

Diversity of the endophytic and rhizospheric fungal communities in *Scrophularia ningpoensis* roots and its correlation with active constituent contents

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Abstract

Scrophularia ningpoensis, a perennial herb native to Southeast China, is used to prepare traditional Chinese medicines for the treatment of various diseases. The root microbiota play pivotal roles in providing hosts with nutrients, promoting growth, suppressing disease, and increasing abiotic stress tolerance. In this study, we investigated the composition of endophytic and rhizospheric fungi in the root microbiomes of *S. ningpoensis* obtained from different habitats and examined their potential effects on the active constituent contents of the host plant using high-throughput sequencing techniques. Our results indicated that 8709 operational taxonomic units (OTUs) and 5780 defined OTUs shared core rhizosphere OTUs. Ascomycota and Basidiomycota were dominant among the endophytic fungi, whereas Mortierellomycota was distributed in the rhizosphere. The fungal community in the rhizospheric compartment was more diverse than the endophytic community. Redundancy analysis and canonical correspondence analysis of the rhizospheric and endophytic samples revealed that the organic matter, total organic carbon, total nitrogen, and Hg levels were well-correlated with the composition of rhizospheric and endophytic fungal communities. Multiple linear regression analyses facilitated the identification of potentially beneficial fungi whose abundance was correlated with active constituent levels. These fungi could potentially provide valuable information regarding the use of *S. ningpoensis* in the medicinal plant industry.

Introduction

The root surfaces of land plants exhibit rich microbial diversity. This facilitates the occurrence of complex interactions among the soil, host plant, and microbiota (Stassen et al., 2020). The microbiota, including the rhizosphere, rhizoplane, and endophytic compartments, play important roles in enhancing plant productivity, nutrient uptake, disease suppression, and abiotic stress tolerance (Jin et al., 2018; Mitter et al., 2017). This is partly because plant roots interact with these microbiota which can produce exudates and deposits (Zahar et al., 2008). For instance, *Serendipita indica* could promote growth through inorganic orthophosphate (Pi) acquisition (Yang et al., 2009), and *Trichoderma* fungi could produce chelating metabolites that solubilize phosphate and increase its acquisition by plants for the promotion of plant growth (Altomare et al., 1999; Nathalie et al., 2011). Microbiota are mainly derived from the surrounding soil and influenced by their geographical location, microhabitat, and soil property (Joseph et al., 2015; Gabriele and Kornelia, 2010). Plants can change the soil microbiota by secreting bioactive molecules, including sugars, amino acids, carboxylic acids, and a diverse set of secondary metabolites, into the rhizosphere (Hu et al., 2018). However, the mechanism by which root microbiota could influence the accumulation of active ingredients is largely unknown.

S. ningpoensis (*Xuanshen*), a traditional medicinal plant, has been used to treat several kinds of diseases, including cardiovascular disease, diabetes, Alzheimer's disease, and cancers. It grows in a specific region with a long history of use and has excellent medicinal efficacy when used in traditional Chinese medicine (TCM) (Wei et al., 2017). The growth area of *S. ningpoensis* has increased to encompass areas such as the Anhui, Hunan, and Guizhou provinces, which has led to variations in the quality of *S. ningpoensis* (Han et al., 2017). Therefore, it is of great significance to study the composition of the microbial community of *S. ningpoensis* roots obtained from different habitats, for promoting plant growth and active component accumulation.

In this study, we sequenced the rhizosphere and endophytic fungal microbiota in 400 *S. ningpoensis* cultivars, which were grown in 16 main areas, including the Zhejiang, Anhui, Henan, Sichuan, and Guizhou provinces of China. We evaluated the effects of the geographic location and microhabitat on the fungal community in the roots and performed a systematic association analysis between rhizospheric fungi and the active ingredient content, and identified a specific fungal genus that was correlated with the active ingredient content of the host plant. Our study provides a basis for facilitating the potential agricultural improvement of *S. ningpoensis* via the modification of microbiota.

Materials And Methods

Sample collection and processing

All samples were collected in December 2019 from 16 main areas, including the Zhejiang, Anhui, and Sichuan provinces. A 20 × 20 m sample plot was selected from each main producing region, and five plants were randomly selected from each site using the five-point sampling method; a total of 25 plant samples were selected from each of the sampling locations. The plant samples for each producing area were collected and included into sterile plastic bags, placed on ice, and transported to the laboratory immediately. The soil attached loosely to the roots was removed via gentle shaking. The soil adhered to the root surface, which formed an approximately 1 cm thick layer, was manually removed and collected in a 50-mL Falcon tube as the rhizosphere compartment, and fibrous roots and other impurities were removed from the soil. The samples were thoroughly pooled to generate a composite sample and divided into three replicates to make the samples more representative (Zhu et al., 2020). The soil attached to the taproots and fibrous roots was collected, and these roots were washed with tap water and then rinsed three times with distilled water. Samples from each plant tissue were successively immersed in 75% ethanol for 1.5 min, a fresh 2.5% sodium hypochlorite solution for 3 min, and 75% ethanol for 30 s for surface disinfection. Then, they were washed with distilled water three times and cut into 2-3 cm sections that were designated as endophytic samples; 6 g of these root sections were pooled in a 10 mL tube (Zhu et al., 2020). Each plant tissue was thoroughly mixed to make a composite sample, which was randomly divided into three subsamples.

The remainder of the roots was dried and used to analyze the harpagide and harpagoside levels. Soil samples obtained in bulk were also collected from an unplanted site far away from the *S. ningpoensis* plants. In total, 96 soil samples (16 bulk soil samples and 16 rhizospheric soil samples; 3 replicates each) and 48 root samples (roots from 16 areas; 3 replicates each) were collected. Collected soils were sieved through a filter with a pore size < 4 mm. All samples were immediately stored at -80°C before DNA extraction.

DNA extraction and high-throughput ITS rRNA amplicon sequencing

DNA was extracted from the rhizospheric and endophytic community derived from soil and root samples using the E.Z.N.A.[®] soil DNA kit (Omega Bio-tek, USA), according to the manufacturer's instructions. PCR amplification was performed using the primers ITS1-F (CTTGGTCATTAGAGGAAGTAA) and ITS2-R (GCTGCGTTCTTCATCGATGC), which amplify the ITS rRNA gene (Eppendorf Mastercycler pro, Germany). The resulting amplicons were separated by

electrophoresis using a 2% agarose gel and extracted using the Axyprep DNA Gel Extraction Kit (Axygen Biosciences, USA). The amplicons were pooled before performing paired-end sequencing (2×300) on an Illumina MiSeq platform (Illumina, USA). The read yield was quantified and purified using a NanoDrop2000 UV-vis spectrophotometer (Thermo Scientific, USA). In addition, the DNA samples were electrophoresed on a 1% agarose gel to assess their quality and integrity further. PCR was performed in triplicate for each DNA sample, resulting in a total of nine PCR products for each plant/soil sample (3 DNA replicates and 3 PCR replicates each). DNA samples were further processed via high-throughput sequencing to analyze the composition and diversity of the fungal community.

Analysis of soil properties

We analyzed the chemical and physical composition of all soil samples. Soil pH was measured using a pH meter at a soil:water ratio of 1:5 after 2 h in a suspension of deionized water (Machado et al., 2014). The soil organic matter (OM) and total organic content (TOC) were measured using Zhu's method (Zhu et al., 2020). The total nitrogen (TN), total phosphorus (TP), and total kalium (TK) levels in the soil were measured by semi-micro Kjeldahl digestion (Nelson and Sommers, 1980), ammonium molybdate (Murphy and Riley, 1962), and atomic absorption spectrophotometric methods (Analyst 00, PerkinElmer, US) (Yao et al., 2017). Heavy metals such as Mn, Cu, Cr, and Hg were detected using flame atomic absorption spectrometry (Chen et al., 2011). All assays were performed in triplicate.

Determination of harpagide and harpagoside levels in *S. ningpoensis* derived from different regions

High-performance liquid chromatography (HPLC) was used to determine the harpagide and harpagoside levels in *S. ningpoensis* roots. The roots of *S. ningpoensis* obtained from different regions were washed using water and then sliced and baked in an oven at 60 °C until the mass of the dried roots remained constant. After crushing the dried roots, the contents were sieved through a filter mesh with a size of 50 mm. We accurately weighed 0.25 g of each *S. ningpoensis* root sample powder, placed the powder into a 25 mL conical flask with a stopper, added 25 mL of 50% methanol solution, and tightly closed the flask with the stopper. After allowing the root powder to soak in the solution for 1 h, ultrasonic treatment was applied for 45 min (300 W, 40 kHz), and the contents were cooled and weighed. The loss in weight was made up with 50% methanol solution. The contents were shaken vigorously and filtered using a 0.45 µm microporous filter, and the filtrate was obtained as a test substance. Appropriate amounts of harpagide and the harpagide reference substance were weighed accurately, and a mixed reference substance solution containing 0.6 mg harpagide and 0.2 mg harpagide per mL was prepared using 30% methanol solution, which was stored in the refrigerator at 4 °C for future use. The mixed harpagoside reference solution (0.34, 0.67, 1.00, 1.40, 1.70 mL) was carefully absorbed and placed in a 2 mL volumetric flask. The volume of the solution was made up to 2 mL with 30% methanol solution. The solution was shaken vigorously and filtered with a 0.45 µm microporous membrane and used as a reference substance.

The harpagide and harpagoside in the reference and test substances were separated on a reversed-phase C₁₈ column (250 mm × 4.6 mm, 5 µm) with an acetonitrile-0.03% phosphate solution via gradient elution (Table S1). The flow rate of the mobile phase was 1 mL/min, the detection wavelengths were set at 210 and 280 nm, and the column temperature was set at 30 °C. All assays were performed in triplicate.

We have performed a methodological examination by carrying out the linear relation test, stability test, precision test, repeatability test, and recovery test, to guarantee the reliability of this analysis method (Table S2). All assays were performed in triplicate.

Bioinformatics analysis

Raw sequence reads were analyzed using Trimmomatic and FLASH software (Zhu et al., 2020). This analysis process involves four steps, including the removal of a low-quality sequence (average quality score < 20) sliding window containing Ns. The remaining paired-end reads were assembled into larger contigs according to their overlaps in such a manner that the minimum overlap length was 10 bp. The maximum mismatch ratio allowed in the overlap area of the merged sequence was 0.2; the directionality of the reads was corrected based on their barcodes and primer sequences, with no mismatches allowed in the barcode and 2 mismatches allowed in the primers (Trimmomatic and FLASH).

Non-chimeric sequences, whose quality was filtered by software, were clustered into the same operational taxonomic units (OTUs) at a minimum pairwise identity of 97% using UPARSE (version 7.1). Non-fungal species, chloroplasts, and archaea were removed before taxonomic classification. The annotated taxonomic information for each representative sequence was selected from each OTU using a ribosomal database project (RDP) classifier (Wang et al., 2007), to generate the OTU table file. Alpha values within samples were analyzed using in-house Perl scripts. Next, the OTU table was used to calculate the observed species, Shannon's index, and Chao 1 index (Zhu et al., 2020). Weighted Bray-Curtis distance matrices were calculated using principal coordinate analysis (PCoA), to determine the beta-diversity (β-diversity). The above methods involved the use of R and QIIME (Quantitative Insights into Microbial Ecology).

Statistical analysis

One-way ANOVA (Tukey's test) was used to evaluate the physicochemical properties of bulk soil as well as the differences in the relative abundance of fungi or the diversity between samples. Spearman correlation analysis was used to evaluate the relationship between the RF of the rhizospheric and endophytic fungi at the genus level. SPSS (version 19.0) was used to determine the multiple linear regression equation between the abundant genus and the active constituent (Yang et al., 2020). Redundancy analysis (RDA) and canonical correspondence analysis (CCA) were performed to investigate the relationships between fungal genus and soil properties. A heatmap was drawn to show the fungal distribution of the top 50 abundant OTUs of each sample. Principle coordinate analysis (PCoA) was performed after taking the relative abundance of OTUs in the rhizospheric and endophytic fungi into consideration (Caporaso et al., 2010). The difference between the genera at sample sites was tested using the Kruskal-Wallis H test. The significance of the index value between the rhizospheric and endophytic fungi was detected using the Wilcoxon signed-rank test; if $p < 0.001$, values were considered significant.

Results

Composition of the endophytic and rhizospheric fungal community in the root

In this study, we collected rhizospheric, endophytic, and corresponding unplanted bulk soil samples from *S. ningpoensis* cultivars in 16 well-separated major regions of China. We sequenced the ITS1F-ITS2R region of the ITS rRNA genes in 96 samples (48 rhizospheric and 48 endophytic samples); after filtering out reads for quality, we obtained 6,055,850 high-quality reads for subsequent analyses, i.e., an average of 63,081 sequences per sample. After discarding operational taxonomic units (OTUs) that were non-fungal and low in abundance, we obtained 8,709 unique OTUs with 97% similarity in the entire S1-S2 region (Table 1).

The rarefaction curves observed using the Chao 1 index indicated that the sequencing depth was sufficient to cover the fungal diversity within individual samples (Fig. S1). The α -diversity decreased from the rhizospheric to endophytic fungi in different compartments (Wilcoxon signed-rank test, $p < 0.001$) (Fig. S2). A Venn diagram revealed that 2,574 OTUs, which represent 29.6% of the total number of OTUs, were common to all rhizospheric and endophytic fungal communities. In addition, a total of 5,780 OTUs (66.4% of the total number of OTUs) were unique to rhizosphere samples, whereas 355 OTUs (4.1% of total) were unique to endophytic samples (Fig. S3). These results suggested that the rhizosphere harbored most of the unique OTUs.

The fungal community consisted of 16 different phyla. The distribution of the rhizospheric and endophytic microbiota obtained from 16 regions at the phylum level has been shown in Fig. 1. The dominant phyla present among all the fungal communities were Ascomycota, Basidiomycota, Mortierellomycota, unclassified_k_fungi, Glomeromycota, and Rozellomycota. In the rhizospheric compartment, Ascomycota and Basidiomycota represented an average of 69.03% and 18.31% of all species, whereas they represented 71.31% and 26.24% of the species in the endophytic community. However, the relative abundance of Mortierellomycota was 9.18% higher in the rhizosphere and 9-fold higher among endophytic fungi. The genus *Plectosphaerella* was found to be highly abundant in areas such as Taiqiu, Zhuzhuang, Nanfeng, Luolong, Yangxi, Yuxi, Guoyang, Qiaocheng, Lixin, and Nanchuan, while the unclassified_f_Schizoporace and *Exophiala* genera were abundant in Zhiwuyuan. The *Guehomyces*, *Ceratobasidium*, and *Cladosporium* genera were found to be abundant in Panan, Linan, and Longdong, respectively. The genera found in abundance in Wulong included unclassified_p_Ascomycota and *Mortierella*.

The Chao richness and Shannon diversity values indicated that the fungal communities in the endophytic compartment were less rich and diverse than the rhizosphere (Table S3). Furthermore, it was apparent that the endophytic fungal community was more selective, as the community exhibited a lower level of richness and diversity than the rhizosphere. Additionally, higher levels of richness were observed in areas such as Longdong and Yongfu, while lower levels of richness were observed in Taiqiu and Zhuzhuang.

We performed a hierarchical clustering analysis involving the 50 most abundant species in the fungal community across rhizospheric and endophytic samples (Fig. S4). The analysis showed that the rhizospheric community was well separated from the endophytic compartment. Fungal communities occurring in the same province were clustered into one category; for example, the rhizospheric samples were clustered together in the RS_NF, RS_ZZ, and RS_TQ categories. Additionally, categories such as RS_WL, EP_WL, RS_PA, and EP_PA were used for the clustering of individual samples; this suggested that the rhizospheric fungal community was similar to the endophytic community. PCoA was performed based on the OTU composition; the results revealed that there were significant variations among the 96 rhizospheric and endophytic fungal samples. The first two axes (PC1 and PC2) showed that 22.63% and 8.57% of the total variance could be observed in the fungal OTUs of the rhizospheric and endophytic samples, respectively (Fig. 2). Based on the different communities, rhizospheric samples were clustered in two regions, while there were more variations in the endophytic communities observed between sampling locations compared to the rhizosphere. Overall, the results indicate a clear division between the endospheric and rhizospheric compartments.

Correlation between soil properties and fungal communities

The enrichment of microbial communities is influenced by biotic and abiotic factors. We estimated the chemical and physical properties of all soil samples (Table 2), and the results showed that the difference in TP in all bulk soil samples was the smallest; conversely, the difference in the Mn content was the largest. In addition, the samples exhibiting lower TP and Mn levels were found in Panan, while those exhibiting higher TP and Mn levels were found in Yangxi and Nanfeng. Meanwhile, we found that the correlation between the rhizospheric and soil physicochemical compartments was stronger than that observed with the endophytic compartment (Table S4, S5). In the rhizospheric community, the *Plectosphaerella* (PL) and *Bulleromyces* (BU) genera exhibited a significant negative correlation with OM ($R_{PL}^2 = 0.320$, $R_{BU}^2 = 0.066$, $p \leq 0.01$), TOC ($R_{PL}^2 = 0.319$, $R_{BU}^2 = 0.065$, $p \leq 0.01$), and TN ($R_{PL}^2 = 0.191$, $R_{BU}^2 = 0.037$, $p \leq 0.05$), and were positively correlated with TK ($R_{PL}^2 = 0.308$, $R_{BU}^2 = 0.007$, $p \leq 0.05$). Conversely, genera such as unclassified_p_Ascomycota (UPA) and *Apiotrichum* (AP) were observed to be positively correlated with OM ($R_{UPA}^2 = 0.288$, $R_{AP}^2 = 0.030$, $p \leq 0.05$), TOC ($R_{UPA}^2 = 0.286$, $R_{AP}^2 = 0.029$, $p \leq 0.05$), and TN ($R_{UPA}^2 = 0.032$, $R_{AP}^2 = 0.027$, $p \leq 0.05$) (Table 4). In the endophytic fungal community, unclassified_f_Ceratobasidiaceae (UFC), *Pyrenochaeta* (PY), and unclassified_f_Melanommataceae (UFM) were positively correlated with OM ($R_{UFC}^2 = 0.555$, $R_{PY}^2 = 0.41$, $R_{UFM}^2 = 0.000$, $p \leq 0.05$), TOC ($R_{UFC}^2 = 0.556$, $R_{PY}^2 = 0.041$, $R_{UFM}^2 = 0.000$, $p \leq 0.01$), and TN ($R_{UFC}^2 = 0.553$, $R_{PY}^2 = 0.066$, $p \leq 0.05$; $R_{UFM}^2 = 0.000$, $p \leq 0.01$).

We then removed the redundant variables and performed detrended correspondence analysis (DCA) for the ten remaining environmental characteristics. The results of DCA showed that the responses of the rhizospheric fungal community composition to the soil properties fit a single peak model (Length = 2.75). Therefore, we further analyzed how fungal communities associated with soil physicochemical factors were based on RDA (Fig. 3a). The first two axes of RDA explain why 45.13% and 12.27% of the total variations were observed in the data. The results of RDA suggested that soil physicochemical factors such as TOM, TOC, TN, and Hg were positively correlated with each other, while TP, TK, pH, Mn, Cu, and Cr levels were positively correlated with each other. Moreover, TOM ($p = 0.001$), TOC ($p = 0.001$), and TK ($p = 0.001$) had a significant correlation with the rhizospheric fungal community structure. Meanwhile, we used the CCA method for the analysis of the association between the endophytic community and soil physicochemical factors (Length = 4.71). We found that the correlation between endophytic fungi and environmental factors was consistent with that between the rhizospheric fungi and environmental factors (Fig. 3b).

Correlation between the root fungal community and active ingredients of *S. ningpoensis*

There are many non-pathogenic microorganisms present inside and outside plants. These microorganisms coexist with plants for a long time. This has a great influence on the formation and medicinal ingredient content in plants (Tello, 2011). According to the 2020 edition of the Chinese pharmacopoeia, harpagide and harpagoside were used to control the quality of *S. ningpoensis*. We measured the levels of harpagide and harpagoside in 16 main producing regions (Table S6), and found that the levels of harpagide isolated from Longdong and Yuxi were higher than those isolated from other areas, while the content isolated from Qiaocheng was lower. The harpagoside content in Panan was higher than that in other areas, and lower levels of harpagoside were found in Yongfu. In addition, the total harpagide and harpagoside content isolated from Nanchuan was higher than that obtained from other areas, while that obtained from Zhiwuyuan was lower. However, the sum of the harpagide and harpagoside levels in *Radix scrophulariae* obtained from 16 areas was higher than 0.45 mg, which indicated that *Radix scrophulariae* obtained from all producing areas met the standards of the 2020 edition of the Chinese Pharmacopoeia.

We analyzed the relationship between the active constituents and the abundance of fungi (RF >1%), including the rhizospheric and endophytic compartments, using correlation and regression analysis (Table S7). The harpagide content (Y_1), harpagoside content (Y_2), the sum of the two (Y_3), and the relative frequencies (X) of the fungal community of *S. ningpoensis* roots were analyzed in table S8. Equation 1 suggests that the highest effect on the harpagide content was observed in unclassified_o_Pezizales, followed by *Cladosporium*, unclassified_p_Basidiomycota, unclassified_f_Melanommataceae, *Pyrenochaeta*, and *Aspergillus*. The harpagide content was positively correlated with the abundance of *Cladosporium*, *Pyrenochaeta*, unclassified_o_Pezizales, unclassified_p_Basidiomycota, and *Aspergillus*; however, a negative correlation with unclassified_f_Melanommataceae was observed. The results obtained using equation 2 showed that the harpagoside content was positively correlated with *Alternaria*, *Apiotrichum*, *Plectosphaerella*, *Minimelanolocus*, *Penicillium*, and unclassified_f_Cordycipitaceae, and was negatively correlated with unclassified_f_Ceratobasidiaceae, *Geotrichum*, and *Trichoderma*. The effect on the harpagoside content was the highest in unclassified_f_Cordycipitaceae, followed by *Alternaria*, *Minimelanolocus*, *Penicillium*, *Apiotrichum*, unclassified_f_Ceratobasidiaceae, *Geotrichum*, *Trichoderma*, and *Plectosphaerella*. In addition, equation 3 suggests that the sum of the harpagide and harpagoside content was positively correlated with the abundance of unclassified_c_Sordariomycetes, and negatively correlated with the abundance of unclassified_f_Schizoporaceae and unclassified_f_Ceratobasidiaceae. The effect on the sum of harpagide and harpagoside content was the highest in unclassified_c_Sordariomycete, followed by unclassified_f_Ceratobasidiaceae and unclassified_f_Schizoporaceae.

Discussion

The presented data provide new insights regarding plant microbial interactions that promote the occurrence of active ingredients in the host plant, while most previous studies have focused merely on the diversity of microbial communities (Hartman et al., 2017). Furthermore, only a few studies have focused on the relationship between *S. ningpoensis* root microbiomes, including rhizospheric and endophytic fungi, and active constituents that have been derived from different areas. To determine the effects of root associated fungal microbiomes of plants on the active ingredients in host plants obtained from different areas, we used ITS rRNA high-throughput amplicon sequencing, to characterize the rhizospheric and endophytic fungal communities, and predicted the association between abundantly present genera and active constituents in 16 major production areas.

Illumina MiSeq of PCR amplicons and sequence analysis revealed that the rhizospheric and endophytic profiles varied considerably across different sampling locations. Our results also showed that the microbiome was mainly composed of rhizospheric and endophytic fungi. In addition, alpha diversity decreased from the *S. ningpoensis* rhizosphere to the endophytic fungal microbiota. These findings were consistent with reports on other plant species such as *Populus deltoides* (Shakya et al., 2013), *Betula celtiberica* (Mes et al., 2017), *Populus tremula*, and *Populus alba* (Beckers et al., 2017), which indicates that the fungal community in the *S. ningpoensis* root follows the general rules for the establishment of microbiota. Our results also revealed that several phyla that were observed commonly in the rhizosphere (Ascomycota, Basidiomycota, Mortierellomycota, Glomeromycota, and Rozellomycota) were almost absent in endophytic fungi. Ascomycota and Basidiomycota have the ability to produce exopolysaccharides (EPSs). These fungal EPSs exhibit many bioactive properties, including anti-oxidative, anti-microbial, immunomodulatory, anti-tumor, hypolipidemic, hypoglycemic, and hepatoprotective activities, and could be used in medical applications (Monika et al., 2015). In addition, among eukaryotes, Ascomycota is uniquely suited for regulatory evolution due to its broad phylogenetic scope, numerous sequenced genomes, and suitability for genomic analysis (Wohlbach et al., 2009). Basidiomycota could maintain soil balance and improve plant productivity in the alpine and temperate grasslands of China (Yang et al., 2019). Differences were detected in the fungal community profile data at a broad taxonomic level, such as at the phylum level. Our results indicated that there was an increase in the relative abundance of Basidiomycota in the endophytic community when compared to the rhizosphere. The ChE-like activity in fungi from the genus Basidiomycota was observed to be an inhibitory activity that aqueous mushroom extracts exhibited against insect acetylcholinesterase. This activity was independent of the presence of ChE-like activity in extracts (Sepi et al., 2019).

Previous studies have shown that soil factors, such as soil pH, organic matter content, and heavy metals, affect the enrichment of bacteria and fungi (Andrew et al., 2012; Ridder-Duine et al., 2005; Inceoglu et al., 2012; Santos-González et al., 2011). The findings of our study showed that the soil organic matter, total organic carbon, total nitrogen, and total potassium levels were most strongly correlated with the fungal community, including the rhizosphere and endosphere of *S. ningpoensis*. The genus *Plectosphaerella* was negatively correlated with the organic matter and total organic carbon content. Some of these correlations, such as the positive correlation between soil organic matter and total organic carbon and the abundance of Actinobacteria, have been suggested previously (Mitter et al., 2017). PCoA results demonstrated that the geographic location was not significantly correlated with the rhizospheric and endophytic fungal communities. Our results indicated that the physicochemical property of the soil was a major factor driving the assembly of the root-associated microbiome (rhizospheric and endophytic).

We analyzed the association between the harpagide, harpagoside, and harpagide + harpagoside levels in *S. ningpoensis* and the abundance of the corresponding rhizospheric and endophytic fungal microbiota using a multilinear regression (MLR) model. We used equations to define the relationship between the active constituent and genus abundance (RF > 0.01%), and found that the levels of active constituents, including harpagide and harpagoside, could be increased by using information regarding the abundance of only 16 fungal genera. The occurrence of most of these genera, including

Plectosphaerella, *Cladosporium*, *Sordariomycetes*, and unclassified_p_Schizoporaceae, was positively correlated with the active constituent content. Thus, an increase in the abundance of these genera may positively contribute to an increase in the active constituent content of *S. ningpoensis*. Our content determination results also confirmed this fact; for example, the abundance of *Cladosporium* was positively correlated with harpagide levels in the Y_1 equation. The harpagide content was found to be the highest in the most abundant genus in samples obtained from Longdong. The genus *Plectosphaerella* was highly abundant in samples obtained from Qiaocheng, Taiqiu, Zhuzhuang, and Nanfeng, and the level of abundance was positively correlated with the harpagoside content. Our results also indicated that the harpagoside content in these samples was higher than that in other samples. These results showed that these MLR equations might be considered to be significant, as it is widely accepted that apart from environmental factors, other factors such as biological factors and abiotic factors also affect the active constituent content. In addition, highly abundant genera, such as unclassified_f_Schizoporaceae and unclassified_f_Ceratobasidiaceae were negatively correlated with the active constituent content. The unclassified_f_Schizoporaceae genus was found to occur abundantly in samples obtained from Zhiwuyuan, while the sum of harpagide and harpagoside was the lowest; this could be associated with the abundance of this genus. However, the most highly correlated genera were not specifically identified and should be examined more thoroughly in future studies. On the other hand, these MLR equations were theoretical and need to be verified in the future.

Taken together, our work has systematically characterized the fungal microbiota in the *S. ningpoensis* root and identified potential root fungi that could provide a basis for the application of beneficial root fungi in applications related to yield improvement that might be developed in the future.

Declarations

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Competing interests

The authors declare no conflicts of interest.

Authors' Contributions

Dan Ren performed the experiments, analyzed the data, wrote and revised the manuscript; Kunyuan Guo and Qing-mei Sun performed the experiments, prepared figures and tables. Bo Zhu and Lu-ping Qin conceived and designed the experiments, authored, or reviewed drafts of the paper, and approved the final draft.

Data Availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

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Tables

Table 1. Summary of data and alpha-diversity of the fungal community of rhizosphere soil and plant endophytic samples.

Sample	Raw reads	Average length	Total bases	OTU (97%)	Shannon index	Chao 1 index
RS_PA	73139	219.57	16123835.67	1089	3.802	872.625
RS_TQ	61162	229.92	14133350.67	985	3.996	677.539
RS_ZWY	70026	235.46	16687022.00	2055	5.197	1445.553
RS_LA	67686	237.97	16209639.67	1366	4.241	990.647
RS_ZZ	73139	230.73	16222463.00	1045	4.197	741.141
RS_NF	57591	231.96	13418415.33	942	3.630	698.771
RS_LL	66710	237.38	16018774.67	1900	4.679	1218.916
RS_YX	66414	231.27	15477698.33	1625	4.672	1076.676
RS_YuX	61226	232.85	14441473.67	1970	5.028	1385.233
RS_LD	65471	227.85	15080974.33	2540	4.953	1871.363
RS_WL	58157	218.82	12982451.00	1680	4.280	1391.928
RS_YF	68263	231.79	16043366.33	2441	5.253	1880.486
RS_GY	57055	223.70	12818702.67	905	3.270	656.946
RS_QC	61541	226.79	14016394.33	1042	3.345	783.611
RS_LX	58534	224.96	13221149.33	1069	3.228	770.026
RS_NC	69103	234.86	16460730.33	2663	5.019	1747.072
EP_PA	60625	223.03	13525587.00	421	3.055	231.971
EP_TQ	56257	218.31	12325367.00	204	1.573	108.367
EP_ZWY	58618	246.24	14484156.67	410	2.044	240.467
EP_LA	47613	231.64	10978289.67	318	2.812	157.619
EP_ZZ	58401	217.96	12722221.00	222	1.649	117.556
EP_NF	61124	215.83	13216139.33	372	1.985	204.413
EP_LL	58913	221.91	13072060.00	439	1.611	240.401
EP_YX	60322	221.87	13363108.33	337	1.099	173.708
EP_YuX	63361	229.38	14582131.67	537	3.630	272.054
EP_LD	60677	234.12	14189659.33	833	3.460	409.252
EP_WL	71132	227.23	16255751.00	768	4.014	376.285
EP_YF	65982	227.80	15045188.67	697	3.516	360.967
EP_GY	57776	220.11	12707841.67	335	1.559	190.600
EP_QC	65596	218.17	14418289.00	337	1.418	157.586
EP_LX	61651	222.27	13698112.00	280	1.417	184.453
EP_NC	67186	225.94	15185097.67	406	1.642	232.618

*PA: Pan' an; ZWY: Zhiwuyuan; LA: Lin' an; TQ: Taiqi; ZZ: Zhuzhuang; NF: Nanfeng; LL: Luolong; YX: Yangxi; YuX: Yuxi; LD: Longdong; WL: Wulong; YF: Yongfu; GY: Guoyang; QC: Qiaocheng; LX: Lixin; NC: Nanchuan; RS: rhizosphere; EP: endophytic.

Table 2. Physical and chemical nature of bulk soil in 16 different area, China (n=3).

Sample	OM(g/kg)	TOC (%)	TN (mg/kg)	TP (mg/kg)	TK (mg/kg)	pH	Mn(mg/kg)	Cu(mg/kg)
BS_PA	40.63±0.31 ^e	2.37±0.03 ^e	1683.33±420.04 ^{efgh}	1066.67±30.55 ^b	18466.67±450.92 ^l	5.79±0.01 ⁱ	85.37±0.57 ^p	18.67±0 55 ^k
BS_TQ	20.13±0.55 ^k	1.18±0.03 ^k	1376.67±41.63 ^{hi}	909.33±13.61 ^b	28233.33±850.49 ^j	8.30±0.04 ^a	392.00±4.58 ⁱ	27.53±0.45 ⁱ
BS_ZWY	29.53±0.74 ^j	1.73±0.06 ⁱ	1380.00±50.00 ^{hi}	760.67±44.66 ^b	12666.67±404.15 ⁿ	8.12±0.05 ^c	167.00±1.00 ^l	37.67±0.35 ^e
BS_LA	35.10±0.56 ^g	2.07±0.05 ^g	1480.00±45.83 ^{ghi}	453.67±44.77 ^b	22833.33±378.59 ^k	7.01±0.03 ^g	111.77±0.68 ⁿ	21.57±0.50 ^j
BS_ZZ	33.37±0.55 ^h	1.94±0.04 ^h	1783.33±66.58 ^{efg}	706.00±7.00 ^b	39266.67±550.76 ^h	7.64±0.06 ^d	538.87±0.61 ^e	31.63±0.40 ^g
BS_NF	34.83±0.70 ^g	2.05±0.05 ^g	2030.00±55.68 ^{de}	1053.33±56.86 ^a	5633.33±568.62 ^d	8.22±0.04 ^b	913.50±0.50 ^a	79.23±0.35 ^a
BS_LL	47.30±0.30 ^d	2.73±0.04 ^d	2220.00±36.00 ^d	863.33±23.29 ^b	16566.67±416.33 ^m	7.23±0.03 ^e	632.07±0.21 ^c	31.23±0.40 ^g
BS_YX	38.37±0.42 ^f	2.23±0.05 ^f	2150.00±60.00 ^d	736.33±7.37 ^b	36366.67±568.62 ⁱ	6.62±0.03 ^h	678.70±1.77 ^b	31.80±0.56 ^g
BS_YuX	33.47±0.60 ^h	1.91±0.04 ^h	1333.53±55.08 ⁱ	404.33±6.51 ^b	46133.33±513.16 ^f	7.20±0.02 ^e	94.33±0.93 ^o	35.20±0.56 ^f
BS_LD	103.97±0.25 ^c	6.04±0.06 ^c	4326.67±534.63 ^b	736.33±5.03 ^b	44633.33±665.83 ^g	7.22±0.04 ^e	331.93±0.70 ^j	37.40±0.50 ^e
BS_WL	106.03±0.65 ^b	6.15±0.06 ^b	2880.00±45.83 ^c	644.47±6.03 ^b	97833.33±404.15 ^a	6.95±0.03 ^g	119.97±0.45 ^m	15.53±0.60 ^l
BS_YF	118.90±0.46 ^a	6.92±0.05 ^a	6480±30.00 ^a	1250.00±60.00 ^b	58600.00±458.26 ^c	6.64±0.08 ^h	602.17±0.47 ^d	63.30±0.56 ^b
BS_GY	20.17±0.40 ^k	1.17±0.03 ^k	1450.00±60.00 ^{hi}	805.00±6.24 ^b	54466.33±650.64 ^e	7.62±0.06 ^d	512.93±0.90 ^f	49.37±0.70 ^d
BS_QC	19.70±0.56 ^k	1.14±0.05 ^k	1180.00±36.06 ⁱ	906.67±13.80 ^b	68500.00±600.00 ^b	7.24±0.05 ^e	484.17±0.38 ^g	29.63±0.61 ^h
BS_LX	24.53±0.67 ^j	1.43±0.04 ^j	1226.67±50.33 ⁱ	943.67±6.66 ^b	44466.67±650.64 ^g	7.01±0.04 ^g	448.23±0.78 ^h	30.17±0.45 ^h
BS_NC	39.17±0.25 ^f	2.27±0.02 ^f	1916.67±20.82 ^{def}	575.67±4.51 ^b	39466.67±351.19 ^h	7.10±0.02 ^f	214.17±0.47 ^k	50.27±0.35 ^c

*Different letters in one column denote significant differences at $P < 0.05$. BS: bulk soil.

Figures

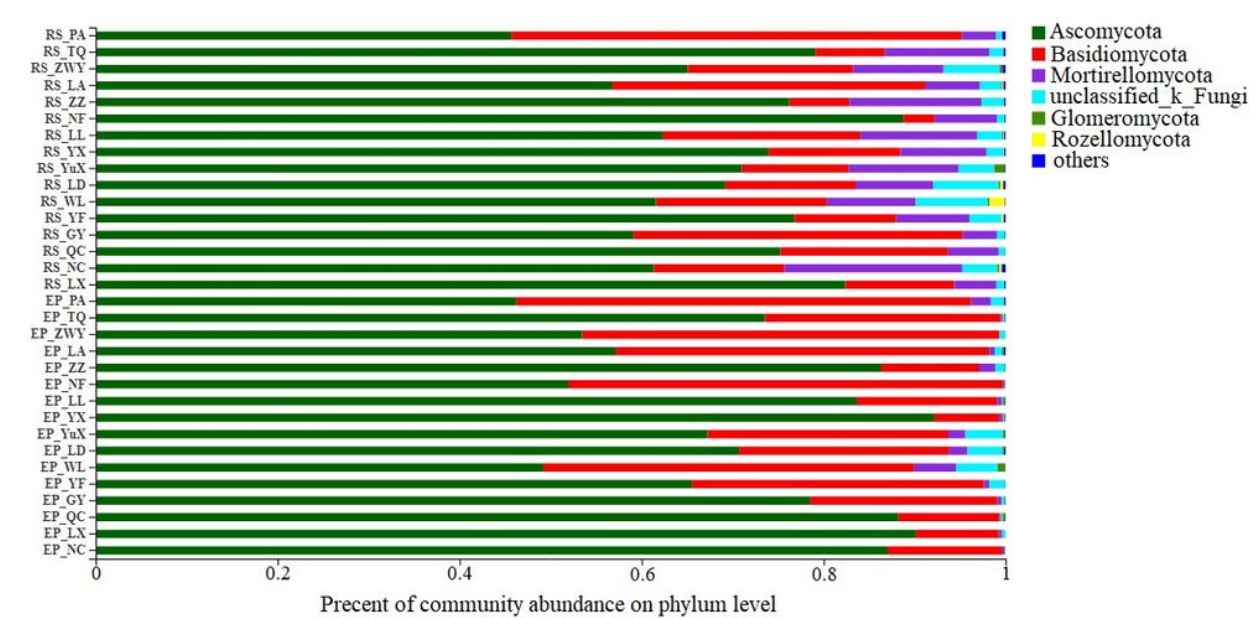


Figure 1

Dominant fungi phyla detected in *S. ningpoensis* root compartments. PA: Pan' an; ZWY: Zhiwuyuan; LA: Lin' an; TQ: Taiqiu; ZZ: Zhuzhuang; NF: Nanfeng; LL: Luolong; YX: Yangxi; YuX: Yuxi; LD: Longdong; WL: Wulong; YF: Yongfu; GY: Guoyang; QC: Qiaocheng; LX: Lixin; NC: Nanchuan; RS: rhizosphere; EP: endophytic.

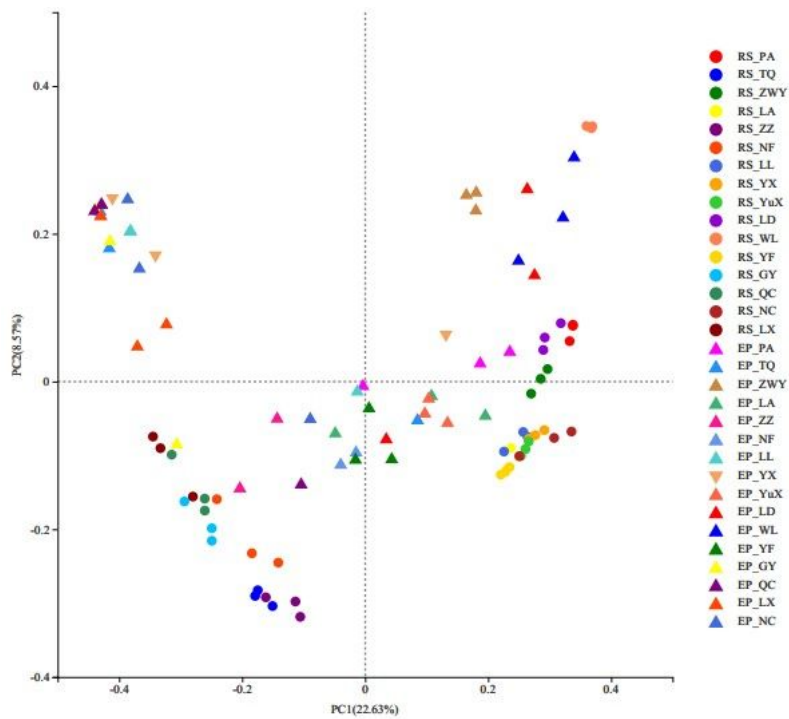


Figure 2

Principal coordinate analysis (PcoA) based on the operational taxonomic unit (OTU) composition. PA: Pan' an; ZWY: Zhiwuyuan; LA: Lin' an; TQ: Taiqiu; ZZ: Zhuzhuang; NF: Nanfeng; LL: Luolong; YX: Yangxi; YuX: Yuxi; LD: Longdong; WL: Wulong; YF: Yongfu; GY: Guoyang; QC: Qiaocheng; LX: Lixin; NC: Nanchuan; RS: rhizosphere; EP: endophytic.

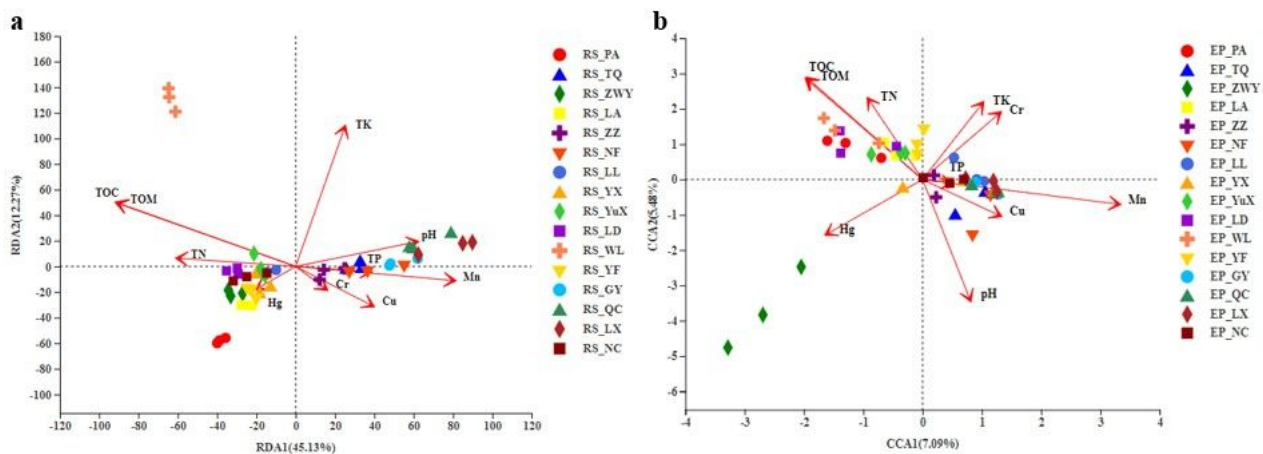


Figure 3

Redundancy Analysis (RDA) between the soil and rhizosphere community (a) and Canonical correspondence analysis (CCA) between the soil and endophytic community (b).

PA: Pan' an; ZWY: Zhiwuyuan; LA: Lin' an; TQ: Taiqiu; ZZ: Zhuzhuang; NF: Nanfeng; LL: Luolong; YX: Yangxi; YuX: Yuxi; LD: Longdong; WL: Wulong; YF: Yongfu; GY: Guoyang; QC: Qiaocheng; LX: Lixin; NC: Nanchuan; RS: rhizosphere; EP: endophytic.

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