromethane (3:2) was added into the centrifuge tube containing sample to extract at 50 mg/ml concentration. After 10 min of Ultrasonic treatment (KL040ST, Shenzhen, China) at room temperature (25 ℃), the supernatant was filtered through 0.22-μm membrane filtration. Two hundred-microliter filter liquor was transferred to the sample bottle prepared for GC-MS analysis.

GC conditions

A Hp-5 (Agilent 19091J-413) column (30 m × 0.25 mm × 0.25 μm) was used. The injection port temperature was 250 ℃. The column temperature was programmed as follow: initial temperature at 80 ℃ (held for 5 min), increased to 100 ℃ at 2 ℃/min, increased to 180 ℃ at 5 ℃/min (held for 1 min), increased to 200 ℃ at 2 ℃/min, and increased to 280 ℃ at 10 ℃/min (held for 15 min). The injection amount of filter liquor was 0.5 μl. High-purity helium was utilized as the carrier gas. The gas flow rate was set as 1.0 ml/min.

MS conditions

An electron ionization (EI) ion source was used with 70 EV ionization energy. Mass spectra scanning range was set as 30~500 m/z. The ion source temperature was 230 ℃, and the GC/MS interface temperature was 280 ℃.

Data processing

NIST 2014 was used in the matching comparison. The screening criteria was that matching rate should be more than 600. The relative content of each component was presented by peak area.

Bioinformatic and statistical analyses

To trim adaptor, remove low-quality reads and short reads, Fastp 0.23.0 were used to deal with FASTQ files. The sequencing raw data were bioinformatically processed using quantitative insights into microbial ecology (QIIME2) software version 2021.11 (Bolyen et al. 2019), with the following process: (i) quality control and de-junctioning of the sequencing raw data using Fastp default parameters (Chen et al. 2018), (ii) importing the sequencing raw files in QIIME2, (ii) importing the sequencing raw files after QC, (iii) using the DADA2 algorithm for noise reduction to obtain the amplicon sequence variants (ASVs) abundance files and representative sequence files for each sample (Callahan et al. 2016), and (iv) using the SILVA reference database (version 138) and the UNITE reference database to annotate the ASVs' taxonomic status (Quast et al. 2012; Nilsson et al. 2019).

To get the exported data and spectra, MSDIAL 4.60 software performed peak alignment, rectification, denoising, and deconvolution on the raw GC-MS downstream data (Tsugawa et al. 2015). The NIST 2014 database selected the GC-MS spectral data from among them for search matching.

R was used for all statistical analyses in this study (version 4.1.1) (R Core Team 2013). For metabolome and microbiome data, Spearman's correlation coefficient (SCC) was determined, the significance level was set at 0.1, and connections with absolute values of correlation coefficients lower than 0.8 were disregarded. Gephi 0.92 software was employed to visualize the network. iTOL was used to visualize the species evolution tree (https://itol.embl.de/) (Letunic and Bork 2019).

Results

Isolation of *Purpureocillium lilacinum*

After being isolated and purified in culture, the strains from *P. oleracea* L. were sequenced for ITS. *P. lilacinum* shared 99.72% homology with the NCBI sequence, according to the comparison. The morphological traits under the microscope allowed for the identification of the strain as *Purpureocillium lilacinum* (Fig. 1).

Microbial diversity analysis

We did 16S and ITS amplicon sequencing on the soil microbiota to see what effect *P. lilacinum* had on the inter-rhizosphere soil microbial community of tobacco. Figure 2 displayed the outcomes of the investigation of bacterial and fungal diversity based on ASV levels.

By conducting a diversity study on a single sample, alpha diversity focused on the number of species in uniform habitats and represented the richness and diversity of microbial communities inside the samples. Colony diversity indicators (Shannon and Simpson), colony abundance indices (Chao1), and sequencing depth indices (observed species) were frequently accessible. In both bacteria and fungi, the pattern of diversity change was comparable, with CK and SmF being the biggest, SSF 30 being the second largest, and SSF 50 being the lowest (Fig. 2).

In the tobacco field, the direct application of SSF products had a bigger effect on the inter-root microbial population, and this effect was related to how much was used. The spore suspension, on the other hand, had less of an effect. Additionally, fungal diversity was much higher than bacterial diversity, indicating that the tobacco field's inter-root soil fungus community was more complex than the community of bacteria.

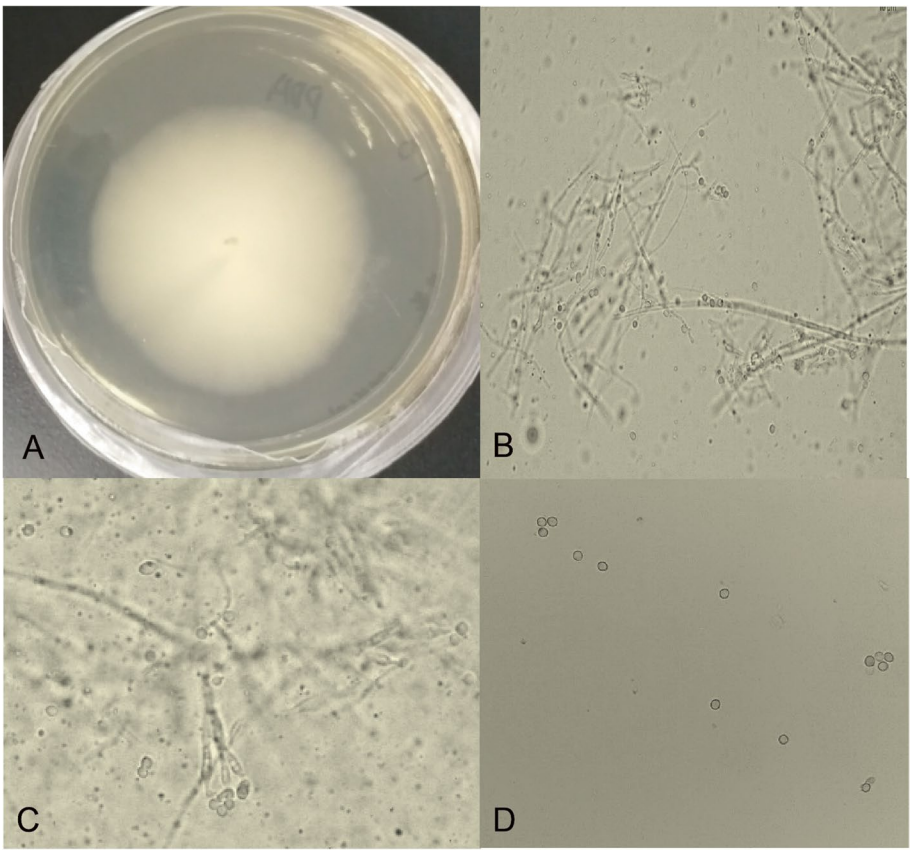

Fig. 1 Morphological characteristics of the *Purpureocillium lilacinum*. **A** The *Purpureocillium lilacinum* on Czapek-Dox Agar; **B** Conidiophore stalk of *P. lilacinum*; **C** Mature conidiophore bearing conidia of *P. lilacinum*; **D** Conidiophore of *P. lilacinum*

Microbial community structure analysis

As can be seen, the bacterial ASV species were much smaller than the fungi, which is consistent with the results of the previous microbial diversity analysis (Fig. 3).

The species of bacteria were mainly *Chloroflexi* (30.81%), *Actinobacteriota* (25.03%), *Proteobacteria* (16.27%), *Acidobacteriota* (8.52%), *Verrucomicrobia* (Verrucomicrobia, 5.24%), *Firmicutes* (4.85%), *Gemmatimonadota* (3.57%), *Bacteroidota* (2.62%), *Myxococota* (1.68%), candidate phylum Radiation taxa (*Patescibacteria*, 0.66%) (average relative abundance share >0.50%) (Fig. 4A), and the fungal species were mainly *Ascomycota* (78.90%), *Basidiomycota* (5.35%), and *Mortierellomycota* (2.68%) (average relative abundance share >0.50%). (Fig. 4B) In addition, unidentified fungi (unidentified, 12.86%) also occupied a high proportion.

Microbe-metabolite networks

We used a co-occurrence network analysis to figure out how the microbial community in the rhizosphere affected the volatile part of tobacco leaves when *P. lilacinum* was present. Bacteria-metabolite network was denser than fungi-metabolite network. Within the same module, metabolite nodes distribute along the edge all the time, and microbe nodes always distribute in the center (Fig. 5).

The network of bacteria and their metabolites had 218 nodes, 649 edges, a graph density of 0.027, a mean degree of 5.954, a modularity score of 0.715, and a positive correlation of 53.78%. The fungal-metabolite network included 222 nodes and 580 edges, a graph density of 0.024, a mean degree of 5.225, a modularity score of 0.726, and a positive correlation of 42.24%. Fungi and metabolites showed stronger connections, but bacteria and metabolites showed more positive associations.

Discussion

In this study, we looked at the structure of inter-rhizosphere soil microbial communities and microbial-metabolite interaction networks in tobacco fields treated with *P. lilacinum* using high-throughput sequencing and gas chromatography-mass spectrometry.

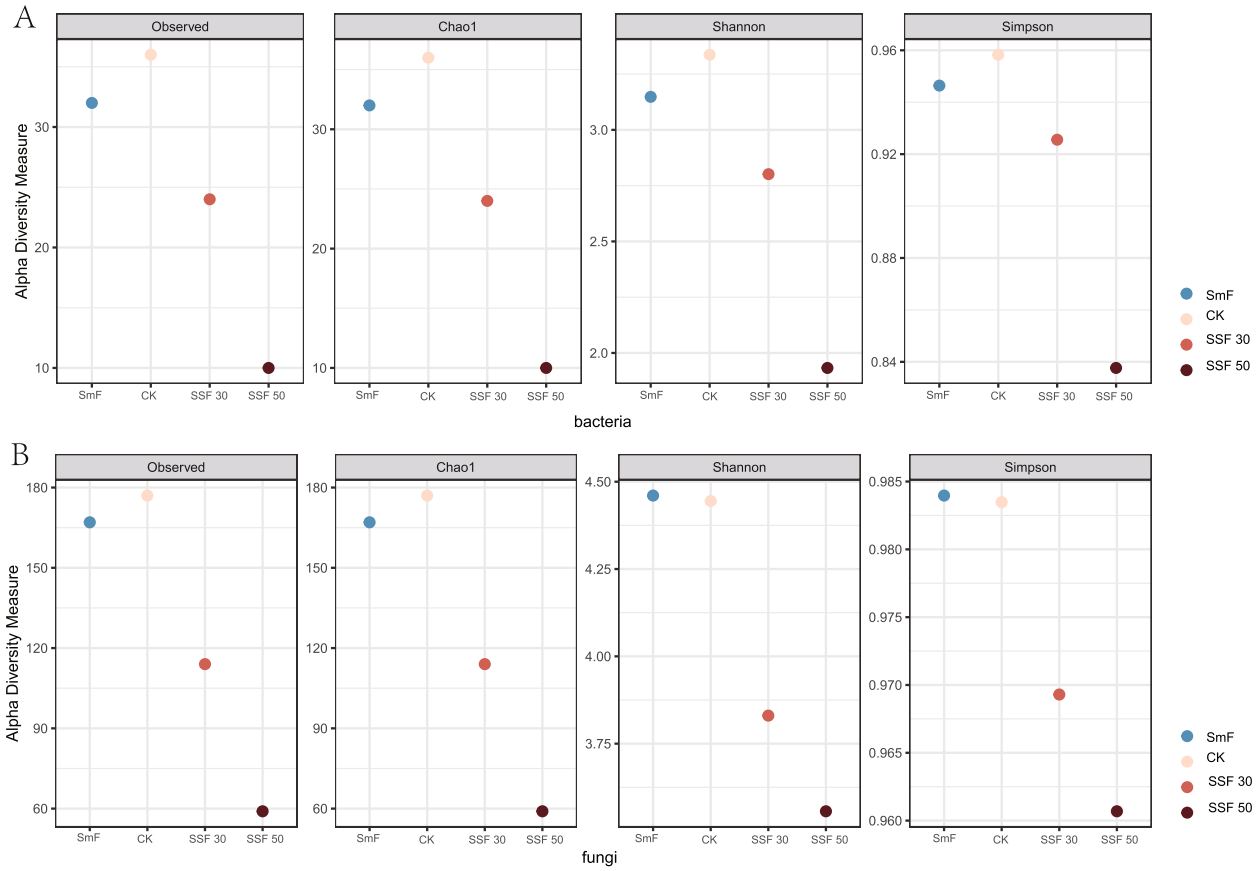

Fig. 2 The *a* diversity under different fermentation methods of *P. lilacinum* (**A** bacteria, **B** fungi)

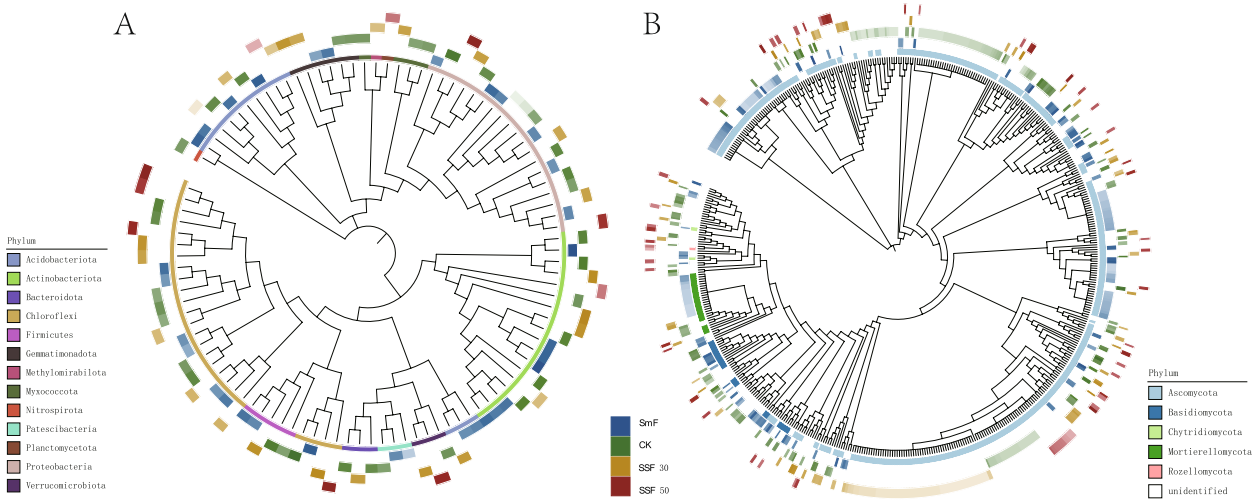

Fig. 3 Evolutionary tree of bacteria (**A**) and fungi (**B**) at ASV level

According to the experimental findings, fungal communities among the inter-root microorganisms were substantially more diverse than bacterial communities. While SmF treatment had no discernible impact on microbial diversity, there was a diminishing trend in microbial diversity as the dose of SSF products increased.

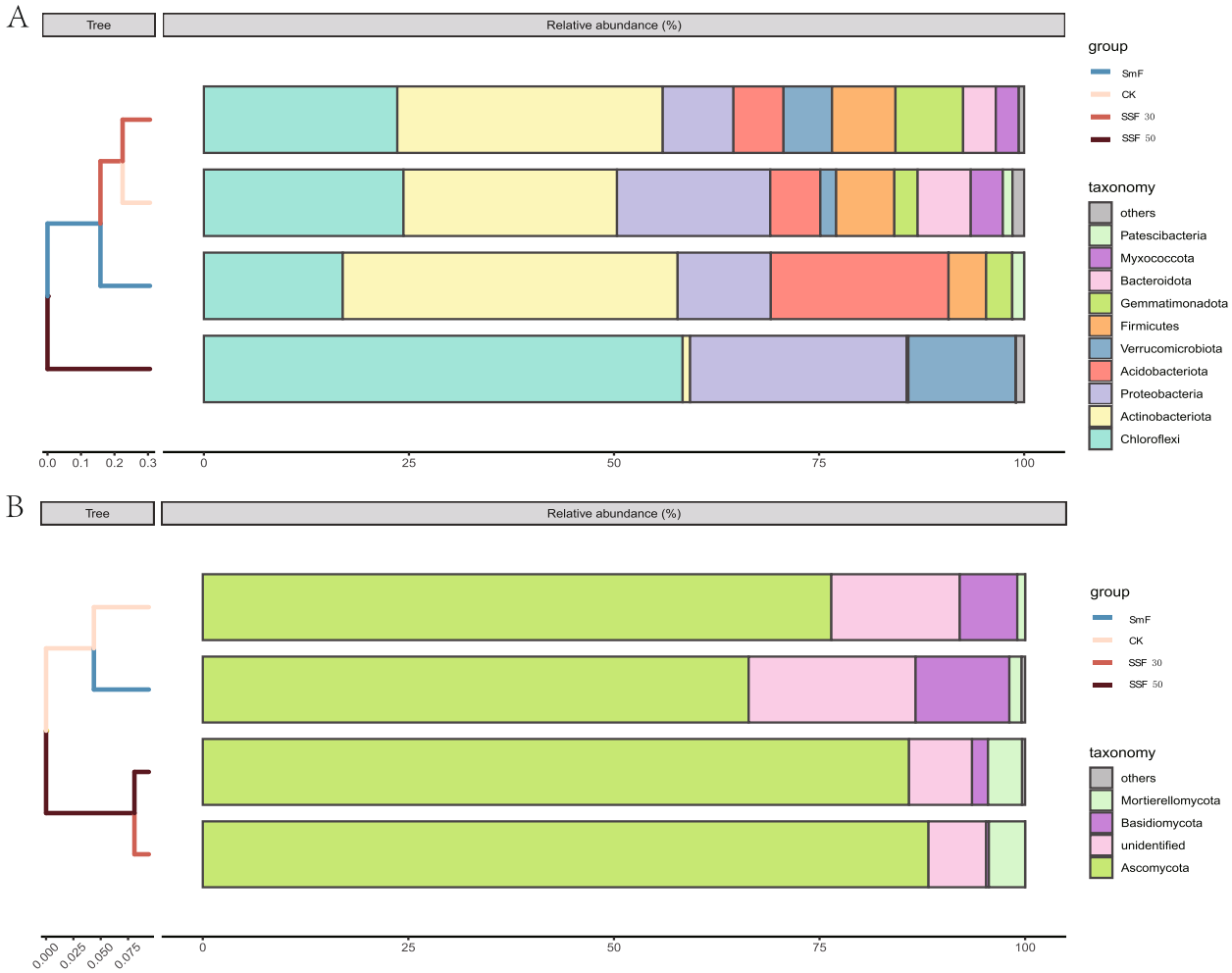

Fig. 4 Community structure of bacteria (**A**) and fungi (**B**) at phylum level

This demonstrated the importance of applying biopesticides to *P. lilacinum*, which were dominated by antagonistic relationships in the inter-rhizosphere microbial community. The benefit of SSF was attributed to the unique physiology of the fungus in the solid media (Barrios-González 2012). In other words, these species spent the majority of their evolutionary time on land. These organisms produced fungal products of biotechnological relevance for use in wet solid substrates, but not in liquids, such as enzymes, secondary metabolites, and spores. SSF systems have been used very successfully in the production of microbial enzymes and secondary metabolites or bioactive compounds (Krishna 2005). According to certain research, filamentous fungus release substantial amounts of enzymes (generally speaking, proteins) in this system that frequently outweigh those secreted during submerged fermentation (SmF) (Acuña-Argüelles et al. 1995; Diaz-Godinez et al. 2001; Elinbaum et al. 2002). For example, in SSF on wheat bran, *Aspergillus oryzae* produced 500-fold-higher yield of heterologous protein (chymosin) than in SmF (Tsuchiya et al. 1994). The results presented in this work further demonstrate the superiority of SSM as a production technique, and it is hypothesized that this technique may result in the creation of enzymes and other compounds that influence soil microbial alterations.

There is solid proof that beneficial microbiological (BM)-plant interactions can promote plant yield and stress resistance. The emission of mVOCs frequently mediates such advantageous interactions. Some mVOCs have been shown to change plant volatile organic molecules and indirectly enhance plant performance by promoting plant growth or inducing resistance to biotic and abiotic stress factors. It is possible that *P. lilacinum* also created some volatiles in this process and so influenced the volatiles of tobacco leaves, as the link between inter-root fungus and volatile compounds in tobacco leaves was more negatively

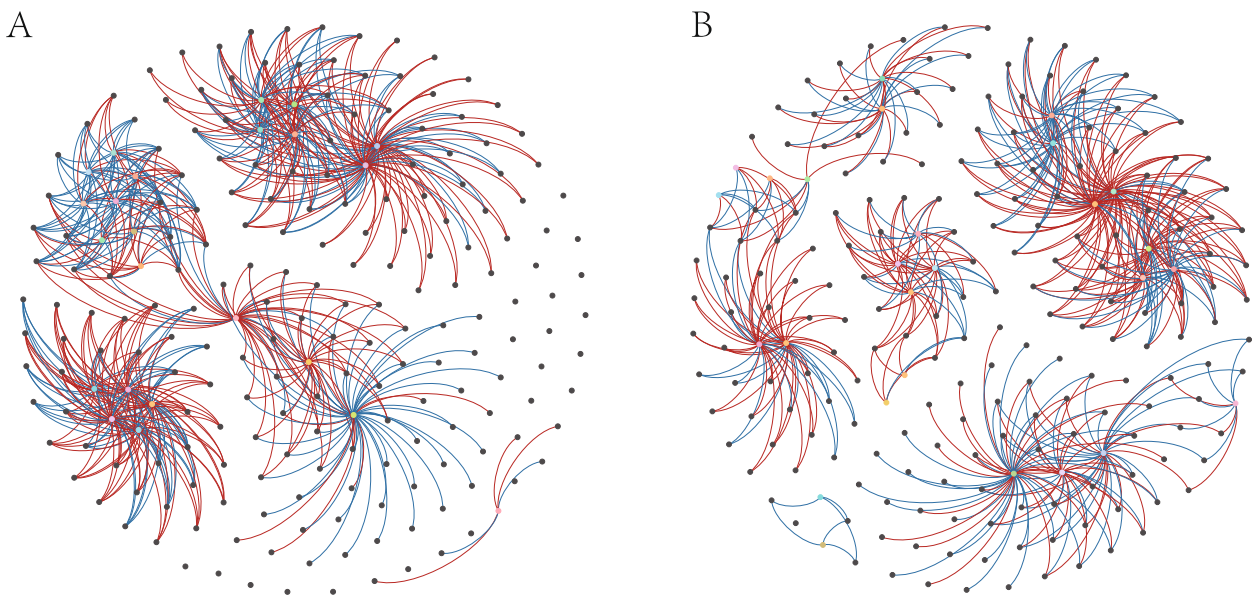

Fig. 5 Network analysis between bacteria (**A**)/fungi (**B**) and metabolite. (Black dots represent volatile compounds. Colored dots represent different family level microbial taxonomy, respectively. Bacterial dots with different colors represent different bacterial phyla. Fungal dots with different colors represent different fungal phyla. Red lines represent positive correlations and blue lines represent negative correlations (absolute correlation ≥ 0.8, *p* value ≤ 0.1)

connected. Meanwhile, the antagonistic effect of *P. lilacinum* on fungal communities has the potential to enrich the volatile compounds of tobacco leaves. By deciphering communication between plants and microorganisms viaVOCs, not only knowledge about agro-ecosystem functioning will be enhanced, but also new sustainable defense practices against biotic and abiotic stresses will be developed (Russo et al. 2022). This study investigated the patterns of interactions between inter-rooted microorganisms and tobacco volatile metabolites during the application of *P. lilacinum* and tobacco rooting from the perspective of co-occurrence networks. This study not only enhanced the microbial information data of tobacco production, but it also served as a crucial reference for research on the impacts of *P. lilacinum* biopesticides on tobacco quality.

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Authors' contributions
All the authors contributed to the final manuscript and approved the submitted version.

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Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
All listed authors consented to the submission of this manuscript for publication.

Competing interests
The authors declare that they have no competing interests.

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