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ORIGINAL PAPER

Variation in soil fungal community structure during successive rotations of *Casuarina equisetifolia* plantations as determined by high-throughput sequencing analysis

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Abstract

Regeneration failure and productivity decline, which is collectively known as consecutive monoculture problem (CMP), were observed during long-term monoculture *Casuarina equisetifolia* plantations. In this study, the high-throughput sequencing method was applied to determine whether the rhizospheric microbial community composition would be significantly degenerated by consecutive monoculture in *C. equisetifolia* plantations. The results showed that the soil fungal community structure exhibited obvious differences among the first rotation plantation (FCP), the second rotation plantation (SCP), and the third rotation plantation (TCP). Both the Shannon and Simpson diversity indices of the soil fungal community in the FCP were significantly higher than in the SCP ($P < 0.05$). Additionally, the relative abundance of *Fusarium, Thelephora, Hortaea* and *Penicillium* were significantly higher in the SCP and TCP soils than in the FCP soils, suggesting that certain fungi gradually became predominant in the continuous monoculture plantation soils. Conversely, the relative abundance of *Tolypocladium and Trichoderma* were significantly lower in the SCP and TCP soils than in the FCP soils, suggesting that some microbes gradually decreased in the continuous monoculture plantation soils. Overall, the results demonstrated that the long-term pure plantation pattern exacerbated the microecological imbalance in rhizospheric soils of *C. equisetifolia* and markedly decreased soil microbial community diversity.

Keywords *Casuarina equisetifolia* · High-throughput sequencing · Successive rotation · Microbial community composition

Abbreviations

CMP	Consecutive monoculture problem
FCP	First rotation plantation
SCP	Second rotation plantation
TCP	Third rotation plantation
TN	Total nitrogen
AN	Alkaline nitrogen
TP	Total phosphorus
AP	Available phosphorus
TK	Total potassium
OTU	Operational taxonomic unit
AK	Available potassium
ACE	Abundance-based coverage estimator
PCoA	Principal coordinate analysis
UPGMA	Unweighted pair-group method with arithmetic means
OTUs	Operational taxonomic units

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Introduction

Casuarina equisetifolia is a she-oak species of the genus *Casuarina*, which is native to the east coast of Australia. *C. equisetifolia* has been widely planted all around the world because of its characteristics of high yield and fast growth (Zhong et al. 2013). This species has adapted to harsh environmental conditions, including high salinity, poor soils, and heavy metal pollution (Scotti-Campos et al. 2016; Vijayabhama et al. 2018). More importantly, *C. equisetifolia* plays significant economic and ecological roles by offering shelter belts for numerous animal species, stabilizing coastal sand dunes, and protecting against storms (Karthikeyan et al. 2013). Indeed, *C. equisetifolia* has been introduced to China's southeastern coastal areas as windbreaks to improve the environment for more than half a century (Liu et al. 2015; Li et al. 2018).

However, studies during the past few decades have shown regeneration failure and productivity decline in long-term monoculture *C. equisetifolia* plantations in a phenomenon referred to as CMP (Wardle et al. 2004). CMP results in slow growth, low output, and aggravation of disease and insect pests of *C. equisetifolia* (Long et al. 2018). This phenomenon has long been a complex problem in forest management; therefore, its formation mechanism and control have been the focus of many studies. Soil nutrients deficiency has been considered one of the reasons for regeneration failure of *C. equisetifolia*, but CMP cannot be solved by application of chemical fertilizer (Karthikeyan 2016). In addition, previous studies have suggested that some root exudates may be responsible for the *C. equisetifolia* replanting problem, such as 12, 13-dihydromicromeric acid, betulinic acid, and catechins (Veluthakkal and Dasgupta 2012; Kelderer et al. 2012). Although much work has been conducted to investigate methods of controlling CMP in *C. equisetifolia* plantations, such as the use of biological organic fertilizer, plant growth hormone, and mycorrhizal inoculation, there has been little success (Hata et al. 2015; Zhang et al. 2016).

Investigations of CMP have gradually come to focus on rhizospheric biological processes. Rhizospheric microorganisms are crucial to forest ecosystems because of their critical role in regulating ecosystem balance via fundamental ecological processes, such as mineralization and decomposition (Bennett et al. 2012). In recent years, studies have suggested that the imbalance of rhizospheric microbial community structure was one of the main reasons for CMP (Shi et al. 2011; Li et al. 2014a, b). Moreover, some studies have shown that root exudates could stimulate harmful fungal pathogens or inhibit beneficial microorganisms within long-term monoculture rotations (Wu et al. 2016a, b). However, few studies have investigated variations in the rhizospheric microbial community in successive rotations of *C. equisetifolia*. In the present study, we investigated whether the soil microbial community composition and diversity would significantly degenerate in response to consecutive monoculture in *C. equisetifolia* plantations. The specific goal of this study was to illuminate shifts in the rhizospheric microbial composition and diversity in successive rotations of *C. equisetifolia* plantations by using high-throughput sequencing technology.

Materials and methods

Field description and soil sampling

The sampling area was located in Hui'an National Forest Farm, on the western ChongWu Peninsula (24°54′N, 118°55′E), which is a barrier island that protects against typhoons in Fujian Province, China. The arid climate consists of scarce precipitation, with a mean of 1000–1500 mm annually, and an annual mean evaporation of 2000 mm. The sandy soils of the sampling site are infertile and vulnerable to erosion.

Rhizospheric soil samples were randomly collected from three sampling positions (20 m × 20 m) on November 10, 2017. Among the three sampling sites, soil samples were collected from 0 to 20 cm depths by a core sampler (diameter of 3.5 cm), with 20 random repetitions samples from each positions at a least distance of 4 m. The 20 soil cores from each sampling positions were mixed into one composite soil sample for analysis. The composite samples were subsequently sieved using 2-mm mesh, after which the DNA was immediately extracted. The remaining sieved soil samples which from each sampling location were divided into two portions, one part stored at − 80 °C for further analysis, and another part were air-dried at room temperature for soil physicochemical properties.

Measurement of soil nutrient

Soil pH was determined by the glass electrode pH meter (1:2.5 soil–water suspensions) (Thomas et al. 1996). The total and available counts of nitrogen, phosphorus and potassium were worked out referring to the methods by Zhao et al. (2017) and Kaur and Garg (2017).

DNA extraction and high-throughput sequencing

The total DNA from individual rhizospheric soil samples was extracted in three replicates using a SoilGen DNA kit (CWBIO, Beijing, China) according to the manufacturer's instructions. The DNA quality was tested on 1% agarose

gels, and the DNA concentration was determined using a Nanodrop 2000C Spectrophotometer (Thermo Scientific, Waltham, MA, USA). The DNA was then diluted with sterile water to 1 ng/μL, after which the internal transcribed spacer (ITS1) was expanded with the designated primers ITS5F and ITS2R (Coyer et al. 2013). The PCR amplification experiments were accomplished in the 30 μL volume involving 15 μL of 2 × Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Beverly, MA, USA), 3 μL of each primer (6 μM), 10 ng DNA and sterile water. The PCR amplification conditions were shown in the supplementary information.

The PCR products of individual rhizospheric soil samples were co-mixed in equal proportions and then purified with a Qiagen Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). Sequencing libraries were developed from a TruSeq DNA PCR-Free Sample Preparation Kit (Illumina, San Diego, CA, USA) after which high-throughput sequencing was carried out on the Illumina HiSeq 2500 platform.

Operational taxonomic units (OTUs) based sequence analysis

After sequencing, the sequences reads were allocated to samples based on their unique barcodes. Paired-end reads were incorporated applying FLASH (V1.2.7) (Tanja and Salzberg 2011). After chimera removal and quality filtering, the remaining sequences named as effective tags ultimately acquired for performing OTU clustering at 97% sequence similarity through Uparse software (Uparse v7.0.1001) (Sun et al. 2014; Edgar 2013). Species annotations were implemented through the Unite Database according to the BLAST algorithm, which was performed with the QIIME software (Version 1.7.0) (Koljalg et al. 2013).

Statistical analyses

Based on the abundances of normalized OTUs, alpha and beta diversity analyses were conducted according to the standardized data. Alpha analyses consisted of determination of the diversity of individual species based on the observed-species, chao1 (http://www.mothur.org/wiki/Chao), abundance-based coverage estimator (ACE) (http://www.mothur.org/wiki/Ace), and Shannon (http://www.mothur.org/wiki/Shannon) and Simpson (http://www.mothur.org/wiki/Simpson) diversity indices. Alpha diversity indices were was determined by QIIME (version 1.7.0) (Caporaso et al. 2010) and displayed with R software (version 2.15.3). Beta diversity analysis consisted of an unweighted UniFrac distance grounded on the abundance of lineages to estimate the diversity between different samples, including principal coordinate analysis (PCoA) and unweighted pair-group method with arithmetic means (UPGMA) clustering. PCoA analysis was displayed by WGCNA package, stat packages and ggplot2 package in R software (version 2.15.3). UPGMA clustering was conducted by QIIME software (version 1.7.0). Statistical analysis was processed by DPS software (version 7.51), and analysis of variance (ANOVA) was used to conclude significance of difference by Tukey's test ($P < 0.05$).

Results

Soil physicochemical properties

The experimental results showed that the SCP and TCP had a higher pH under the successive rotations of *C. equisetifolia*. The total nitrogen (TN) in rhizospheric soils of FCP was significantly higher than in SCP and TCP ($P < 0.05$). In the FCP, alkaline nitrogen (AN) was significantly higher than in the TCP ($P < 0.05$), while it was higher in the SCP (not significant, $P > 0.05$). Total phosphorus (TP) was significantly higher in the FCP and SCP than in the TCP. The available phosphorus (AP), total potassium (TK), and available potassium (AK) did not differ significantly among treatments ($P > 0.05$) (Table S1).

OTUs cluster and species annotation

The impact of successive rotations of *C. equisetifolia* was assessed by using ITS1 deep pyrosequencing to investigate the soil fungal community is concerned. Following filtration analysis, a total of 359,640 effective sequences were found from nine rhizospheric soil samples with a mean of 39,960 effective tags. The sequences from the nine soil samples were clustered into 5399 OTUs at a 97% similarity cut-off level. The average number of OTUs in the FCP, SCP, and TCP was 654, 600, and 545, respectively (Fig. S1). On average, more than 90% of the effective tags could be classified at the class level, but only 15.6% at the genus level (Fig. S2). Rarefaction curve analyses indicated that the observed species number trended to be stable in 30,000 sequences (Fig. S3).

Alpha diversity indices

To explain the species diversity, alpha diversity indices were evaluated. The richness and diversity indices of the fungal communities in individual rhizospheric soil samples were obtained with a cutoff of 35,692 sequences. The observed species, Chao1 and ACE were significantly higher in the FCP than in the TCP ($P < 0.05$), and higher than in the SCP (not significant, $P > 0.05$). In the FCP, the fungal community showed significantly higher Shannon and Simpson diversity indices than in the SCP ($P < 0.05$). There was no significant

Table 1 Variance analysis of alpha diversity indices in individual rhizospheric soil samples

Categories	Observed species	Shannon	Simpson	Chao1	ACE
FCP	616 ± 46.972a	5.767 ± 0.319a	0.929 ± 0.013a	673.028 ± 45.218a	664.262 ± 50.070a
SCP	537 ± 55.194ab	3.980 ± 0.763b	0.693 ± 0.109b	592.876 ± 57.207ab	601.043 ± 49.994ab
TCP	494 ± 20.207b	5.819 ± 0.044a	0.960 ± 0.001a	520.143 ± 17.091b	532.046 ± 19.089b

The different letters in each column represent significant differences ($P \leq 0.05$, $n = 3$)

difference in the Shannon and Simpson diversity indices between the FCP and TCP (Table 1).

Beta diversity indices

The results of the unweighted Unifrac distances can reveal differences in community composition and structure of total fungal groups between different plantation treatments. The unweighted Unifrac distance between the FCP and SCP was 0.393, while it was 0.513 between the FCP and TCP. When compared with the FCP, the unweighted Unifrac distance increased with the aggravation of successive rotations.

PCoA and UPGMA clustering

According to the unweighted UniFrac distance, both PCoA analysis and the UPGMA clustering were conducted to compare fungal community structures across all rhizospheric soil samples. The goal of PCoA and UPGMA was to identify distinct differences among generations and similarities within the same generation of rhizospheric soil samples. The first two principal components of identified by PCoA accounted 42.95% (PC1) and 26.67% (PC2) of the overall rhizospheric soil fungal community alterations, respectively (Fig. 1a). Additionally, PCoA revealed the fungal community in FCP and SCP were divided from that in TCP by PC1, and the community in FCP was divided from that in SCP by PC2 (Fig. 1a). Furthermore, the results of hierarchical clustering showed that fungal community structure from FCP and SCP were gathered together, and then clustered together as a single group with TCP (Fig. 1b).

Venn diagram analysis

The exclusive and shared OTUs among different sets were observed based on Venn diagram analysis. The results revealed that the percentage of OTUs exclusively constructed in the FCP was 17.9% at the species level (201 species), while the percentage of OTUs exclusively constructed in the SCP was 10.2% (114 species). The proportion of species-level OTUs exclusively constructed in the TCP was 9.7% (109 species). Furthermore, the number of OTUs shared in FCP and SCP was 158 (14.1%), revealing a similar community structure in the FCP and SCP. The number of OTUs shared in the FCP, TCP, and SCP was 35.0% (393 species) (Fig. 2a).

Alterations in rhizospheric soil fungal community composition and structure under successive rotations

According to the Ribosomal Database Project classifier through the Unite Database, all of the OTUs in individual rhizospheric soil samples were classified into different clusters. The results showed that the predominant fungal phyla in three different treatments, listed from most to least common,

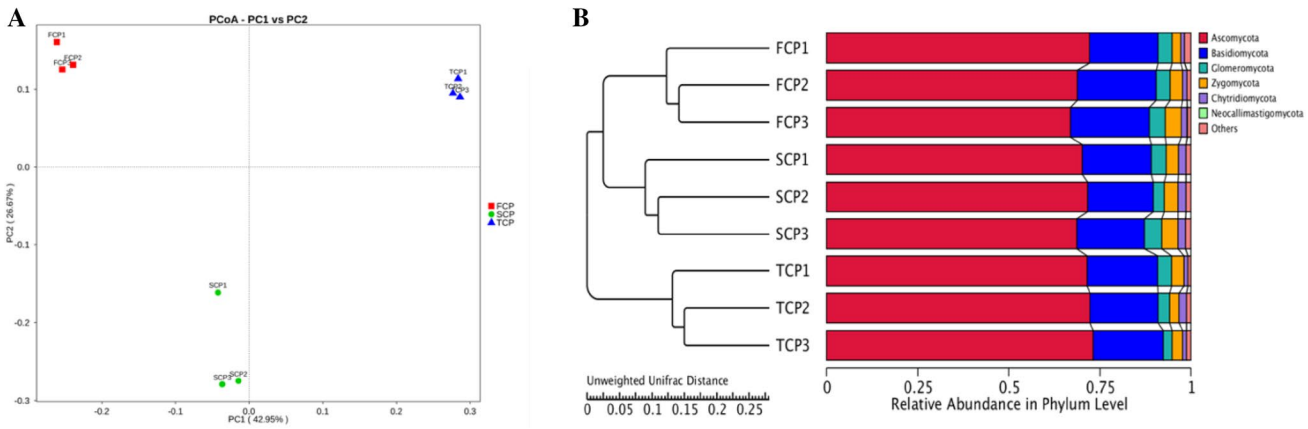

Fig. 1 PCoA plot of fungal communities according to unweighted Unifrac arithmetic (**a**) and hierarchical clustering of fungal communities according to unweighted Unifrac arithmetic from three different rhizospheric soil samples (**b**)

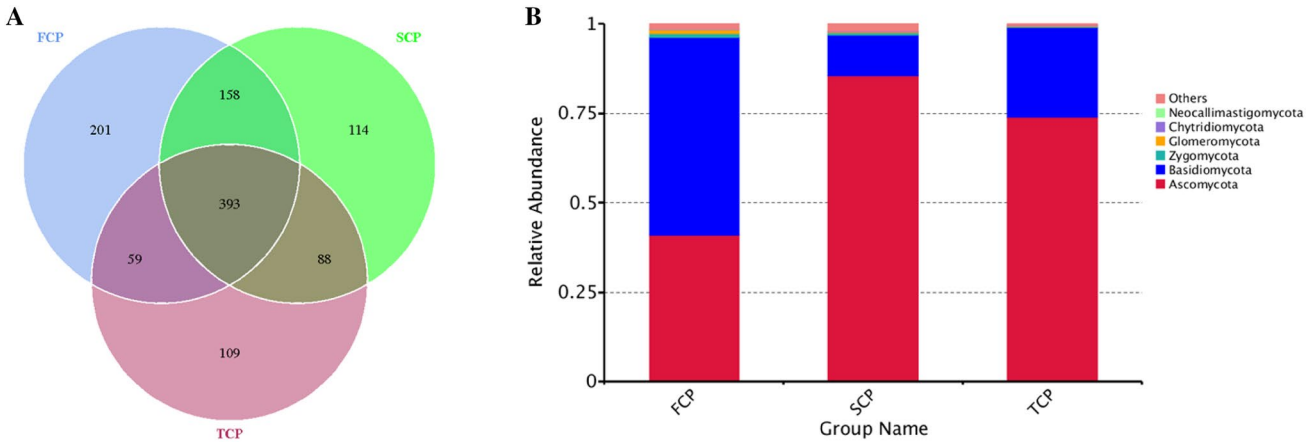

Fig. 2 Venn diagram of exclusive and shared OTUs among the FCP, SCP, and TCP at species-level (**a**) and **t**he relative abundance of predominant fungi in three different rhizospheric soil samples at the phylum level (**b**)

were *Ascomycota, Basidiomycota, Zygomycota, Glomeromycota, Chytridiomycota*, and *Neocallimastigomycota*. Of these phyla, *Ascomycota* was the dominant phylum, accounting for 40.9%, 85.6%, and 73.8% of the populations in the FCP, SCP, and TCP, respectively (Fig. 2b).

At the genus level, the relative abundance of *Fusarium, Thelephora, Hortaea*, and *Penicillium* significantly increased, while the relative abundance of *Tolypocladium, Trichoderma, Melanconiella*, and *Polyporus* decreased significantly. Among these, *Fusarium* was considered one of the dominant plant pathogens for many crucial plantations. The top fungal genera, which are listed in Table 2, included *Fusarium, Tolypocladium, Trichoderma*, and *Penicillium*.

As shown in the heat map of the 35 most abundant fungi (relative abundance > 1%) at the genus level, there were distinct alterations in the fungal community composition and structure in replanted soils at three time scales. Moreover, when compared with FCP, the dissimilarity of the community structure of fungi was enhanced under the extended monoculture regime of *C. equisetifolia*, implying that the rhizosphere fungal community gradually changed with increasing years of successive rotations (Fig. 3).

Discussion

Consecutive monoculture problem, also known as soil disease or replant failure, is a pivotal factor threatening sustainable and stable development of a variety of vegetations. The rapid growth and high yield of cultivated tree species, including *C. equisetifolia, Eucalyptus robusta*, and Chinese fir plantations, suffer from a significant decline under successive rotations. Studies have shown that successive rotations culture problem is caused by many factors, including soil nutrient deficiency, the autotoxicity of root exudates, and the imbalance of soil microorganisms (Wu et al. 2016a, b). In this study, the results of soil chemical properties also showed that TN, AN, and TP were higher in the FCP than in the SCP and TCP (Table S1). Previous studies suggested that successive rotations could lead to the consumption of soil available nutrients in *C. equisetifolia* stands, but the

Table 2 Information regarding the top fungi at the genus level in three individual rhizospheric treatments under successive rotations

Genus	Class	Phylum	Relative abundance (%)		
			FCP	SCP	TCP
Thelephora	Agaricomycetes	Basidiomycota	0.0831b	0.0467b	9.6586a
Archaeorhizomyces	Archaeorhizomyces	Ascomycota	0.0458b	3.2454a	0.1121b
Fusarium	Sordariomycetes	Ascomycota	0.2428b	1.5503a	1.9052a
Tolypocladium	Sordariomycetes	Ascomycota	1.8688a	0.0252b	0.0205b
Trichoderma	Sordariomycetes	Ascomycota	1.4382a	1.4438a	0.3474b
Polyporus	Agaricomycetes	Basidiomycota	1.3112a	0.0486b	0.0177b
Hortaea	Dothideomycetes	Ascomycota	0.0532b	0.3054b	1.1151a
Penicillium	Eurotiomycetes	Ascomycota	0.2587b	0.0943b	0.9479a

The different letters in each column represent significant differences ($P \leq 0.05$, $n = 3$)

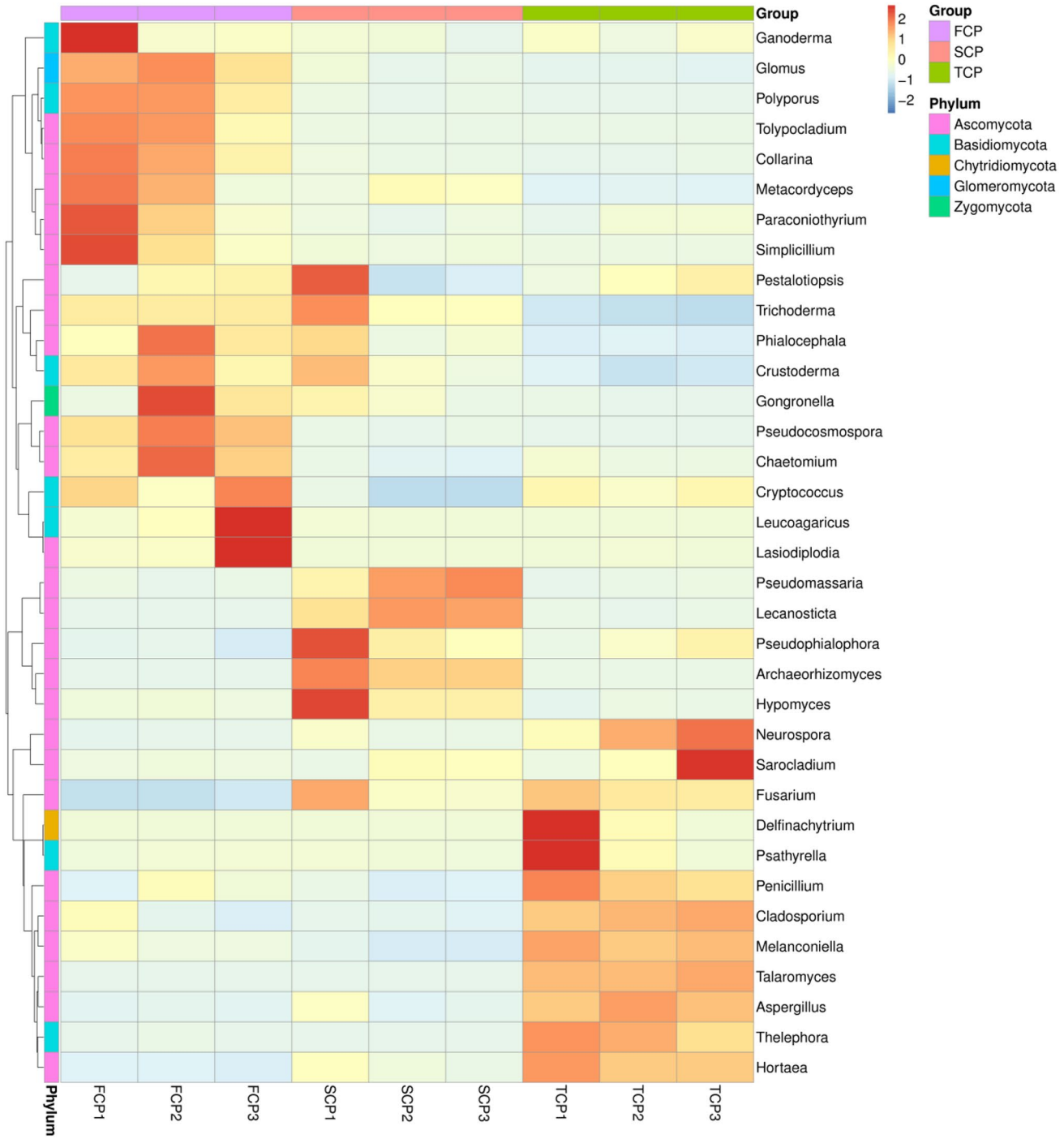

Fig. 3 Heat map analysis of the main fungi in three different soil samples at the genus level

application of fertilizer does not prevent replant disease, indicating that CMP is more closely associated with autotoxicity and soil microbes (Berendsen et al. 2012).

Moreover, many scholars have revealed that allelochemicals released by roots did not possess sufficient conditions to directly impact the health of adjacent plants or host plants. Therefore, the belowground microbial community structure and functional diversity has attracted increasing attention (Haney and Ausubel 2015; Xiong et al. 2016). Numerous species of microorganisms come into contact with plant roots, resulting in a complicated plant-related microbial community regarded as the second genome of plants, which is essential to their growth and productivity. Fungi, the major decomposers, play a role in biogeochemical cycles in the

soil ecosystems (Li et al. 2014a, b). In this study, the results of deep pyrosequencing demonstrated that the composition and diversity of the soil fungal community was significantly altered in the rooting zone after successive rotations of *C. equisetifolia*. Moreover, both PCoA and UPGMA clustering revealed an obvious partition among the three rhizospheric soil samples of FCP, SCP, and TCP. Additionally, the fungal community diversity indices of the FCP were significantly higher than those of the SCP. This results showed that the richness and diversity of soil fungal community in *C. equisetifolia* decreased significantly with the increase of CMP. Generally, bacteria will increase when soil nutrients are enough. Conversely, fungi will increase when soil nutrient decline, because fungi are more able to adapt to the bad soil environment. In this study, we found that in FCP soil, adequate soil nutrition resulted in the reproduction of bacteria. However, in SCP and TCP soil, nutrient content decrease resulted in the reproduction of fungi. Similar result was also reported by Zhao et al. (2013) in which soil fungal diversity increased in *Eucalyptus* monocultures.

Our findings exhibited that successive rotations of *C. equisetifolia* resulted in a significant increase in the relative abundance of *Fusarium, Penicillium, Thelephora*, and *Hortaea*, while *Trichoderma, Polyporus, Tolypocladium*, and *Melanconiella* decreased significantly. It is well known that most *Fusarium* are causative agents of root rot and wilt in a number of important plants (Van Wees et al. 2008), such as Chinese fir, *Eucalyptus robusta*, cucumber and strawberry (Bashir et al. 2012; Zhao et al. 2017). In this study, *Fusarium* showed a significantly higher relative abundance in SCP or TCP than in FCP. The increase in genus *Fusarium* from the FCP to SCP and TCP was primarily a result of the increase of *Fusarium oxysporum* by approximately 682% and 830%, respectively. It was also reported that many *Penicillium* could strengthen protection against pathogenic agents and facilitate the growth of plants, but some could also induce plant sickness. For example, Leelasuphakul et al. (2008) revealed that inoculation of a suspension of the *Penicillium digitatum* conidia into injured citrus fruit resulted in signs and symptoms at day 3 and corrupted at day 5. However, the relative abundance of *Trichoderma* showed a reduction from 1.44% in the FCP to 0.35% in the TCP. According to previous reports, some *Trichoderma* have been exploited as biocontrol agents against fungal diseases of the soil (Harman 2006).

Based on the above discussion, regeneration failure and productivity decline of *C. equisetifolia* could be because of the rapid reproduction of possible pathogenic agents and the consumption of plant beneficial fungi in replant failure soils. Many studies have shown that the quantity of fungal pathogens gradually increased under monoculture regime (Zhou et al. 2012). Moreover, studies have demonstrated that alterations in the rhizospheric soil microbial community play a far more important role in the successive rotation problem of plants than allelopathic effects (Haichar et al. 2008; Chen et al. 2018). Additionally, there is increasing evidence that plant root exudates can significantly reshape rhizospheric soil microbial community structure and microflora could then affect plant healthy growth in plant-soil ecosystems (Huang et al. 2014). Some studies demonstrated that the phenolic compounds that were released during dissolution and then accumulated in soils around the rhizosphere had a negative impact on the growth of Chinese fir plantations (Kõljalg et al. 2013; Peiffer et al. 2013). Additionally, many researchers detected that the inhibition or promotion of certain rhizospheric microorganisms induced by root exudates might be the dominant factor influencing soil disease within peanut crops, particularly *F. oxysporum*.

Conclusion

The successive rotations of *C. equisetifolia* can alter rhizosphere fungal communities by reducing plant beneficial fungi and enriching host-specific pathogens, which might be the key factor triggering soil disease of this tree species. Nevertheless, the impact of pathogens and beneficial microbes mentioned in this study on *C. equisetifolia* need to be verified in subsequent experiments. In addition, further studies are required to detect the roles of root exudations in the rhizosphere to address the consecutive productivity problem associated with *C. equisetifolia*, as well as the mechanisms underlying the rhizospheric mutual impact between plant beneficial microbes and pathogenic microbes.

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Author contributions WZY and LWX conceived and directed the project. WZY, ZLT and LWX designed all experiments. LY, LJJ, WJY, CJ, LSY and BY did all of experiments. ZLT and LJJ performed the integrated data analysis. ZLT and WZY wrote the manuscript with the assistance and approval of all authors.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing financial interests.

Ethical approval This study has been approved by the Hui'an National Forest Farm Management Committee, which takes care of the planning and protection of Hui'an National Forest Farm. The study did

not involve any endangered or protected species. All of the data in this study can be published and shared.

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