Diversity of Rhizosphere and Endophytic Fungi in *Atractylodes macrocephala* During Continuous Cropping

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Rhizospheric and endophytic fungi are key factors which influence plant fitness and soil fertility. *Atractylodes macrocephala* is one of the best-known perennial herbs used in traditional Chinese medicine. Continuous cropping has been shown to have a negative effect on its growth and renders it more susceptible to microbial pathogen attacks. In this study, we investigated the effects of continuous cropping on the endophytic and rhizospheric fungi associated with *A*. *macrocephala* using culture-independent Illumina MiSeq. Continuous cropping was found to decrease fungal diversity inside plant roots, stems, leaves and tubers. Additionally, we found that the structure and diversity of rhizospheric and endophytic fungal communities were altered by root-rot disease. *Fusarium* was overrepresented among root-rot rhizospheric and endophytic fungi, indicating that it has a major negative impact on plant health during *A*. *macrocephala* monocropping. Canonical correspondence analysis of the control and diseased samples revealed that pH, hydrolysis N, electrical conductivity and Hg content were well-correlated with fungal community composition during continuous cropping. Taken together, these results highlight the ecological significance of fungal communities in maintaining plant fitness and will guide the development strategies to attenuate the negative impacts of *A*. *macrocephala* continuous cropping.

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Abbreviations: 1-year fallow soil (FS_1); 2-year fallow soil (FS_2); 1-year healthy rhizospheric soil (RS_H1); 2-year healthy rhizospheric soil (RS_H2); 2-year root-rot diseased rhizospheric soil (RS_D); blank control soil with no *A. macrocephala* planted (S_CK); 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1); 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2); 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D); OTU, operational taxonomic unit; PCA, principle component analysis; CCA, canonical correspondence analysis; DCA, detrended correspondence analysis.

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Abstract

Rhizospheric and endophytic fungi are key factors which influence plant fitness and soil fertility. *Atractylodes macrocephala* is one of the best-known perennial herbs used in traditional Chinese medicine. Continuous cropping has been shown to have a negative effect on its growth and renders it more susceptible to microbial pathogen attacks. In this study, we investigated the effects of continuous cropping on the endophytic and rhizospheric fungi associated with *A. macrocephala* using culture-independent Illumina MiSeq. Continuous cropping was found to decrease fungal diversity inside plant roots, stems, leaves and tubers. Additionally, we found that the structure and diversity of rhizospheric and endophytic fungal communities were altered by root-rot disease. *Fusarium* was overrepresented among root-rot rhizospheric and endophytic fungi, indicating that it has a major negative impact on plant health during *A. macrocephala* monocropping. Canonical correspondence analysis of the control and diseased samples revealed that pH, hydrolysis N, electrical conductivity and Hg content were well-correlated with fungal community composition during continuous cropping. Taken together, these results highlight the ecological significance of fungal communities in maintaining plant fitness and will guide the development strategies to attenuate the negative impacts of *A. macrocephala* continuous cropping.

Keywords

Continuous cropping, *Atractylodes macrocephala*, Fungal endophytes, Rhizosphere fungi, *Fusarium*

Introduction

Plants and rhizospheric soil are colonized by fungal communities that can impact their fitness by influencing nutrient acquisition, causing soil-borne diseases and affecting the activity of plant pathogens (Tan et al. 2017a). Many previous studies have shown that studying plant-microorganism interactions can lead to improvements in several agronomic processes, including crop rotation and tillage (Somenahally et al. 2018), pesticide application (Regar et al. 2019), irrigation (Dang et al. 2019), fertilizer application (Nguyen et al. 2018) and continuous cropping (Ali et al. 2019; Xiong et al. 2015). Continuous cropping is widely adopted in Chinese agricultural production and is defined as cultivating the same or similar crop species for a long period of time (Shipton 1977). Studies have shown that continuous cropping leads to compromised growth, yield loss, disease susceptibility and quality deterioration (Hontoria et al. 2019). The detrimental effects of continuous cropping have been demonstrated in a variety of crop species, including *Atractylodes macrocephala* (Zheng et al. 2018).

A. macrocephala is a perennial herb that has been cultivated for over 700 years in temperate and subtropical regions. The tuber of *A. macrocephala* is commonly referred to as "*Baizhu*", and has been used to treat cancer, osteoporosis, gastrointestinal dysfunction, obesity and fetal irritability in traditional Chinese medicine in East Asia (Zhu et al. 2018b). It has recently been shown that continuous cropping of *A. macrocephala* can lead to reduced yield and quality (Zheng et al. 2018), possibly due to alterations in soil enzymatic activities, allelochemical substance enrichment, soil microbial community changes or soil-borne pathogen accumulation (Xiong et al. 2015). Change in soil microbiota communities has been singled out as one of the major causes of yield loss during *A. macrocephala* continuous cropping (Chen et al. 2014; Shi

2018). Root-rot diseases, which are often associated with *A. macrocephala* continuous cropping, also play a major role in loss of yield and quality during *A. macrocephala* continuous cropping (Zheng et al. 2018). Most of these diseases are associated with fungal pathogens such as *Fusarium oxysporum*, *Rhizoctonia solani*, and *Ceratobasidium* sp. (Liu 2012; You et al. 2013; Zhang 2015).

Rhizospheric fungi play key roles in organic matter decomposition, nutrient cycling and soil fertility maintenance (Miao et al. 2016; Zhou et al. 2017). Some rhizospheric fungi have also been associated with pathogen growth inhibition, leading to their application as biocontrol agents (Venneman et al. 2019). Additionally, the overall level of endophytic fungi has been shown to be a good indicator of host plant health (Zhu et al. 2018a). Recent studies have proposed that continuous cropping results in imbalances in endophytic and rhizospheric soil fungal community diversity and structure, and rapid accumulation of fungal pathogens (Liu et al. 2019; Qin et al. 2017). Thus, a healthy and stable endophytic and rhizospheric fungal community may be essential for maintaining long-term continuous cropping and stable crop yields.

Both the mechanism by which continuous cropping affects rhizospheric soil fungal communities and how these changes influence soil productivity are still largely unknown. To date, there has been no culture-independent studies of rhizospheric soil and endophytic fungal community diversity during monocropping of *A. macrocephala* (Xiong et al. 2015; Zhou & Wu 2012). In this study, we used molecular characterization to examine changes in rhizospheric fungal communities, rotation soils, roots, leaves, stems, and tubers in *A. macrocephala* grown in continuous cropping fields. We aimed to investigate the link between soil physiochemical

properties and the structure, composition and diversity of fungal communities. Additionally, we examined the underlying mechanism by which continuous cropping of *A. macrocephala* influenced endophytic and rhizospheric fungal communities.

Materials & methods

Study site and experimental design

Samples were collected from a grassland located in a hilly area in Lin'an County, Zhejiang Province, China, with a typical mid-subtropical monsoon climate (30°14′ N, 119°25′ E, 155 m elevation). The study area was divided into five zones according to different cropping practices (no *A. macrocephala* planted, *A. macrocephala* planted for 1 year, *A. macrocephala* planted for 2 years, 1-year fallow, and 2-year fallow). Each zone consisted of a 20 × 20 m plot, which was managed as uniformly as possible. Due to limited land access, only one plot was used for each zone. However, we randomly collected 15 soil cores from the plow layer (0–30 cm in depth), thoroughly pooled them as a composite sample and divided the pooled samples into three replicates to make the samples more representative (Hu et al. 2017; Tan et al. 2017a). Fallow soil zones were left unplanted for the specified number of years after an initial planting and harvesting of *A. macrocephala*. For the fallow zones, three soil samples were randomly selected, with each soil sample composed of 15 soil cores randomly collected from the plow layer (0–30 cm in depth).

Plants were sampled by inspecting them for root rot symptoms, and were then placed either into the group with or without symptoms. Symptoms were recorded regarding the general appearance of the entire plant, and more specifically regarding the health of the roots, the stems,

and the foliage. In the absence of overt symptoms in above-ground parts, roots were checked for necrotic flecking, dead, broken roots, and water-soaked lesions (Sims et al. 2019). For the 1-year and 2-year *A*. *macrocephala* planted zones, 15 plant samples for each plant type (1-year healthy plant, 2-year healthy plant, and 2-year diseased plant) were collected and put into sterile plastic bags, placed on ice and transported to the laboratory immediately. The soil which was loosely attached to the roots was removed by gentle shaking. Tightly bound soil was considered rhizospheric and was obtained by firmly shaking the root system in a sterile plastic bag (Kobayashi et al. 2015; Tan et al. 2017b). The rhizospheric soil samples were thoroughly mixed, and randomly divided into three subsamples, as described previously (Yu et al. 2019). The plants were washed with tap water and then rinsed three times with distilled water. They were then separated into leaf, stem, tuber, and root tissues. For surface disinfection, 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, then washed with distilled water three times (Zhu et al. 2018a). Each plant tissue was thoroughly mixed to make a composite sample, and randomly divided into three subsamples. In total, there were 18 soil samples (3 fallow soils and 3 rhizospheric soils, with 3 replicates each) and 36 plant samples (3 plant types, with 4 tissue types and 3 replicates each). Collected soils were sieved through a <4 mm filter and stored at 4 °C prior to biochemical characterization, or frozen at −80°C less than two weeks before DNA extraction.

The soil and plant conditions were as follows: 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizospheric soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-

112 year root-rot diseased rhizospheric soil (RS_D), blank control soil with no *A. macrocephala*
113 planted (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy
114 root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)
115 (Fig. S1).

116 **Soil physicochemical properties**

117 The chemical and physical properties of all soil samples were analyzed. Soil pH was measured
118 by a pH meter and soil electrical conductivity by an electric conductivity bridge, according to the
119 manufacturers' protocols (Satapute et al. 2019). The moisture content of the soil was determined
120 after dry weights were obtained by drying the samples at 105 °C. The soil organic matter content
121 was measured using Yang's method (Yang et al. 2019). We used a nickel crucible to digest soil
122 total K with sodium hydroxide at 750 °C, and extracted soil available K using 1 mol/L
123 ammonium acetate (Zhao et al. 2014). All K contents were determined by an atomic absorption
124 spectrophotometer (AAnalyst 400, PerkinElmer, US) (Yao et al. 2017). Soil available P and total
125 P and were determined by the ammonium molybdate method (Murphy & Riley 1962). Soil total
126 N was determined using the semi-micro Kjeldahl method, according to Nelson and Sommers
127 (Nelson & Sommers 1980). Soil hydrolysis N was determined by the alkali-hydrolyzed
128 distillation method described by Zhang et al. (Zhang et al. 2018). Heavy metals Pb, Cu, Hg, As,
129 Cr and Cd were detected using flame atomic absorption spectrometry (Analytikjena AAS vario6,
130 Germany) according to the method of Ye et al. (Ye et al. 2011). All assays were performed in
131 triplicate.

132 **DNA extraction, PCR amplification, and sequencing**

Genomic DNA was isolated from the rhizosphere, fallow soil, blank control soil samples and plant organs using the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, USA) according to the manufacturer's instructions. The DNA extractions for each sample were conducted in triplicate. The quantity and purity of the DNA samples were assayed by a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, USA). The DNA samples were also run on a 1% agarose gel by electrophoresis to further assess their quality and integrity. The DNA samples were diluted to 1 ng/μL with sterile water and stored at −80 °C until further processing. The ITS1 region was amplified from fungal genomes with primers ITS1-F (CTTGGTCATTTAGAGGAAGTAA) and ITS2-F (GCTGCGTTCTTCATCGATGC) in a thermocycler PCR system (Eppendorf Mastercycler pro, Germany). The A adaptor-containing end was the sequencing end, and a 10 bp barcode was added between the adaptor and the forward primer sequence to allow for dis-crimination of the samples during sequencing (Bai et al. 2015). The PCR mixtures contain 5 × *TransStart* FastPfu buffer 4 μL, 2.5 mM dNTPs 2 μL , forward primer (5 μM) 0.8 μL, reverse primer (5 μM) 0.8 μL, *TransStart* FastPfu DNA Polymerase 0.4 μL, template DNA 10 ng, and finally ddH_2O up to 20 μL. The reaction program was: initial denaturation at 95 ℃ for 3 min, followed by 27 cycles of denaturing at 95 ℃ for 30 s, annealing at 55 ℃ for 30 s and extension at 72 ℃for 45 s, and single extension at 72 ℃ for 10 min, and end at 4 ℃. The PCR reactions for each DNA sample were conducted in triplicate, resulting in nine total PCR products for each plant/soil sample (3 DNA replicates, with 3 PCR replicates each). The resulting amplicons were separated by electrophoresis using a 2% agarose gel and extracted with the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, USA). The

nine purified PCR products were then pooled before paired-end sequencing (2×300) on an Illumina MiSeq platform (Illumina, USA) (Saenz et al. 2019).

Bioinformaitics analysis

The raw ITS rDNA gene sequencing reads were demultiplexed, quality filtered by Trimmomatic and merged by FLASH with the following criteria: 1) Reads were removed if any site had an average quality score <20 over a 50 bp sliding window. Reads containing Ns or with a length of less than 50 were also removed. 2) The remaining pair-end reads were assembled into larger contigs according to their overlaps, with a minimum overlap length of 10 bp. 3) The maximum mismatch ratio allowed in the overlap area of the merged sequence was 0.2. 4) The directionality of reads was corrected based on their barcodes and primer sequences, with no mismatches allowed in the barcode and 2 mismatches allowed in the primers (FLASH and Trimmomatic).

We assigned sequences with ≥ 97% similarity to the same Operational Taxonomic Units (OTUs) in UPARSE (version 7.1, Table 1) and then selected a representative OTU for each community in order to perform taxonomic annotation using a Ribosomal Database Project classifier. The OTU clustering process and criteria were as follows: 1) In order to reduce the amount of redundant calculation in the process of analysis, non-repetitive sequences were obtained from optimized data (http://drive5.com/usearch/manual/dereplication.html). 2) Singletons were removed (http://drive5.com/usearch/manual/singletons.html). 3) With 97% clustering, an OTU sequence was required to be at least 3% different from all other OTUs, and OTU representative sequence were the most abundant sequences in their neighborhood. During the clustering process, chimeric sequences were discarded. 4) All optimized sequences were

175 mapped to OTU representative sequences, and sequences with a similarity＞97% were selected
176 to generate an OTU table (Usearch, vsesion 7.0, http://drive5.com/uparse/). Alpha within
177 samples was analyzed by in-house Perl scripts.

178 Next, we rarified the OTU table and calculated Observed Species, Shannon's index, and
179 Chao1to calculate Alpha Diversity (Tan et al. 2017a). QIIME and the
180 compute_core_microbiome.py script were used to identify OTUs that occurred within 95% of
181 the *A. macrocephala* or soil samples (Mahoney et al. 2017). A species was defined as dominant
182 if $P_i > 1/S$, where S represents species richness, and P_i is the relative abundance of a species i,
183 defined as the number of competing species present in the community (Rivera-Orduna et al.
184 2011; Wu et al. 2013).

185 **Statistical analysis**

186 One-way ANOVA of soil physical and chemical properties as well as the changes in dominant
187 fungal genera of rhizospheric and fallow soil samples were performed using SPSS (version 19.0;
188 SPSS, Chicago, IL, USA). Significance was calculated by Duncan's mean test ($P < 0.01$). Mean
189 values of replicates are expressed as mean ± standard error (SE). False discovery rate (FDR) was
190 used to adjust the significance value in multiple comparisons.

191 A heatmap was drawn to show the relative abundances of the 25 predominant genera in each
192 sample. Principle component analysis (PCA) was conducted using all OTUs relative abundances
193 in the plant and soil samples.

194 We performed a canonical correspondence analysis (CCA) using normalized OTU
195 abundance and soil physicochemical data using the vegan package implemented in R

196 (http://cran.rproject.org/) (Dixon 2003). Significant correlations between soil properties and the
197 fungal communities at OTU level were determined via Spearman's correlations using > 0.8 or <
198 −0.8 as the threshold, with a cutoff of $P < 0.01$ (Faust et al. 2012). All the data analysis for
199 correlations was finished in online 'i-sanger' (http://www.i-sanger.com/) developed by Majorbio
200 Bio-Pharm Technology Co. Ltd (Lin et al. 2018).

201 ## Results

202 ### Soil properties

203 Continuous cropping practice noticeably decreased soil pH compared with the S_CK and fallow
204 soil. Electrical conductivity, total P, total K, hydrolysis N, available P, and available K contents
205 were much higher in the continuous cropping soil relative to the S_CK, while organic matter and
206 total N contents were higher in the S_CK than in continuous cropping soil samples. In addition,
207 the continuous cropping soil exhibited much higher Pb, As, Cd, Cr, and Cu contents, while
208 S_CK had higher Hg levels (Table 2).

209 ### Composition and alpha diversity of the fungal communities

210 A total of 484,844 and 251,911 reads were obtained from *A. macrocephala* plant and soil
211 samples, respectively. A total of 48,550 reads mapped to unidentified phylum, while 271,914
212 reads mapped to unidentified genera. As indicated by the rarefaction curves, all samples reached
213 the saturation phase with a satisfactory level of confidence and a Good's coverage index ≥ 99.59%
214 (Table 1, Fig. 1). Sequences from plant samples clustered into 2,545 OTUs using a 3%
215 dissimilatory threshold. The core fungi of plant samples consisted of 276 OTUs, each of which
216 had a relative abundance of greater than 1% and were found in 95% of plant samples, and

included Pleosporales (with 73 OTUs), Hypocreales (45), Capnodiales (81), Helotiales (33), Tremellales (19), Cantharellales (5) and Sporidiobolales (20) (Fig. 2a). Samples T_D and T_H2 contained the highest levels of Hypocreales (93.43%) and Pleosporales (72.57%) sequences, respectively (Fig. 2c).

Sequences from soil samples clustered into 3,596 OTUs, and their core fungal composition (431 OTUs) included Eurotiales (63), Hypocreales (104), Pleosporales (65), Chaetothyriales (51), Mortierellales (27), Sordariales (44), Helotiales (42), Geminibasidiales (3), Chaetosphaeriales (12) and Capnodiales (20) (Fig. 2b). Samples S_CK and FS_1 contained the highest levels of Eurotiales (40.08%) and Hypocreales (37.04%), respectively (Fig. 2c). At the phylum level, Zygomycota, Ascomycota, and Basidiomycota were the dominant fungal phyla in both continuous cropping and fallow samples. During continuous cropping, rhizospheric soil showed a significant increase of *Talaromyces*, *Fusarium*, *Cotylidia* and *Acremonium*, and significant decrease of *Penicillium* ($P<0.01$) (Fig. 2d). A higher abundance of the genera *Cotylidia*, *Phialophora*, and *Fusarium* in rhizospheric fungi was observed in root-rot diseased plants than the S_CK (Fig. 2d). Additionally, rhizospheric soil fungal alpha-diversity decreased with long-term *A. macrocephala* cropping. The fungal diversity index was the highest in blank control soil, and the fungal diversity of the fallow soil samples was also higher than that in samples from continuous cropping. Moreover, root-rot disease decreased the endophytic fungal diversity of *A. macrocephala* (Table 1).

Put Figs. 1, 2 here.

Structure of the fungal communities

We performed a hierarchical clustering analysis on the top 25 most abundant fungal genera across 18 plant and soil samples (Fig. 3a). The analysis showed that *A. macrocephala* aerial samples clustered into one category (except for T_D), while all rhizospheric and fallow soil samples clustered into another category. R_D, T_H1, R_H1, and T_H2 endophytic samples clustered together, but were separate from the R_H2 sample. PCA analysis based on the OTU composition revealed obvious variations in fungal communities among the 18 plant and soil samples (Fig. 3b). The first two axes (PC1 and PC2) explained 41.59% and 19.86% of the total variance in the fungal OTUs of the plant and soil samples, respectively, in *A. macrocephala* continuous cropping fields.

Put Fig. 3 here.

Correlation between soil properties and the fungal communities

We then removed the redundant variables, and the eight remaining environmental characteristics were subjected to detrended correspondence analysis (DCA). The DCA showed that the responses of fungal community composition to soil properties fit a single-peak model (Length=6.32). Therefore, we further analyzed how fungal communities associated with soil physicochemical factors based on CCA (Fig. 4). Soil physicochemical factors Hg, electrical conductivity, available P, Cr, pH, and hydrolysis N positively correlated with each other, while total N and organic matter positively correlated with each other. Moreover, pH (p=0.001), hydrolysis N (p=0.001), electrical conductivity (p=0.001), and Hg (p=0.001) had a significant correlation with fungal community structure (Fig. 4). Spearman's correlation analysis revealed that the relative abundance of the genus *Acremonium* was significantly negatively corelated with

soil electrical conductivity and Hg content, while *Penicillifer* genus abundance had a negative correlation with soil Cr content (Table S1, S2).

Put Fig. 4 here.

Discussion

Sustainable *A. macrocephala* production management calls for a deep understanding of how continuous cropping alters the structure and diversity of fungal communities. To gain more insight in this, we assessed the effects of continuous cropping on fungal communities in rhizospheric soil, rotation soil and endophytes of *A. macrocephala*. We found that the diversity, structure and composition of rhizospheric soil and endophyte fungal communities were greatly affected by continuous cropping. Rhizospheric soil fungal alpha-diversity indices, including Chao1 and Shannon indices, decreased after continuous cropping practices. In addition, fallow soils showed relatively higher overall fungal activity, whereas fungal diversity was similar between continuously cropped soil and the S_CK, suggesting that fungal diversity gradually recovers to unplanted control soil with time of fallowing. Severe root-rot disease has long been known as a major problem in continuous cropping (Tan et al. 2017a). Consistently, we detected a relatively higher fungal community diversity in healthy tissues and rhizospheric soils compared to diseased samples. Therefore, the decrease of soil and endophytic fungal diversity may play a role in disease development during *A. macrocephala* continuous cropping.

Rhizospheric soil fungal communities were also strongly influenced by continuous cropping of *A. macrocephala*. Zygomycota, Ascomycota, and Basidiomycota were the dominant fungal phyla in both continuous cropping and fallow samples. At the genus level, the relative abundance

of *Talaromyces* and *Fusarium* increased significantly with cropping time. *Talaromyces* and *Fusarium* genera fungi contain several potential pathogens, such as *Fusarium oxysporum* and *Talaromyces helices* (Wu et al. 2016). Thus, increases in these genera may negatively contribute to *A. macrocephala* continuous cropping, eventually leading to increased disease pressure. Moreover, the fungal community profile of fallow soil became more similar to the control soil profile as the number of years of fallowing increased, indicating that recovery of a healthy soil profile is possible.

Endophytic fungi usually inhabit different plant tissues without harming their hosts. However, we observed reduced abundance of endophytic fungal OTUs in diseased samples, signifying reduced endophytic fungal diversity. Ascomycota, Basidiomycota, and Zygomycota were the top three fungal phyla detected in both healthy and diseased *A. macrocephala* plants, which was similar to the profile of soil fungi. This pattern fits with earlier publications, which have shown that endophytic fungi are primarily derived from soil fungi that enter the plants via roots, tubers, leaves and stems (Dai et al. 2010; Tan et al. 2017b). At the genus level, *Fusarium* and *Alternaria* abundance significantly increased in the root-rot diseased *A. macrocephala* samples. This may be due to increases in other root-rot disease associated species, such as *F. oxysporum*, *F. solani*, and *Alternaria gansuense*. Increases in these pathogenic fungi likely caused a decrease in endophytic fungi due to limited availability of space and nutrients (Zeng 2016; Zhang et al. 2015). These findings warrant future studies on the changes in microbial communities, as they can be used as indicators of overall soil and plant health during continuous cropping.

A deeper understanding of soil properties during *A. macrocephala* continuous cropping systems is key to improving soil productivity. Long-term monoculture of *A. macrocephala* has been reported to reduce organic matter content and soil pH, due to the return of organic material and the application of fertilizer to the soil (Geng et al. 2015). Increased soil hydrolysis N, available P, and available K contents often result from fertilizer application (Shi 2018). The optimum pH range for *A. macrocephala* growth is 5.1 to 6.6 (Zhu et al. 2018b), and the pH decline seen during continuous cropping may therefore increase the disease susceptibility of *A. macrocephala*.

In agricultural ecosystems, soil microbial communities have a major impact on soil organic matter accumulation and nutrient cycling, which are often used as indicators of soil quality (Ashworth et al. 2017). In our study, soil pH, hydrolysis N, electrical conductivity, and Hg content were most strongly correlated with fungal community structure during continuous cropping of *A. macrocephala*. Soil pH may directly alter fungal community composition by inhibiting fungal survival and growth, as seen in earlier publications indicating fungal taxa were unable to grow under a certain soil pH (Zhang et al. 2016). In addition, soil communities can affect soil N dynamics, while hydrolysis N has a major impact on the composition of fungal community under continuous cropping. The change in fungal composition may be a result of both fertilization and the observed increase in hydrolysis N cycling (Thompson & Kao-Kniffin 2019). Agricultural management practices, such as high fertilizer usage and restricted irrigation can immensely influence electrical conductivity variation (Adviento-Borbe et al. 2006; Kim et al. 2016). Electrical conductivity is associated with soil salinity and our results suggest it may be an

important predictor of fungal community compositions in continuous cropping soils of *A. macrocephala*. Moreover, *Acremonium* and *Penicillifer* show a negative correlation with heavy metal contents in the soil, especially Hg and Cr.

Conclusions

Overall, we found that continuous cropping and severe root-rot disease could both significantly affect the structure and diversity of *A. macrocephala* endophytic and soil fungal communities. Rhizospheric soil pH and organic matter content decreased with increasing continuous cropping time. Moreover, the abundance and diversity of fungal communities decreased, while the prevalence of severe root-rot disease increased with prolonged continuous cropping. Of all root-rot rhizospheric and endogenous fungal species, *Fusarium* was most significantly enriched upon continuous cropping. Further, soil pH, hydrolysis N, electrical conductivity, and Hg were most strongly correlated with fungal community composition. Simultaneously examining both endophytic fungal populations (from surface-sterilized plant tissues) and the rhizospheric samples (including soils from the roots) allowed us to gain a deeper understanding of how continuous cropping alters fungal populations. Our results suggest that changes in fungal diversity can be used to predict disease outbreaks in *A. macrocephala* continuous cropping systems. The findings of this research can also guide the development of management strategies to improve *A. macrocephala* production.

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Competing Interests

All authors declare that there is no conflict of interest.

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497

498 **Figure legends**

499 Figure 1: Rarefaction curves of fungal communities based on observed operational taxonomic
500 units (OTUs) for 18 plant and soil samples under an *Atractylodes macrocephala* continuous
501 cropping system. Each plant and soil samples (18 samples) were randomly divided into three
502 subsamples (18×3), and were analyzed using the average data of their subsamples, respectively.
503 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-
504 year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank
505 control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber
506 (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot
507 diseased root/stem/leaf/tuber (R/S/L/T_D)

508 Figure 2: Fungal composition of 18 plant and soil samples under an *A*. *macrocephala* (AM)
509 continuous cropping system: Each plant and soil samples (18 samples) were randomly divided
510 into three subsamples (18×3), and were analyzed using the average data of their subsamples,
511 respectively. The "core fungi" of *A*. *macrocephala* (a) and soil (b). The relative abundance at
512 order level of 18 plant and soil samples (c), and the dominant genera of six soil samples (d),
513 different letters above the bars in one genus denote significant differences at $P<0.01$. 1-year
514 fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year
515 healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank
516 control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber
517 (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot
518 diseased root/stem/leaf/tuber (R/S/L/T_D).

519 Figure 3: The top 25 most abundant fungal communities across 18 plant and soil samples (a) and
520 principle component analysis (PCA) based on the operational taxonomic unit (OTU)
521 composition (b). Each plant and soil samples (18 samples) were randomly divided into three
522 subsamples (18×3), and were analyzed using the average data of their subsamples, respectively.
523 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-
524 year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank
525 control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber
526 (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot
527 diseased root/stem/leaf/tuber (R/S/L/T_D).
528 Figure 4: Canonical correspondence analysis (CCA) between the soil and fungal community.
529 Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3),
530 and were analyzed using the average data of their subsamples, respectively. 1-year fallow soil
531 (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy
532 rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil
533 (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber
534 (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot
535 diseased root/stem/leaf/tuber (R/S/L/T_D).
536 Figure S1: Study sites and samples: 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year
537 healthy rhizospheric soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot
538 diseased rhizospheric soil (RS_D), blank control soil with no A. macrocephala planted (S_CK),
539 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber

(R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D).

Figure 1

Rarefaction curves of fungal communities based on observed operational taxonomic units (OTUs) for 18 plant and soil samples under an *Atractylodes macrocephala* continuous cropping system

Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3), and were analyzed using the average data of their subsamples, respectively. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)

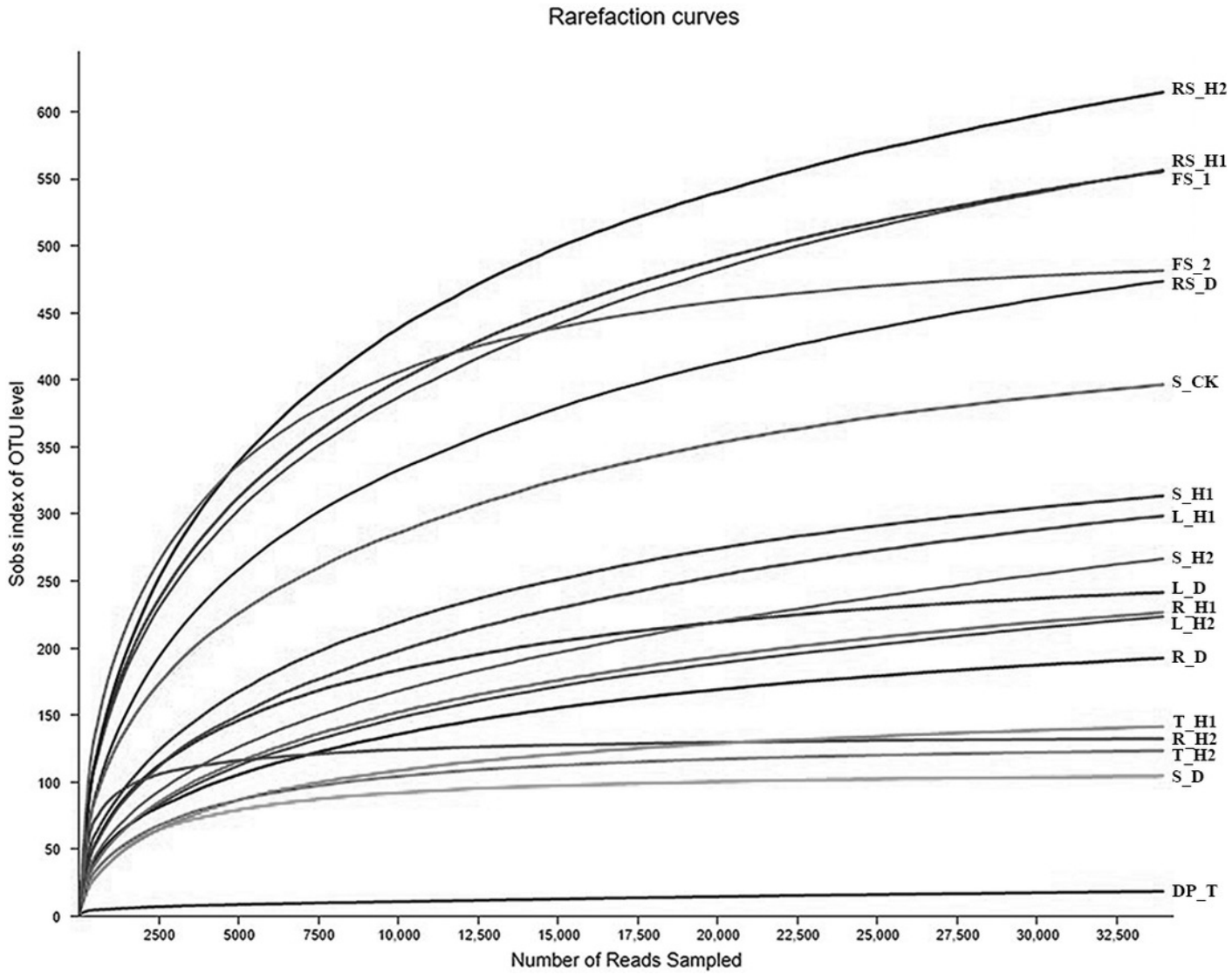
Rarefaction curves
Sobs index of OTU level
Number of Reads Sampled
0
50
100
150
200
250
300
350
400
450
500
550
600
2500
5000
7500
10,000
12,500
15,000
17,500
20,000
22,500
25,000
27,500
30,000
32,500
RS_H2
RS_H1
FS_1
FS_2
RS_D
S_CK
S_H1
L_H1
S_H2
L_D
R_H1
L_H2
R_D
T_H1
R_H2
T_H2
S_D
DP_T

Figure 2

Fungal composition of 18 plant and soil samples under an *A*. *macrocephala* (AM) continuous cropping system

Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3), and were analyzed using the average data of their subsamples, respectively. The “core fungi” of *A*. *macrocephala* (a) and soil (b). The relative abundance at order level of 18 plant and soil samples (c), and the dominant genera of six soil samples (d), different letters above the bars in one genus denote significant differences at $P<0.01$. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D).

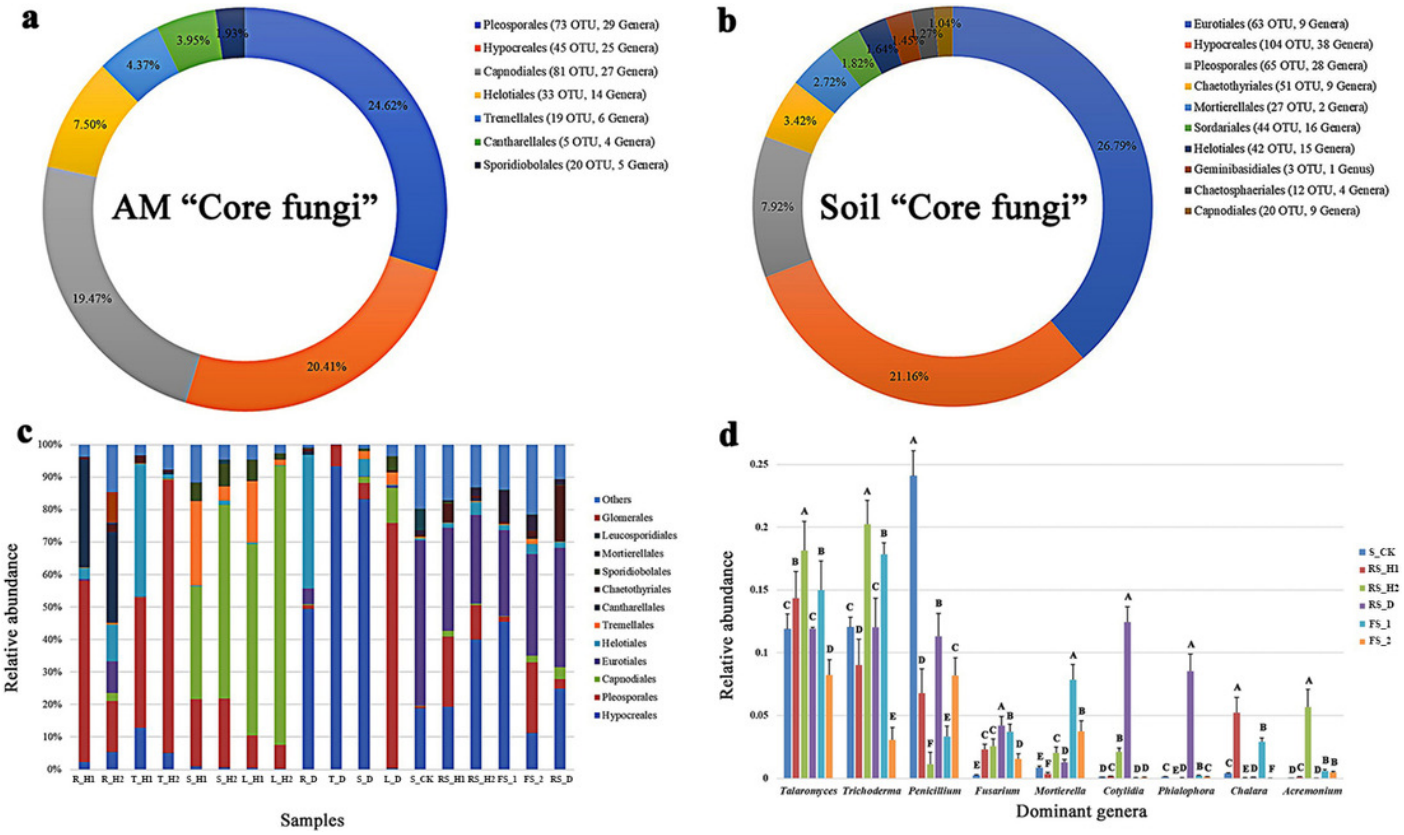
a
AM "Core fungi"
24.62%
20.41%
19.47%
7.50%
4.37%
3.95%
1.93%
Pleosporales (73 OTU, 29 Genera)
Hypocreales (45 OTU, 25 Genera)
Capnodiales (81 OTU, 27 Genera)
Helotiales (33 OTU, 14 Genera)
Tremellales (19 OTU, 6 Genera)
Cantharellales (5 OTU, 4 Genera)
Sporidiobolales (20 OTU, 5 Genera)
b
Soil "Core fungi"
26.79%
21.16%
7.92%
3.42%
2.72%
1.82%
1.64%
1.45%
1.27%
1.04%
Eurotiales (63 OTU, 9 Genera)
Hypocreales (104 OTU, 38 Genera)
Pleosporales (65 OTU, 28 Genera)
Chaetothyriales (51 OTU, 9 Genera)
Mortierellales (27 OTU, 2 Genera)
Sordariales (44 OTU, 16 Genera)
Helotiales (42 OTU, 15 Genera)
Geminibasidiales (3 OTU, 1 Genus)
Chaetosphaeriales (12 OTU, 4 Genera)
Capnodiales (20 OTU, 9 Genera)
c
Relative abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
R_H1 R_H2 T_H1 T_H2 S_H1 S_H2 L_H1 L_H2 R_D T_D S_D L_D S_CK RS_H1 RS_H2 FS_1 FS_2 RS_D
Samples
Others
Glomerales
Leucosporidiales
Mortierellales
Sporidiobolales
Chaetothyriales
Cantharellales
Tremellales
Helotiales
Eurotiales
Capnodiales
Pleosporales
Hypocreales
d
Relative abundance
0.25
0.2
0.15
0.1
0.05
0
Talaromyces
Trichoderma
Penicillium
Fusarium
Mortierella
Cotylidia
Phialophora
Chalara
Acremonium
Dominant genera
S_CK
RS_H1
RS_H2
RS_D
FS_1
FS_2

Figure 3

The top 25 most abundant fungal communities across 18 plant and soil samples (a) and principle component analysis (PCA) based on the operational taxonomic unit (OTU) composition (b)

Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3), and were analyzed using the average data of their subsamples, respectively. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A. macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)

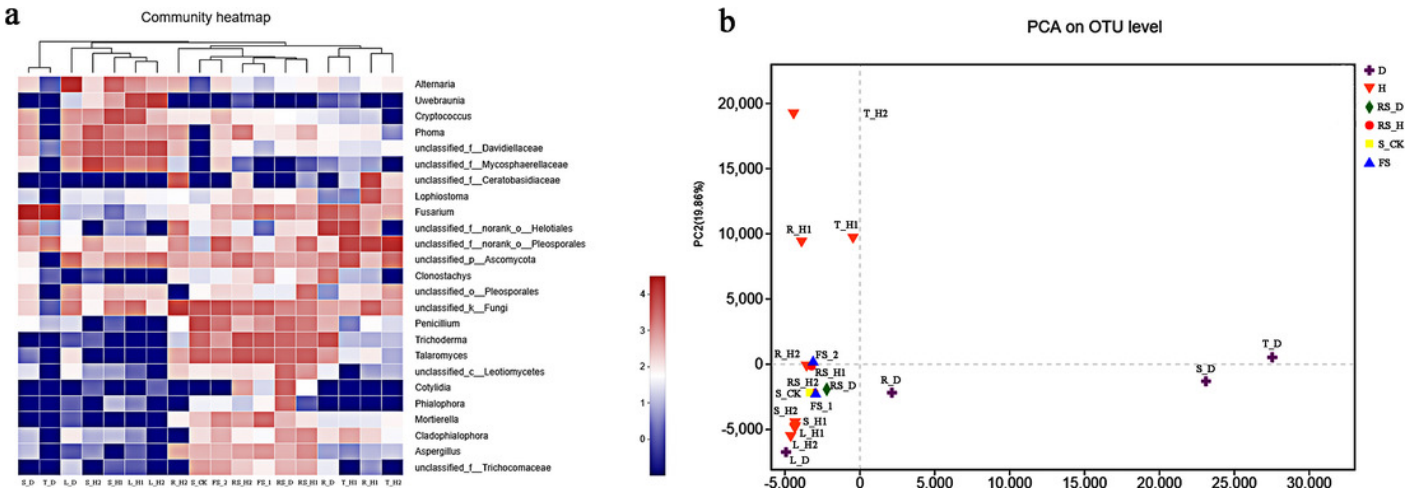

Figure 4

Canonical correspondence analysis (CCA) between the soil and fungal community

Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3), and were analyzed using the average data of their subsamples, respectively. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A. macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)

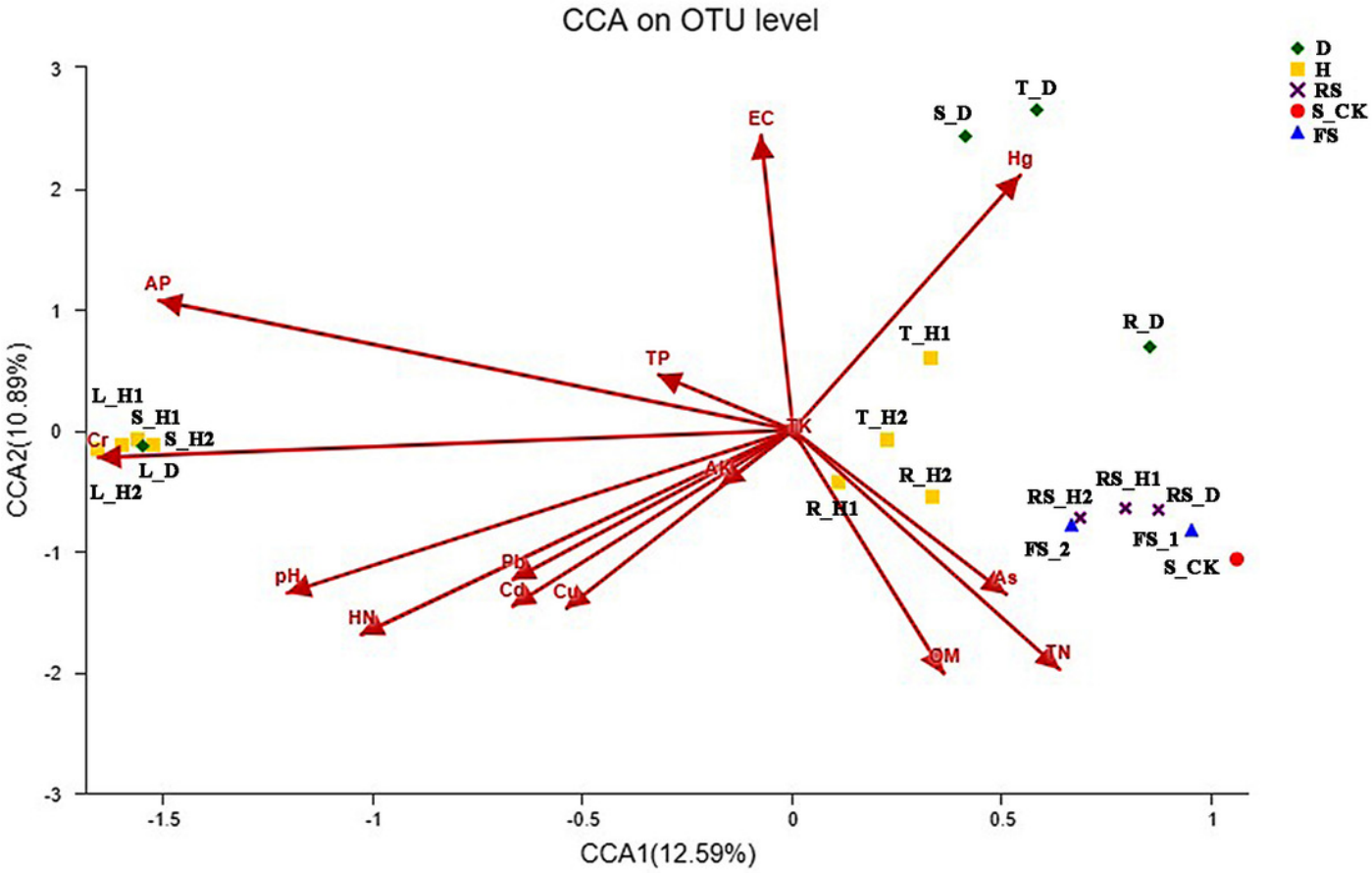

Table 1(on next page)

Summary of data and alpha-diversity of the fungal community of rhizospheric soil, plant endophytic and fallow soil samples

Each plant and soil samples (18 samples) were randomly divided into three subsamples (18 ×3), and were analyzed using the average data of their subsamples, respectively. Different letters in one column denote significant differences at $P<0.01$ among the samples from same source. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)

Table 1 Summary of data and alpha-diversity of the fungal community of rhizospheric soil, plant endophytic and fallow soil samples

Source of sample	Sample	Raw reads	Average length	Total bases ($\times 10^7$)	Q30	Q20	Nseqs	Coverage	OTU (97%)	OTU (95%)	Chao1 (97%)	Shannon (97%)
Soil	FS_1	58691	265.37	1.56	97.87	99.31	33919	0.9965	684	613	736.649C	5.264C
	FS_2	42763	270.10	1.16	97.66	99.24	33919	0.9991	586	527	782.019B	5.573B
	RS_H1	58643	270.71	1.59	97.61	99.19	33919	0.9961	614	498	645.101D	4.213D
	RS_H2	51435	269.26	1.38	97.77	99.28	33919	0.9959	555	451	598.489E	3.646F
	RS_D	48644	273.85	1.33	97.39	99.14	33919	0.9968	500	411	545.750F	3.722E
	S_CK	40379	272.72	1.10	97.59	99.21	33919	0.9979	657	562	828.137A	5.813A
Root	R_H1	59575	263.53	1.57	98.49	99.51	33919	0.9983	252	209	255.724B	2.089C
	R_H2	34464	257.25	0.89	98.50	99.41	33919	0.9999	132	119	313.500A	3.359A
	R_D	44330	268.88	1.19	98.11	99.40	33919	0.9988	205	176	218.941C	2.612B
Tuber	T_H1	48878	256.17	1.25	98.64	99.56	33919	0.9994	153	132	154.353A	1.867B
	T_H2	48295	265.07	1.28	98.21	99.41	33919	0.9997	168	134	151.000B	1.905A
	T_D	57338	244.44	1.40	98.72	99.57	33919	0.9998	23	15	26.200C	0.314C
Stem	S_H1	58464	249.31	1.46	98.67	99.58	33919	0.9979	345	275	350.143B	3.109A
	S_H2	66439	245.48	1.63	98.60	99.55	33919	0.9971	321	240	393.500A	2.519B
	S_D	55367	244.13	1.35	98.70	99.55	33919	0.9998	106	91	106.750C	1.211C
Leaf	L_H1	62977	252.25	1.59	98.54	99.53	33919	0.9975	332	247	337.893A	2.942A
	L_H2	54317	251.72	1.37	98.35	99.45	33919	0.9980	258	202	322.500B	2.348B
	L_D	46142	257.35	1.19	98.63	99.55	33919	0.9990	250	205	251.667C	2.191C

-Each plant and soil samples (18 samples) were randomly divided into three subsamples (18×3), and were analyzed using the average data of their subsamples, respectively. Different letters in one column denote significant differences at $P<0.01$ among the samples from same source. 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A*. *macrocephala* was planted) (S_CK), 1-year-old healthy root/stem/leaf/tuber (R/S/L/T_H1), 2-year-old healthy root/stem/leaf/tuber (R/S/L/T_H2), 2-year-old root-rot diseased root/stem/leaf/tuber (R/S/L/T_D)

Table 2(on next page)

Physical and chemical nature of rhizospheric and fallow soils in Lin'an, China (n=3)

Different letters in one column denote significant differences at $P<0.01$, 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A. macrocephala* was planted) (S_CK)

Table 2 Physical and chemical nature of rhizospheric and fallow soils in Lin'an, China (n=3)

Sample	pH	Organic matter (g/kg)	Total N(g/kg)	Total P(g/kg)	Total K(g/kg)	Hydrolysis N(mg/g)	Available P(mg/g)	Available K(mg/g)	Electrical conductivity (mS/m)	Pb(mg/g)	As(mg/g)	Hg(mg/g)	Cd(mg/g)	Cr(mg/g)	Cu(mg/g)
S_CK	5.84±0.16A	44.0±2.8AB	1.73±0.16AB	0.24±0.04B	15.2±0.7B	163.2±6.9A	2.6±0.4E	68±7D	3.9±0.3C	18.6±0.5BC	2.84±0.20C	0.084±0.002AB	0.12±0.01BC	14±2C	22±2A
RS_H1	5.55±0.25AB	41.4±1.4BC	1.60±0.21AB	0.30±0.05AB	16.8±0.8AB	175.0±5.0A	24.2±1.3B	83±3C	6.0±0.4B	18.0±0.8C	3.01±0.15BC	0.075±0.005AB	0.13±0.02B	21±3AB	22±2A
RS_H2	5.31±0.21BC	37.2±2.0CD	1.49±0.16AB	0.35±0.05AB	16.5±0.7AB	170.2±4.9A	28.9±0.8A	142±6B	5.7±0.5B	23.7±0.7A	2.92±0.11C	0.064±0.008B	0.17±0.02A	24±3A	26±1A
FS_1	5.01±0.15C	42.5±2.0ABC	1.70±0.04AB	0.40±0.03A	17.3±0.9A	143.8±3.5B	12.9±0.6C	198±8A	4.1±0.6C	22.0±3.0AB	3.35±0.25B	0.061±0.008B	0.19±0.03A	15±2BC	25±3A
FS_2	5.47±0.27ABC	47.5±3.2A	1.83±0.03A	0.26±0.04AB	18.2±0.2A	103.5±3.3C	7.6±0.4D	45±3E	3.5±0.3C	15.5±1.2C	4.07±0.07A	0.064±0.007B	0.07±0.01D	16±2BC	23±3A
RS_D	4.49±0.08D	33.8±0.7D	1.41±0.06B	0.33±0.05AB	16.6±0.6AB	98.3±2.1C	24.3±0.9B	96±6C	9.7±0.4A	16.6±0.5C	2.77±0.14C	0.100±0.020A	0.09±0.01CD	18±2ABC	21±4A
Mean	5.28	41.1	1.63	0.31	16.8	142.3	16.8	105	5.5	19.1	3.16	0.075	0.13	18	23

-Different letters in one column denote significant differences at $P<0.01$, 1-year fallow soil (FS_1), 2-year fallow soil (FS_2), 1-year healthy rhizosphere soil (RS_H1), 2-year healthy rhizospheric soil (RS_H2), 2-year root-rot diseased rhizospheric soil (RS_D), blank control soil (no *A. macrocephala* was planted) (S_CK).