# Temporal and spatial pattern of endophytic fungi diversity of *Camellia Sinensis* (cv. Shu Cha Zao)

**Zhen zhen Wu**
Anhui Agricultural University
**Qing qing Su**
Anhui Agricultural University
**Yu chen Cui**
Anhui Agricultural University
**Hong zhu He**
Anhui Agricultural University
**Jia li Wang**
Anhui Agricultural University
**Yong Zhang**
Anhui Agricultural University
**Yu Zhao**
Anhui Agricultural University
**Hassan Abul**
Anhui Agricultural University
**Yun qiu Yang**
Anhui Agricultural University
**Yan hua Long** ( longyanhua@ahau.edu.cn )

**Research article**

**Keywords:** Shu Cha Zao, Endophytic fungi, Spatial and dynamic distribution, High-throughput sequencing

**Posted Date:** May 7th, 2020

**DOI:** https://doi.org/10.21203/rs.3.rs-26432/v1



**Version of Record:** A version of this preprint was published at BMC Microbiology on August 28th, 2020.
See the published version at https://doi.org/10.1186/s12866-020-01941-1.

# Abstract

**Background:** The experimental materials were a 60-year-old tea tree (cv. Shu Cha Zao; SCZ) (the mother plant), and 1-year-old and 20-year old plants of SCZ that originated as cuttings from the mother plant. The aim of this study was to explore the spatial and dynamic distribution of endophytic fungi in different leaf niches (upper leaves, middle leaves, lower leaves) and rhizosphere soil on tea plants (*Camellia sinensis*) which with different ages in the same garden by high-throughput sequencing.

**Results:** In this study, Ascomycota (83.77%), Basidiomycota (11.71%), and Zygomycota (3.45%) were the three dominant fungal phyla in all samples. *Cladosporium* (12.73%), *Zymoseptoria* (9.18%), and *Strelitziana* (13.11%) were the dominant genera in the leaf. Alpha diversity analysis revealed that endophytic communities in leaves differed from those in rhizosphere soil and different leaf niches had similar fungal diversity. Shannon's indices and NMDS analysis indicated significant differences in fungal diversity and composition among the SCZ trees of different ages ($p \leq 0.01$). The abundance of *Cladosporium* and *Zymoseptoria* decreased with increasing SCZ age, whereas the abundance of *Strelitziana* increased.

**Conclusions:** Our results illustrate the variation in endophytic fungi among different niches on tea plants of different ages. The distribution of endophytic fungi in leaves of *C. Sinensis* (cv. Shu Cha Zao) has a certain spatiotemporal variation.


# Background

Endophytic fungi live in healthy plant tissues at certain stages or throughout their life history. They cause no apparent symptoms of disease to plant tissues, latent pathogens that temporarily do not harm the host, and mycorrhizal fungi [1]. A strain of endophytic fungus (*Taxomyces andreanae*), which can produce anti-cancer substance, was isolated from Pacific yew (*Taxus brevifolia*) firstly [2]. Researches about endophytic fungi aroused general interest from that on.

Kinds of endophytic fungi have been isolated from different plants, such as moss [3], fern [4], grass [5], shrub [6], conifer [7] and deciduous tree [8]. But most of the researches focus on the isolation, identification, diversity, metabolites and the interaction with hosts [9, 10, 11].

In recent researches, the endophytic community diversity of plants varied spatially was highlighted. Using *Populus* as a model ecosystem, Cregger found that the fungal microbiome varied among plant habitat classes (leaves, stems, roots, and soils) regardless of the plant genotype, and differed markedly between stems and soils [12]. A study on sugarcane identified 23,811 bacterial operational taxonomic units (OTUs) and 11,727 fungal OTUs inhabiting the endophytic and exophytic compartments of roots, shoots, and leaves [13]. These communities originated from the native soil surrounding the plants, and plant organs were colonized via different patterns. The dynamics of endophytic fungal communities were found to be influenced by plant genotype and plant growth stage in sugar beet. Endophytic fungal diversity in stages of seedling growth (18 OTUs) and rosette formation (58 OTUs) were lower than during

sucrose accumulation (84 OTUs) and tuber growth (74 OTUs) [14]. However, investigation about the dynamics of endophytic fungi diversity in different leaf niches and rhizosphere soils of *C. sinensis* with different ages has not been reported until now.

*C. sinensis* is an evergreen, non-deciduous, perennial shrub which belongs to the Theaceae family [15, 16]. The tea prepared from its leaves is a popular non-alcoholic beverage with some important health benefits. Many studies have focused on endophytic fungi of other economically important plants, but few have focused on those of tea plants. Recent studies on the endophytic fungi of *C. sinensis* have focused on diversity and universality [17, 18], the distribution of endophytic fungi in different tissues (leaves, stems, roots) [19], and how their distribution is affected by season, habitat [20], and leaf age [21]. In those studies, fungi were isolated using traditional culture methods. While with the development of molecular biology, sequence analysis on 18S rDNA [22, 23, 24] and internal transcribed spacer (ITS) region [25, 26, 27] have been widely used in classification and identification of fungi. And based on the advantages of high throughput sequencing, this method has been the core choice in studies on fungal community diversity [28, 29].

The endophytic fungi diversity, especically their dynamic changes during tea plants' growth and development, have not been systemically analyzed and reported until now. Therefore, using high-throughput sequencing technology and uncommon plant materials, we investigated the fungal community structure and diversity in the upper leaves, middle leaves, lower leaves, and rhizosphere soil of all samples, determined the discipline of changes in different leaf niches or ages of tea plants. The results of this study may lay the foundation for further studies on the co-evolution and adaptation mechanisms of *C. sinensis* and leaf endophytic fungi.

# Results

OTU clustering and species annotation

The raw sequence data of all samples consisted of 5,060,529 reads prior to quality checking and assigning the reads to their respective samples. The average read length (± standard deviation) of reads before processing was 243.77 ± 11.20 bp. After quality trimming and assigning reads to different samples, 4,489,368 high-quality reads remained in the data set, with an average length (± standard deviation) of 246.45 ± 15.36 bp. A total of 3,753 OTUs were generated after clustering at a 97% similarity level. Representative sequence for each OTU was screened for further annotation. 7 phyla, 33 classes, 117 orders, 271 families, 480 genera, and 762 species were identified from these sequences.

Composition of fungal communities

At the phylum level, Ascomycota (83.77%), Basidiomycota (11.71%), and Zygomycota (3.45%) were the three dominant phyla (Fig. S1). At the class level, Dothideomycetes (52.8%) and Eurotiomycetes (32.67%) own a higher proportion compared to rhizosphere soil (11.41% and 6.95%, respectively) in all ages' leaf samples. Sordariomycetes and Leotiomycetes were more abundant in rhizosphere soil (23.76% and

14.40%, respectively) than that in leaf niches (1.25% and 1.45%, respectively) (Fig. 1). Notably, Cystobasidiomycetes and Microboiryomyceres were more abundant in the upper leaf than other leaf niches. Capnodiales (25.35%) and Chaetothyriales (13.93%) were the dominant orders in leaf niches (Fig. S1), while Mortierellales (12.10%), Hypocreales (11.47%) and Helotiales (8.53%) were the dominant orders in rhizosphere soil. The dominant families in the leaf niches were Davidiellaceae (14.65%), Incertae_sedis_Chaetothyriales (13.31%), Incertae_sedis_Dothideomycetes (12.71%) and Mycosphaerellaceae (9.86%). But Mortierellaceae (11.21%), Nectriaceae (4.86%) and Amphisphaeriaceae (4.27%) show a certain high proportion in the rhizosphere soil (Fig. S1). *Cladosporium* (12.73%), *Zymoseptoria* (9.18%), and *Strelitziana* (13.11%) were the dominant genera in the leaves (Fig. S1).

A Venn diagram was constructed to highlight the similarities and differences in communities among different ages of plants and leaf/soil niches. The communities in YS, ES and LS had 565 OTUs in common; the upper leaves, middle leaves, lower leaves, and rhizosphere soil had 487 OTUs in common (Fig. 2). Some OTUs appeared in the leaf endophytic fungi community were also detected in the rhizosphere soil, which showed the colonization probability of soil fungi in leaves. The large number of common OTUs among samples from different-aged trees indicated that colonization patterns may be conserved during long-term evolution. The leaf endophytic fungal communities differed between LS and YS/ES (Fig. 3) remarkably. According to the relative abundance, we found that the abundance of some families among the top 35 OTUs, such as Dothioraceae, Taphrinaceae, Wallemiaceae, Amanitaceae, Herpotrichiellaceae, Incertae_sedis_Capnodiales, Incertae_sedis_Sporidiobolales, Incertae_sedis_Dothideomycetes, Davidiellaceae, Teratosphaeriaceae, Incertae_sedis_Erythrobasidiales, Geoglossaceae, Incertae_sedis_Pleosporales, Rutstroemiaceae, and Pleosporaceae, decreased with the increasing age of the plant. In contrast, the abundance of Incertae_sedis_Chaetothyriales, Elsinoaceae, Mycosphaerellaceae, Tuberaceae, Glomeraceae, and Ramalinaceae increased with the tree age, and gradually became dominant in LS.

Alpha rarefaction curves and alpha diversity

The rarefaction curves approached the plateau phase, indicating that it would be unlikely for more fungal taxa to be detected with additional sequencing (Fig. 4). And these curves showed the endophytic fungi communities were less diverse in leaves than in the rhizosphere soil, as evidenced by differences in number of OTUs between these communities.

Community richness and diversity were analyzed using five alpha diversity indices: Chao1, Shannon's, Simpson's, ACE, and Goods_coverage (Table S1). The depth index (Goods_coverage) of each sample library was over 99% (99.2–99.8%), indicating that the sampling was reasonable. The Chao1 and ACE indices are indicative of fungal community richness, and Shannon's and Simpson's indices are indicative of fungal community diversity. The fungal richness and diversity were significantly higher in rhizosphere soil than in leaf samples ($p < 0.0001$) (Fig. 5a). Especially, the richness and diversity of fungi in the rhizosphere soil of the mother plant were highest among all samples (Table S1). The different leaf niches had similar fungal alpha diversity (Fig. 5a). The Shannon's indices indicated that fungal diversity was

greater in younger plants (YS and ES) than in the mother plant (LS) ($p \leq 0.01$), but did not differ significantly between YS and ES (Fig. 5b).

Beta diversity

Applying NMDS analysis, the degree of difference between groups or in-group can be reflected through a multidimensional space. Here, the NMDS analysis revealed clearly that the mycobiomes between rhizosphere soil samples and leaf samples were significantly distinguished ($R^2 = 0.23$, $p < 0.001$) (Fig. 6, left).

Furthermore, when analyzing the mycobiome composition of all samples, no matter the different leaf niches or the different tree ages, the samples could be clearly separated ($R^2 = 0.022$, $p < 0.001$) (Fig. 6, right). And independent analysis of YS, ES and LS revealed significant differences respectively in the composition of endophytic fungi in the upper, middle and lower niche ($p < 0.001$). Among them, the composition of endophytic fungi in the upper leaves of YS was not significantly different from that in the middle ($R^2 = 0.178$, $p = 0.092$), but the differences between the upper and lower layers and the middle and lower layers were quite significant ($R^2 = 0.394$, $p = 0.007$; $R^2 = 0.274$, $p = 0.006$). This maybe the reason that plant height of annual plant was not high enough and endophytic fungi distributed in different height leaves evenly. In the comparative analysis of the endophytic fungi composition of the three leaf niches of ES, the pairwise differences between them were found to be significant ($p < 0.05$). This indicated that as the plant has grown for 20 years in this study, the colonization of leaf endophytic fungi at different heights has formed a significant difference. Growth time has a certain effect on the distribution of endophytic fungi. However, in the LS sample, the differences between the upper, middle, and lower layers were not significant. This may be the reason that the plant growth period was so long enough that the endophytic fungi distributed in the three niches widely.

A horizontal comparison of tree age based on different leaf niches showed that there was a significant difference between different samples in middle and lower leaf layers ($p < 0.001$). However, in upper leaf layer, although the fungi composition between YS, ES, and LS existed significant difference, the difference between YS and ES was not significant ($p = 0.341$). This indicated that the leaves of upper layer of the tea branch that have grown for 20 years were consistent with the growth situation and distribution of endophytic fungi of the same layer leaves of the one-year-old plant. This could also be explained by the number of shared OTUs among YS and ES (789 OTUs), which was much higher than that of YS and LS (678 OTUs) or ES and LS (704 OTUs) (Fig. 2).

All these analysis showed a certain discipline of the distribution of endophytic fungi in different leaf niches of the same plant. At the same time, the composition and distribution of endophytic fungi in plants also showed certain differences with the growth time. That is to say, the distribution of endophytic fungi in leaves of *C. Sinensis* (cv. Shu Cha Zao) has a certain spatiotemporal variation.

# Discussion

During the long period of co-existence and evolution, some endophytic fungi have evolved as integral partners of plants [36]. C. *sinensis* and its endophytic fungi may have formed stable relationships during evolution [20]. Many fungal species colonize the SCZ leaf, and gradually form a dynamic equilibrium through continuous interspecific competition under the influence of environmental factors. In the tea plants studied here, the abundance of *Cladosporium* and *Zymoseptoria* decreased with increasing SCZ age, while the abundance of *Strelitziana* increased with increasing SCZ age (Fig. 7). This may indicate that there is competition among *Cladosporium, Zymoseptoria*, and *Strelitziana* after their colonization.

The fungal community composition differed significantly among niches and among tea plants of different ages. The composition of fungal communities is determined more by the soil they originated from than by the host plant species [37]. By comparing OTU abundance among the niches, we found that fungal community structure differed markedly between rhizosphere soil and the leaf niches (Fig. 1). Sordariomycetes and Leotiomycetes were more abundant in rhizosphere soil than in leaf niches. Perhaps members of Sordariomycetes were regarded as decomposers in soil and fungal parasites and pathogens of plants could explained this phenomenon well [38, 39].

The alpha diversity indices as well as the richness accumulation curves showed significantly larger values for rhizosphere soil than for the leaf niches (Fig. 4). The rhizosphere soil fungi were much more diverse than the leaf endophytic fungi (Fig. 5). An NMDS analysis also indicated that the community composition differed markedly between leaf niches and rhizosphere soil (Fig. 6). However, we detected significant differences in both fungal diversity and composition among SCZ trees of different ages. There were a considerable number of OTUs in the leaves of younger plants (YS and ES) than the mother plant (LS) (Fig. 3). The diversity difference of fungal communities between YS and ES is not significant while it showed a contrary result among YS, ES, and the mother plant (Fig. 5 and Fig. 6). The degree of difference in endophytic fungal diversity is directly proportional to the difference in plant age. Plants with a long growing period can show low species diversity and abundance of endophytic fungi. These results may indicate that young SCZ leaves are more susceptible to infection and colonization by endophytic fungi. The differences in fungal communities among the SCZ plants of different ages may be related to the geographical location distance between plants and their stage of growth and development.

Fungal endophytes are often horizontally transmitted in the healthy foliage of woody plants [40]. Endophytic fungi that spread horizontally come from fungal spores in the air during the growing season [41]. Horizontal gene transfer between the host plant and its endophytic fungi can occur during host-symbiont interactions or directly in a living environment such as soil over a long period of time. Direct contact can result in the transmission and absorption of genetic material [42]. Some OTUs shared among the leaf niches were also present in the rhizosphere soil, which may be the result of colonization caused by rhizosphere soil fungi in leaves (Fig. 2). Notably, in this study, the YS and ES plants were derived from cuttings of the mother plant in the same tea garden. We used this sampling strategy to avoid seasonal, genetic, and geographical variations, so that we could assess mycobiome diversity solely in relation to different leaf niches and rhizosphere soil in SCZ plants of different ages. Some shared OTUs were also found in endophytic fungal communities of different ages (Fig. 2). Parts of the endophytic fungal

communities of the 1-year-old and 20-year-old SCZ plants may have been inherited from the mother plant.

Ascomycota, Basidiomycota, and Zygomycota were the three dominant fungal phyla on tea plants in this study, which were also reported as dominant phyla on other plants [14]. In this study, Dothideomycetes, Eurotiomycetes, Sordariomycetes, Leotiomycetes were the dominant classes, consistent with the results of a previous study of vascular plants in the high arctic zone [4]. In tropical and temperate plants, the major class of endophytes was reported to be Sordariomycetes, followed by Dothideomycetes and Leotiomycetes [43, 44, 45].

Most studies on the endophytic fungi of tea plants have been conducted in China. However, there are still some shortcomings in analyses of the population diversity of endophytic fungi. Traditional isolation and identification methods cannot detect microorganisms that are difficult or impossible to culture. Here, we analyzed the species richness, composition, and distribution of SCZ endophytic fungi in different leaf niches and in trees of different ages based on high-throughput sequencing data, and compared them with those of rhizosphere soil fungal communities. In previous studies, the dominant endophytic fungi of tea trees were found to be *Colletotrichum* sp., *Pestalotiopsis* sp., *Guignardia* spp., *Phomopsis* sp., *Macrophoma* sp., *Aspergillus* sp., *Candida* sp., *Thamidium* sp., *Alterinaria* sp., and *Fusarium* spp. [36, 37, 38]. However, the dominant endophytic fungi on SCZ identified from the sequencing data in our study were *Cladosporium*, *Strelitziana*, *Zymoseptoria*, *Pseudeurotium*, *Pseudoramichloridium*, *Penicillifer*, *Trichoderma*, *Paraconiothyrium*, *Melanconiella*, and *Saccharomycopsis*. The differences in results between our study and previous studies may be related to the limitations of traditional separation and culture methods, different geographical and environmental conditions, and/or differences among tea varieties.

Our results demonstrate that the SCZ fungal community significantly differs across the soil-upper leaf-middle leaf-lower leaf landscape and among trees of different ages. These findings provide information about the composition and diversity of endophytic fungi communities in tea plants of different ages, and lay the foundation for further research on the co-evolution and adaptation of endophytic fungi and tea trees. The data in this study also provide a reference for research on endophytic microbes in other plants.

# Conclusion

In summary, all these analyses showed a certain discipline of the distribution of endophytic fungi in different leaf niches of the same plant. At the same time, the composition and distribution of endophytic fungi in plants varied with the growth time. *Cladosporium*, *Zymoseptoria* and *Strelitziana* were the dominant genera in the leaf. The abundance of *Cladosporium* and *Zymoseptoria* decreased with increasing SCZ age, whereas the abundance of *Strelitziana* increased. That is to say, the distribution of endophytic fungi in leaves of *C. Sinensis* (cv. Shu Cha Zao) has a certain spatiotemporal variation.

# Methods

Sample collection

In this study, to avoid the variation caused by geographical differences in sampling, the variety Shu Cha Zao (SCZ) of *Camellia Sinensis* located in the 916 tea plantation (Shucheng county, Lu'an, China) were chose as the main object. In this tea garden, there is one tea tree (regarded as mother plant) had lived for over 60 years and was named as 60-year-old SCZ (LS). Cuttings origined from the mother plant were cultivated from 1997 until now and were named as 20-year-old SCZ (ES). 1-year-old SCZ (YS) plants were cultivated as cuttings from the mother plant in 2016. The upper leaves, middle leaves, lower leaves, and rhizosphere soil (Table 1) were collected from YS, ES and LS in 2017. For YS, five plants were randomly selected. According to the height, the upper leaves, middle leaves and lower leaves were collected respectively. Five ES plants were also randomly selected. Each ES was divided into five equal parts from top view and leaves were collected from proximal, median and distal parts (the upper leaves, middle leaves, lower leaves) of the same branch in each region. Leaves from the same niche of one ES plant were mixed. For the only one 60-year-old plant (LS, mother plant), samples were collected using the same method as described on ES. Leaves from each region were regarded as replicants. The rhizosphere soil was sampled 15–20 cm deep and near 6 mm away from the rhizoplane of each plant with five repetitions. All samples were stored in dry ice immediately, transported to laboratory and stored at -80°Cfor further experiments.

Table 1
Names (coordinates), parts, and serial numbers of materials

| Sample name (coordinates) | Material position | Number |
|---|---|---|
| 1-year-old Shu Cha Zao/YS (31°19'7"N, 117°1'25"E) | Rhizosphere soil | YS1 |
| | Upper leaf | YS2 |
| | Middle leaf | YS3 |
| | Lower leaf | YS4 |
| 20-year-old Shu Cha Zao/ES (31°19'13"N, 117°1'21"E) | Rhizosphere soil | ES1 |
| | Upper leaf | ES2 |
| | Middle leaf | ES3 |
| | Lower leaf | ES4 |
| 60-year-old Shu Cha Zao/LS (31°19'38"N, 117°1'57"E) | Rhizosphere soil | LS1 |
| | Upper leaf | LS2 |
| | Middle leaf | LS3 |
| | Lower leaf | LS4 |

DNA extraction and PCR amplification

To isolate microorganisms, all leaf samples were washed with tap water, soaked in 75% ethanol for 5 min, rinsed three times with sterile water, surface sterilized for 5 min in 1% sodium hypochlorite, washed three times with sterile water, and then dried with sterile filter paper. The samples were ground into powder in liquid nitrogen using mortar and pestle. Powder were transferred into 50 mL tube containing 10 mL sterile water and centrifuged gradiently at 200 × *g* for 20 min, 500 × *g* for 20 min and 16,500 × *g* for 15 min at 4 ℃. Total genomic DNA was extracted from precipitates using CTAB method with some modifications [30].

Polymerase chain reaction (PCR) was performed to amplify the ITS1 regions of the fungal ITS rRNA genes using primers ITS5-1737F (5'-GGA AGT AAA AGT CGT AAC AAG G-3') and ITS2-2043R (5'-GCT GCG TTC TTC ATC GAT GC-3'). Each 30 μl PCR reaction mixture contained 15 μl Phusion Master Mix (2×), 3 μl Primer (2 μM), 10 ng DNA, and 2 μl dd$H_2O$. All samples were replicated three times. The PCR was performed using GeneAmp PCR System 9700 with the following standard procedure: initial denaturation at 98 °C for 1 min, followed by 30 cycles of 98 °C for 10 s, annealing at 50 °C for 30 s, 72 °C for 30 s, and final extension at 72 °C for 5 min. Then, the PCR products were analyzed by electrophoresis on a 2% (v/v) agarose gel (100 V, 40 min). The gel was stained with ethidium bromide and bands were photographed on an ultraviolet light transilluminator.

Library preparation and sequencing

cDNA libraries were generated using the TruSeq®DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) with index codes following the manufacturer's recommendations. The library quality was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific, Waltham, MA, USA) and Agilent Bioanalyzer 2100 system (Agilent, Palo Alto, CA, USA). At last, the library was sequenced on the Illumina HiSeq 2500 platform and 250 bp paired-end reads were generated at Novogene (Beijing, China) [31].

Statistical analysis

Sequences were analyzed using Uparse software (v7.0.1001, http://drive5.com/uparse/) [32]. Sequences with ≥ 97% similarity were assigned to the same OTU. A representative sequence for each OTU was screened for further annotation. Each representative sequence was annotated with taxonomic information from the Unite Database (https://unite.ut.ee/) [33] based on BLAST algorithm which was calculated using QIIME (Version 1.9.1) (http://qiime.org/scripts/assign_taxonomy.html). To obtain the phylogenetic relationships among different species and differences in dominant species among different samples (groups), multiple sequence alignments were conducted using the MUSCLE software (Version 3.8.31, http://www.drive5.com/muscle/) [34]. The OTUs abundance information was normalized using a standard sequence number corresponding to the sample with the fewest sequences. Subsequent analyses of alpha and beta diversity were performed on these normalized output data.

Five indices, such as Chao1, Shannon, Simpson, ACE and good-coverage, were calculated with QIIME (Version 1.9.1, http://qiime.org/index.html) [35] and displayed using R software (Version 2.15.3). Chao1 (http://www.mothur.org/wiki/Chao) and ACE (http://www.mothur.org/wiki/Ace) indices were selected to

identify community richness. Shannon (http://www.mothur.org/wiki/Shannon) and Simpson (http://www.mothur.org/wiki/Simpson) indices were used to identify community diversity. Good's coverage (http://www.mothur.org/wiki/Coverage) indice to characterize sequencing depth. Beta diversity analysis is used to evaluate differences in species diversity among samples. Beta diversity values (weighted and unweighted unifrac) were calculated using QIIME (Version 1.9.1). A non-metric multidimensional scaling (NMDS) analysis was conducted using the vegan software package in R software (Version 2.15.3). The paired t-test was used for statistical comparisons between groups. Differences were considered significant at $p \leq 0.01$.

# Abbreviations

SCZ: Shu Cha Zao; DNA: Deoxyribonucleic acid; cDNA: Complementary deoxyribonucleic acid; PCR: Polymerase chain reaction; NMDS: Non-metric multidimensional scaling; ITS: Internal transcribed spacer; OUTs: Operational taxonomic units; CTAB: Cetyl Trimethyl Ammonium Bromide; *p*: Level of significance; $R^2$: Determination coefficient.

# Declarations

Acknowledgements

Not applicable.

Author contributions

All authors contributed to the study conception and design. ZZW analyzed the data of this project; HZH, JLW and Y Zhao helped the sample collection, extraction of DNA and prepared the PCR; Y Zhang gave instructions for analysis of data; YQY helped the samples collection and designed the project with YHL; ZZW, QQS, YCC, YHL, YQY and HA drafted the manuscript.

Funding

The financial support from the National Natural Science Foundation of China (grant no. 31300426 and 31870635) and the Open fund of State Key Laboratory of Tea Plant Biology and Utilization (SKLTOF201801109).

Availability of data and materials

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

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] School of Life Sciences, Anhui Agricultural University, Hefei, 230036, P.R.China.

[2] State Key Laboratory of Tea Plant Biology and Utilization, Anhui Agricultural University, Hefei, 230036 P.R.China.

# References

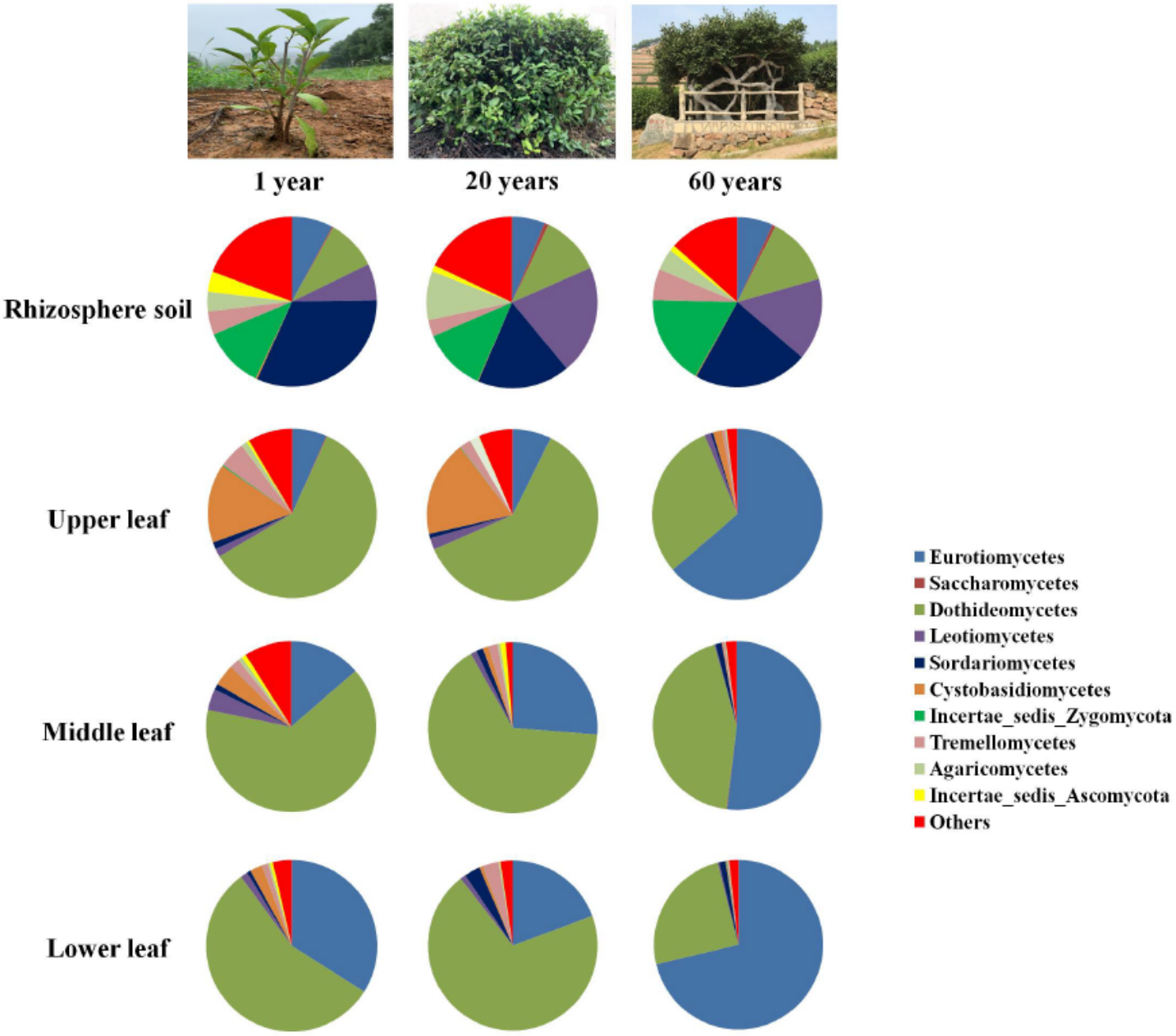
1. Petrini O. Fungal endophytes of tree leaves. In: Andrews JH, Hirano SS, editors. Microbial Ecology of Leaves. Brock/Springer Series in Contemporary Bioscience. Springer, New York; 1991. p. 179-197. https://doi.org/ 10.1007/978-1-4612-3168-4_9
2. Strobel G, Stierle A, Stierle D, Hess WM. *Taxomyces andreanae*, a proposed new taxon for a bulbilliferous hyphomycete associated with Pacific yew (*Taxus brevifolia*). Mycotaxon. 1993;47(71):71-80.
3. Davey ML, Currah RS. Interactions between mosses (Bryophyta) and fungi. Can J Bot. 2006;84(10):1509-1519. https://doi.org/ 10.1139/b06-120
4. Zhang T, Yao YF. Endophytic fungal communities associated with vascular plants in the high arctic zone are highly diverse and host-plant specific. PLoS One. 2015;10(6):e0130051. https://doi.org/10.1371/journal.pone.0130051
5. Su YY, Guo LD, Hyde KD. Response of endophytic fungi of Stipa grandis to experimental plant function group removal in Inner Mongolia steppe, China. Fungal divers. 2010;43(1):93–101. https://doi.org/1007/s13225-010-0040-6
6. Olsrud M, Michelsen A, Wallander H. Ergosterol content in ericaceous hair roots correlates with dark septate endophytes but not with ericold mycorrhizal colonization. Soil Biol and Biochem. 2007;39(5):1218-1221. https://doi.org/ 10.1016/ j.soilbio.2006.11.018
7. Jae-Joon Y, Ahn-Heum E. Molecular identification of endophytic fungi isolated from needle leaves of conifers in bohyeon mountain, Korea. Mycobiology. 2012;40(4):231-235. https://doi.org/10.5941/MYCO.2012.40.4.231
8. Albrectsen BR, Björkén L, Varad A, Hagner A, Wedin M, Karlsson J, et al. Endophytic fungi in European aspen (*Populus tremula*) leaves-diversity, detection, and a suggested correlation with herbivory resistance. Fungal Divers. 2010;41(1):17-28. http://doi: 10.1007/s13225-009-0011-y

9. Aly AH, Debbab A, Kjer J, Proksch P. Fungal endophytes from higher plants: a prolific source of phytochemicals and other bioactive natural products. Fungal Divers. 2010;41(1):1-16. http://doi: 10.1007/s13225-010-0034-4
10. Bills GF, González-Menéndez V, Martín JJ, Platas G, Fournier J, Peršoh D, et al. *Hypoxylon pulicicidum* nov. (Ascomycota, Xylariales), a Pantropical Insecticide-Producing Endophyte. PLoS One. 2012;7(10):e46687. https://doi.org/10.1371/journal.pone.0046687
11. Waqas M, Khan AL, Hamayun M, Shahzad R, Kang SM, Kim JG, et al. Endophytic fungi promote plant growth and mitigate the adverse effects of stem rot: an example of *Penicillium citrinum* and *Aspergillus terreus*. J Plant Interact. 2015;10(1):280-287. https://doi.org/10.1080/17429145.2015.1079743
12. Cregger MA, Veach AM, Yang ZK, Crouch MJ, Vilgalys R, Schadt CW, et al. The *Populus* holobiont: dissecting the effects of plant niches and genotype on the microbiome. 2018;6(1):31. https://doi.org/ 10.1186/s40168-018-0413-8.
13. de Souza RSC, Okura VK, Armanhi JSL, Jorrín B, Lozano N, da Silva MJ, et al. Unlocking the bacterial and fungal communities assemblages of sugarcane microbiome. Sci Rep. 2016;6:28774. https://doi.org/ 10.1038/srep28774
14. Shi YW, Li C, Yang HM, Zhang T, Gao Y, Zeng J, et al. Endophytic fungal diversity and space-time dynamics in sugar beet. Eur J Soil Biol. 2016;77:77-85. https://doi.org/10.1016/j.ejsobi.2016.09.005
15. Gupta RK, Sharma, C. Diversity of arbuscular mycorrhizal fungi in *Camellia sinensis* in Uttarakhand State, India. Afr J Biotechnol. 2013;9(33):5313-5319. https://doi.org/10.1186/1471-2180-10-219
16. Mondal TK, Bhattacharya A, Laxikumaran M, Ahuja PS. Recent advance of tea (*Camellia sinensis*) Biotechnology. Plant Cell Tiss Org. 2004;76(3):195-254. https://doi.org/10.1023/b:ticu.0000009254.87882.71
17. Chen HQ, Xu YP, Xie LH, Wang GH, Yang MH. Isolation of endophytic fungi in tea plant (*Camellia sinensis*), and their distribution patterns in different tissues. Journal of Laiyang Agricultural College. 2006;23(4):250-254. (in chinese)
18. Lu D S, Wang JP, Wu XQ, Ye JR. The species and distribution of endophytic fungi in tea trees. Journal of Henan Agricultural Sciences. 2007;10:54-56.(in Chinese)
19. You JM. Isolation of endophytic fungi from tea plant. Hubei Agricultural Sciences. 2009;48(5):1168-1170. (in Chinese)
20. Fang WP, Yang LC, Zhu X J, Zeng L, Li XH. Seasonal and Habitat Dependent Variations in Culturable Endophytes of *Camellia sinensis*. J Plant Pathol Microb. 2013;4:3. https://doi.org/10.4172/2157-7471.1000169
21. Osono T. Endophytic and epiphytic phyllosphere fungi of *Camellia japonica*: seasonal and leaf age-dependent variations. Mycologia. 2008;100(3):387-391. https:// doi.org/10.3852/07-110R1
22. Cheung MK, Au CH, Chu KH, Kwan HS, Wong CK. Composition and genetic diversity of picoeukaryotes in subtropical coastal waters as revealed by 454 pyrosequencing. ISME J. 2010;4(8):1053-1059. https://doi.org/ 10.1038/ismej.2010.26

23. Oros-Sichler M, Smalla K. Semi-Nested PCR approach to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. In: Gupta V, Tuohy M, Ayyachamy M, Turner K, O'Donovan A, Laboratory Protocols in Fungal Biology, Fungal Biology. Springer, New York; 2013. p. 289-298. https://doi.org/10.1007/978-1-4614-2356-0_23
24. Stoeck T, Behnke A, Christen R, Amaral-Zettler L, Rodriguez-Mora MJ, Chistoserdov A, et al. Massively parallel tag sequencing reveals the complexity of anaerobic marine protistan communities. BMC Biol. 2009;7(1):72. https://doi.org/1186/1741-7007-7-72
25. Bachy C, Dolan JR, López-García P, Deschamps P, Moreira D. Accuracy of protist diversity assessments: morphology compared with cloning and direct pyrosequencing of 18S rRNA genes and ITS regions using the conspicuous tintinnid ciliates as a case study. ISME J. 2013;7(2):244-255. http://doi: 10.1038/ismej. 2012.106
26. Bengtsson-Palme J, Ryberg M, Hartmann M, Branco S, Wang Z, Godhe A, et al. Improved software detection and extraction of ITS1 and ITS2 from ribosomal ITS sequences of fungi and other eukaryotes for analysis of environmental sequencing data. Methods Ecol. Evol. 2013;4(10):914-919. https://doi.org/10.1111/2041-210X.12073
27. Findley K, Oh J, Yang J, Conlan S, Deming C, Meyer JA, et al. Topographic diversity of fungal and bacterial communities in human skin. Nature. 2013;498(7454):367-370. https://doi.org/ 10.1038/nature12171
28. Degnan PH, Ochman H. Illumina-based analysis of microbial community diversity. ISME J. 2012;6(1):183-194. https://doi.org/10.1038/ismej.2011.74
29. Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, et al. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012;6(8):1621-1624. https://doi.org/ 10.1038/ismej.2012.8
30. Attitalla IH. Modified CTAB method for high quality genomic DNA extraction from medicinal plants. Pak J Biol Sci. 2011;14(21):998-999. http://doi:10.3923/pjbs. 2011.998.999
31. Li JJ, Jiang X, Jing Z, Li G, Chen ZZ, Zhou LB, et al. Spatial and seasonal distributions of bacterioplankton in the pearl river estuary: the combined effects of riverine inputs, temperature, and phytoplankton. Mar Pollut Bull. 2017;125:199-207. https://doi.org/10.1016/j.marpolbul.2017.08.026
32. Edgar RC. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 2013;10(10):996–998.https://doi.org/10.1038/2604
33. Kõljalg U, Nilsson RH, Abarenkov K, Tedersoo L, Taylor AF, Bahram M, et al. Towards a unified paradigm for sequence–based identification of fungi. Mol Ecol. 2013;22(21):5271-5277. https://doi.org/ 10.1111/mec.12481
34. Edgar RC. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004;32(5):1792-1797. https://doi.org/10.1093/ nar/gkh340
35. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. QIIME allows analysis of high-throughput community sequencing data. Nat methods. 2010;7(5):335-336. https://doi.org/ 10.1038/nmeth.f.303

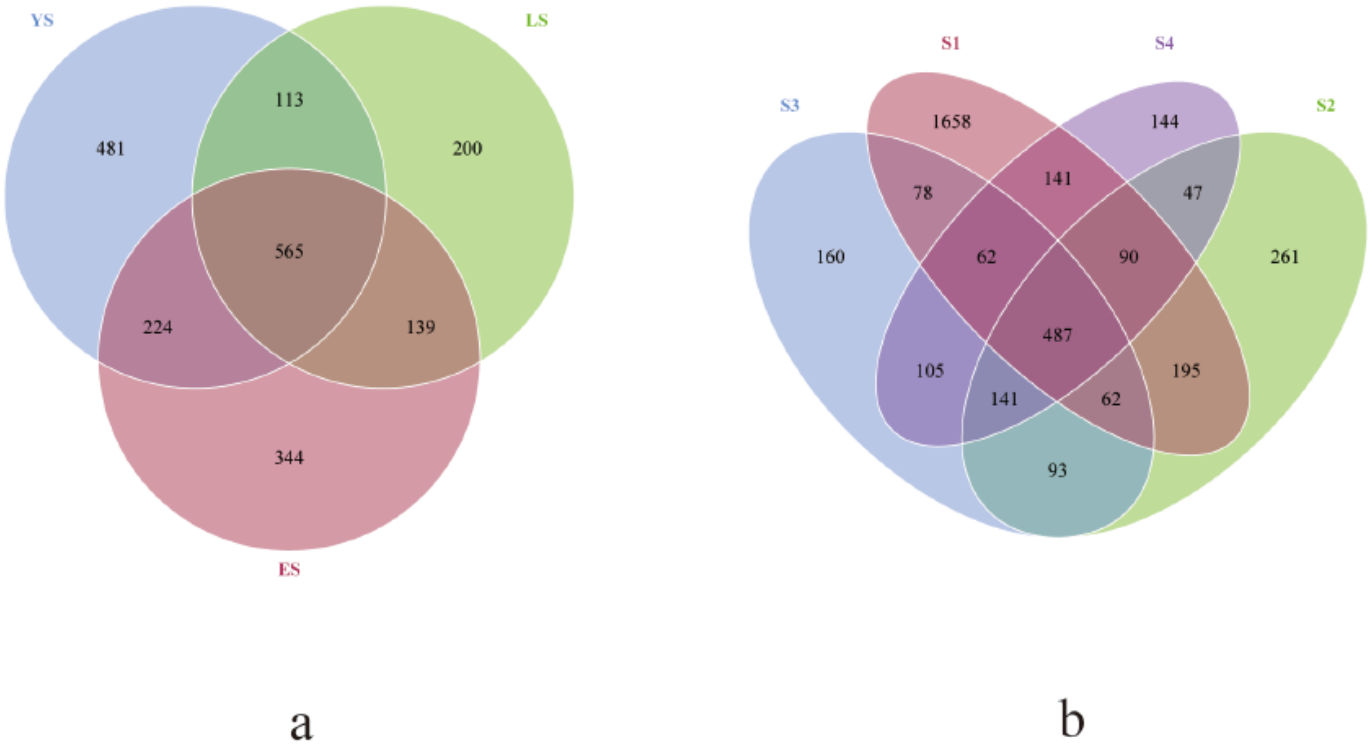
36. Jia M, Chen L, Xin HL, Zheng CJ, Rahman K, Han T, et al. A friendly relationship between endophytic fungi and medicinal plants: a systematic review. Front Microbiol. 2016;7:906. https://doi.org/10.3389/fmicb.2016.00906
37. Bonito G, Reynolds H, Robeson II MS, Nelson J, Hodkinson BP, Tuskan G, et al. Plant host and soil origin influence fungal and bacterial assemblages in the roots of woody plants. Mol Ecol. 2014;23(13):3356-3370. https://doi.org/10.1111/mec.12821
38. Berbee ML, Taylor JW. Two ascomycete classes based on fruiting-body characters and ribosomal DNA sequence. Mol Biol Evol. 1992;9(2):278-284. https://doi.org/10.1093/oxfordjournals.molbev.a040719
39. Celio GJ, Padamsee M, Dentinger BT, Bauer R, McLaughlin DJ. Assembling the Fungal Tree of Life: constructing the structural and biochemical database. Mycologia. 2006;98(6):850-859. https://doi.org/10.1080/2006.11832615
40. Gazis R, Miadlikowska J, Lutzoni F, Arnold AE, Chaverri P. Culture-based study of endophytes associated with rubber trees in Peru reveals a new class of Pezizomycotina: Xylonomycetes. Mol Phylogenet Evol. 2012;65(1):294-304. https://doi.org/10.1016/j.ympev.2012.06.019
41. Carroll G. Forest endophytes: pattern and process. Can J Bot. 1995;73(S1):1316-1324.
42. Nisbet LJ. The importance of microbial biodiversity to biotechnology// Biodiversity of Microorganisms & Invertebrates: Its Role in Sustainable Agriculture First Workshop on the Ecological Foundations of Sustainable Agriculture. 1991.
43. U'Ren JM, Lutzoni F, Miadlikowska J, Laetsch AD, Arnold AE. Host and geographic structure of endophytic and endolichenic fungi at a continental scale. Am j bot. 2012;99(5):898-914. https://doi.org/10.3732/ajb.1100459
44. Arnold AE, Lutzoni F. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? Ecology. 2007;88(3):541-549. http://doi: 10.2307/27651135
45. Arnold AE. Endophytic fungi: hidden components of tropical community ecology. In: Schnitzer S, Carson W, editors. Tropical Forest Community Ecology, Wiley-Blackwell; 2008. p. 254-271.

# Figures



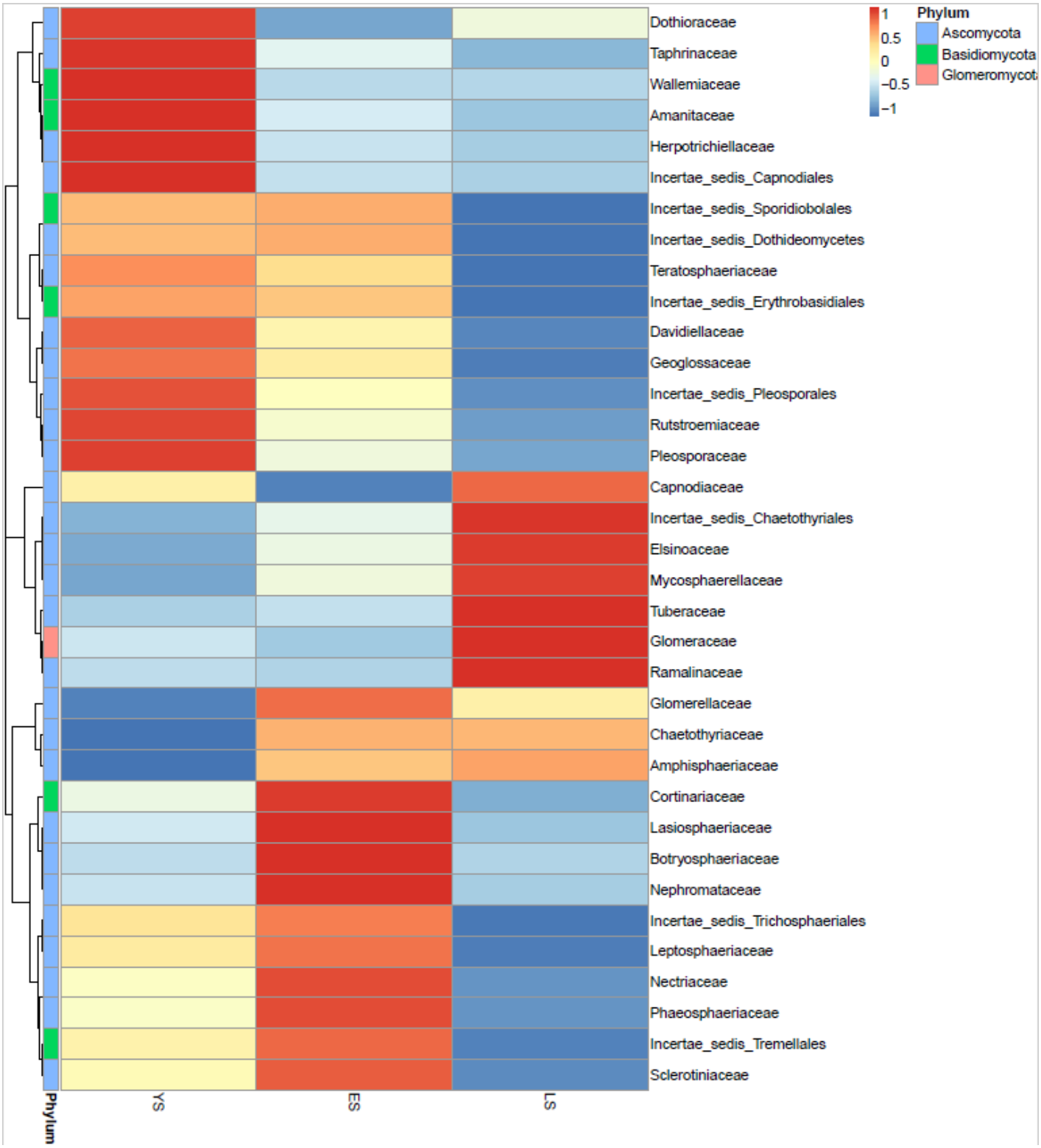

## Figure 1

Relative abundance of dominant (>0.1%) fungal classes in leaf niches (upper leaf, middle leaf, lower leaf) and rhizosphere soil from plants of different ages.




## Figure 2

Venn diagrams showing number of shared OTUs among sample groups. a. Number of shared OTUs among tea plants of different ages. b. Number of shared OTUs among different niches (S1, rhizosphere soil; S2, upper leaf; S3, middle leaf; S4, lower leaf).




## Figure 3

Heat maps of leaf endophytic fungal families in tea trees of different ages, Square colors shifted from dark blue towards dark orange indicate higher abundance. (YS, 1-year-old tea plant; ES, 20-year-old tea plant; LS, 60-year-old mother plant).

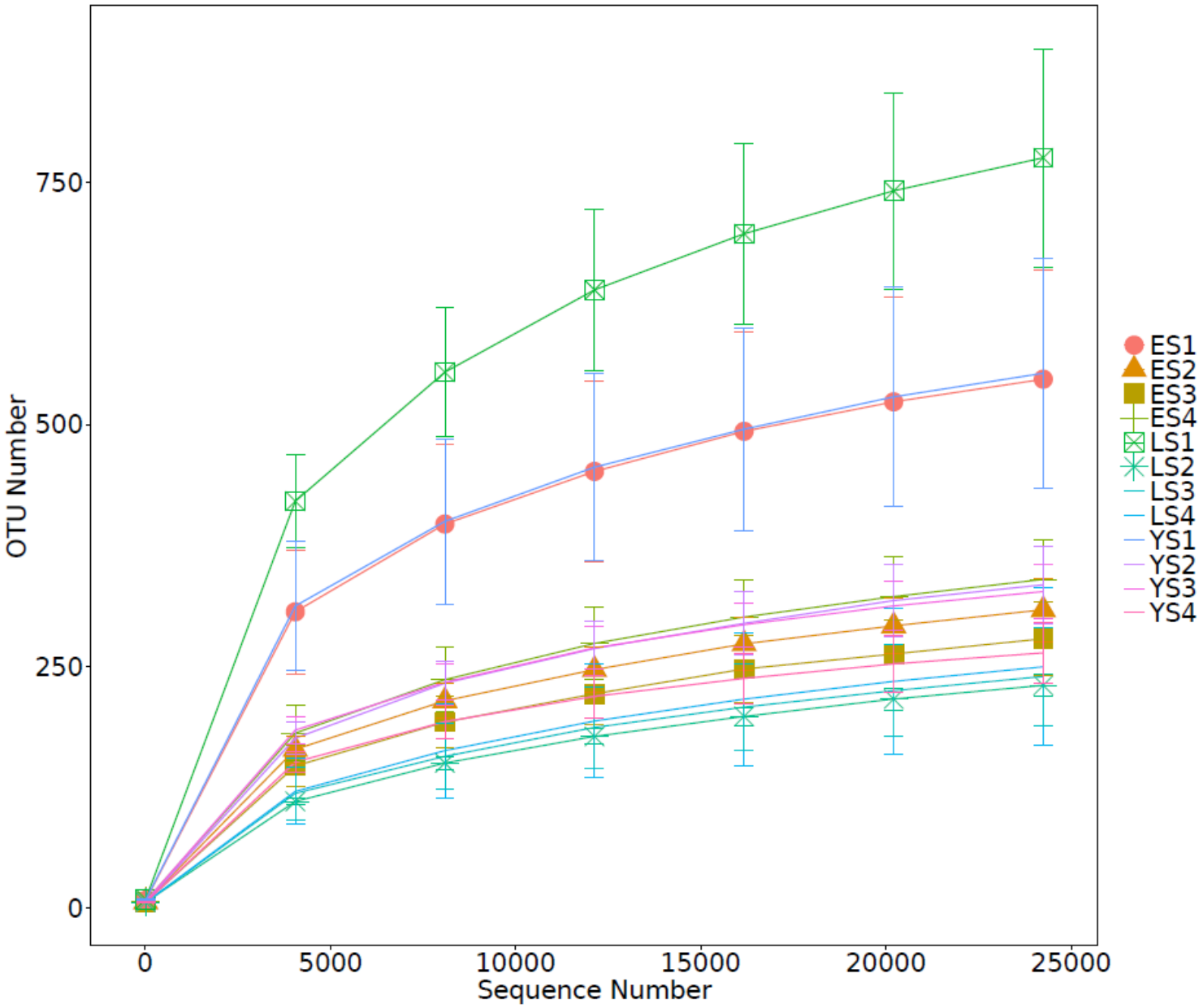



**Figure 4**

Rarefaction curves of OTUs in different samples (YS, 1-year-old tea plant; ES, 20-year-old tea plant; LS, 60-year-old tea plant; 1, rhizosphere soil; 2, upper leaf; 3, middle leaf; 4, lower leaf).

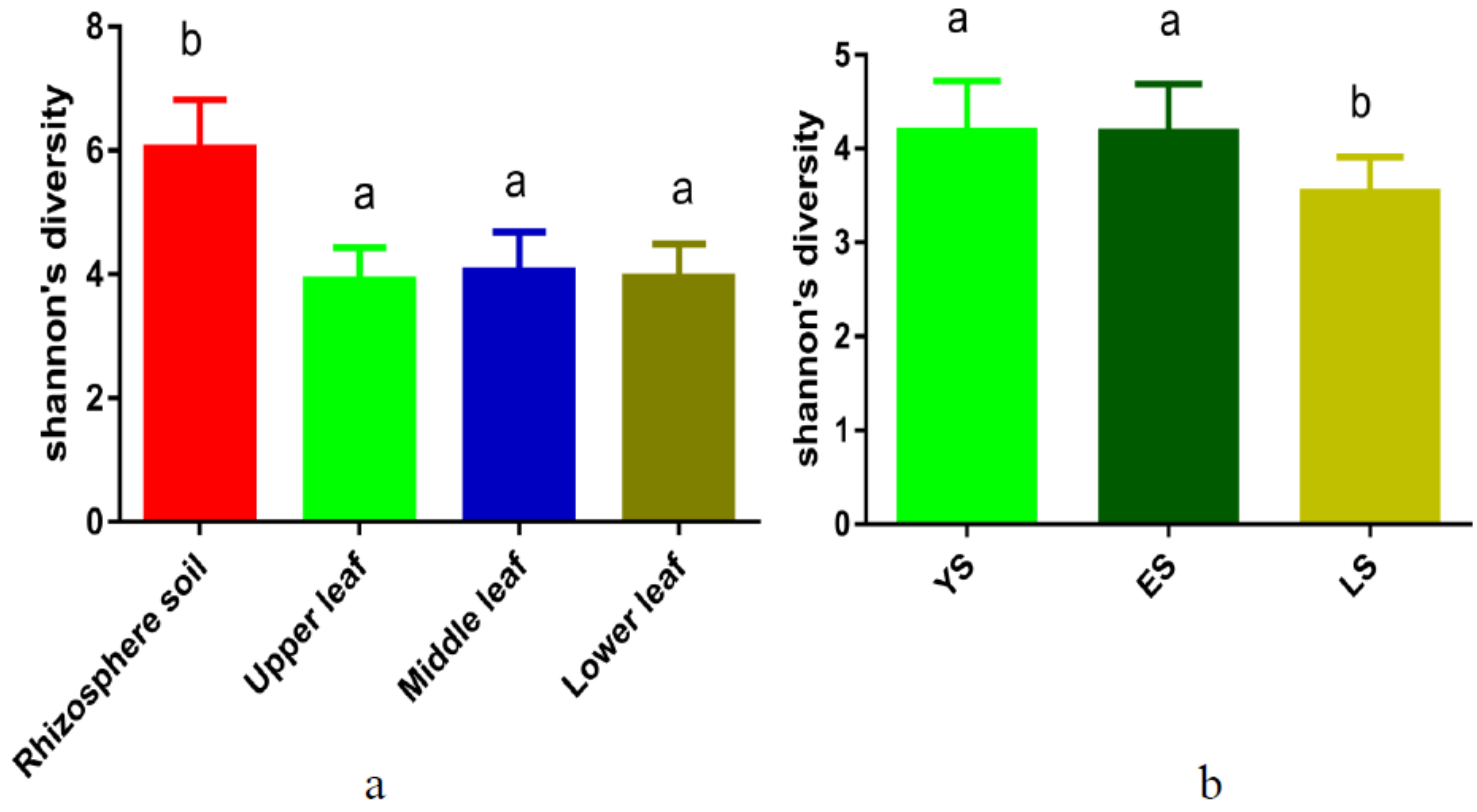
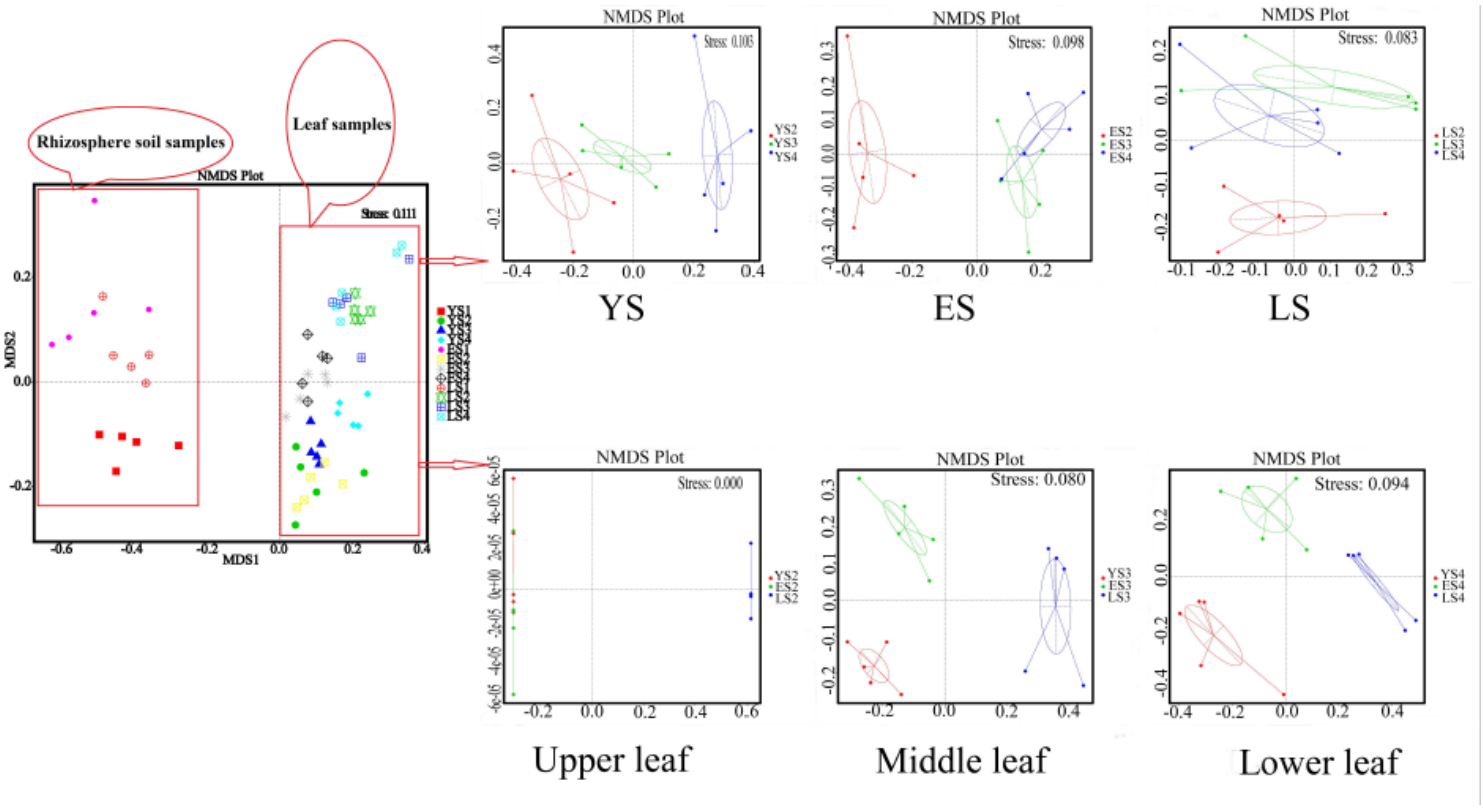



**Figure 5**

a. Shannon's diversity values for fungal communities across samples in categories of niche (rhizosphere soil, upper leaf, middle leaf, lower leaf); b. Shannon's diversity values for fungal communities across samples in different plant ages. Different letters above bars within plots represent significant differences (pairwise t tests, $p \leq 0.01$). Bars represent means ±SEM (n=5). YS, 1-year-old tea plant; ES, 20-year-old tea plant; LS, 60-year-old mother plant.




## Figure 6

NMDS ordination of soil (1: rhizosphere soil) and leaf niches (2: upper leaf, 3: middle leaf, 4: lower leaf) in plants of different ages (YS, 1-year-old tea plant; ES, 20-year-old tea plant; LS, 60-year-old mother plant).

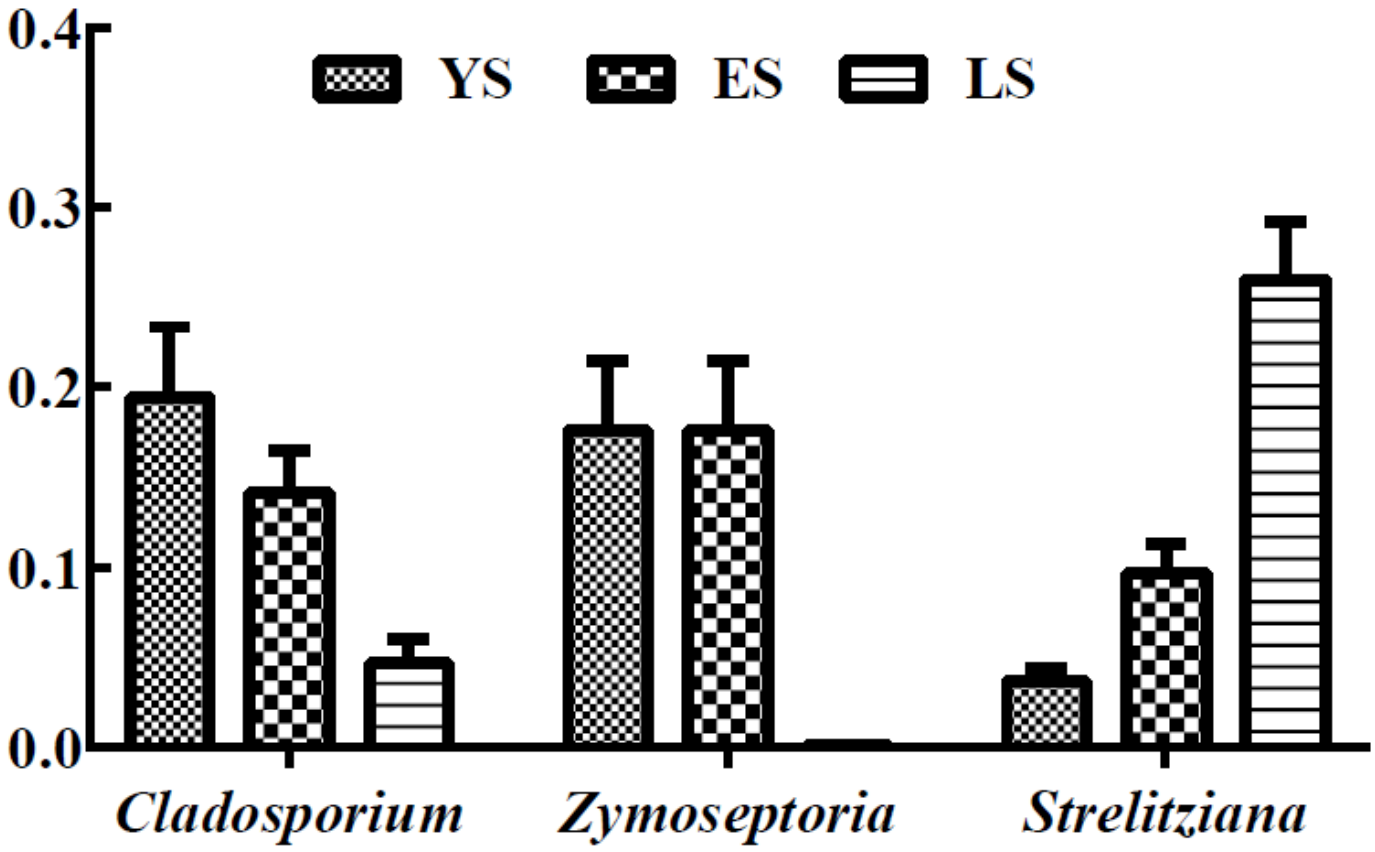



**Figure 7**

Competition map among Cladosporium, Zymoseptoria, and Strelitziana. (YS, 1-year-old tea plant; ES, 20-year-old tea plant; LS, 60-year-old mother plant).

## Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- supplementarymaterial.pdf