



Characterization of AMF-diversity of endosphere versus rhizosphere of tea (*Camellia sinensis*) crops

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ABSTRACT

Tea [*Camellia sinensis* (L.) Kuntze] crops are globally one of the important commercial crops grown predominantly on acidic soils, a natural ally in form of soil arbuscular mycorrhizal fungi (AMF). The present study was carried out to characterize AMF-diversity in roots and rhizosphere of 20-yr-old *Camellia sinensis* cv. Yichang Dayecha grown in Xingshan, Hubei, China. Small subunit ribosomal RNA (SSU rRNA) was used to identify the diversity. Tea roots were colonized by native AMF species, with $32.71 \pm 3.50\%$ of root mycorrhizal colonization. As many 163 and 177 operational taxonomic units (OTUs) were observed in the clone library of rhizosphere soils and roots respectively, suggesting that roots inhabited more AMF species than rhizosphere soils, with as many 111 OTUs overlapped between them. There were only *Glomus* species and *Claroideoglomus* / *Glomus* predominantly observed in roots and rhizosphere soils, respectively, with *Glomus* Glo20 having highest relative abundance in rhizosphere (>75%) and roots (>25%). Soil and root samples were grouped with the high taxonomic similarity, indicating good group diversity in AMF associated with tea crops.

Key words: *Glomus*, Mycorrhiza, OUT, SSU rRNA, Tea

Tea [*Camellia sinensis* (L.) Kuntze] plants, one of the most commercial crops grown on alfisols and ultisols of China, are beverages (with better use in preparing) due to strong nutraceutical value aiding in health and nutrition (Li *et al.* 2017). Evidences, accrued from different researches had shown that root colonization (6.6%~44.0%) by native arbuscular mycorrhizal fungi (AMF), were highly dependent on the tea clones for optimum economic output (Balasuriya *et al.* 1991). In fact, AMF are a kind of soil beneficial fungi that can associate with roots of land's plants to form arbuscular mycorrhizas (AMs) (Wu *et al.* 2013, Wang *et al.* 2012). AMs have been shown to display multiple beneficial impacts on nutrient and water absorption, plant stress tolerance, changes in soil aggregate size of the physical soil environment, and soil microbiodiversity structurally as well functionally (Wu *et al.* 2013, Srivastava *et al.* 2015, Powell and Rillig 2018). Earlier studies by Singh *et al.* (2008) showed presence of variety of

AMF species within rhizosphere of both natural (*Glomus*, *Acaulospora*, *Gigaspora*, and *Scutellospora*) and cultivated (*Glomus* and *Acaulospora*) tea plants which opened the door for greater in depth studies on AMF associated with tea rhizosphere. Subsequently Karthikeyan *et al.* (2012) reported that inoculation with *Acaulospora scrobiculata*, *Glomus aggregatum*, *G. fasciculatum*, *G. intraradices*, *G. geosporum*, and *Scutellospora calospora* colonized the roots of tea to varying propitious, besides improving plant growth and nutritional status. Later, Shao *et al.* (2018) reported a positive effect on root total length and volume and leaf nutrients concentration, but a negative effect on root-hair length and number in *Camellia sinensis* Fuding Dabaicha. In addition to favorable responses on growth and nutrition, mycorrhizal inoculation significantly increased quality of tea in terms of higher concentration of amino acids, total protein content, total polyphenols, caffeine content, and sugar content (Singh *et al.* 2010).

Tea crops are primarily planted on acidic soils of hilly and mountainous regions with a low pH, high Al concentration, and marginal availability of nutrients, which seriously limit the productivity of tea plants (Singh *et al.* 2010). Under such agro-pedological conditions, role of AMF becomes a natural choice, regardless of crops (Wu *et al.* 2017a) for improving the performance of tea, with soil microorganisms further playing an important role towards soil nutrient cycling vis-a-vis plant health (Yang *et al.* 2017). High-throughput pyrosequencing is considered

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as a good method to study soil microbial diversity, with high quality sequencing of DNA specific sections of soil microbial communities. In this background, we attempted a technique using small subunit ribosomal RNA (SSU rRNA) gene sequences to characterize the community diversity of AMF in roots and associated rhizosphere of tea crops under natural habitat.

MATERIALS AND METHODS

A 20-year-old tea plantation (31°15'N and 111°05'E) with *Camellia sinensis* cv. Yichang Dayecha was used as the experimental plant material. The tea field was located in the Shuiyueshi town, Xingshan, Yichang, Hubei, China, at 1090 m altitude with a subtropical monsoon humid climate, having four distinct seasons (99 kcal/cm² annual total solar radiation, 1682.8 h annual sunshine hour, 15.3°C annual average air temperature, and 900–1200 mm annual precipitation). The physico-chemical properties of the yellow-brown soil consisted of: pH 5.4, organic matter 13.1 g/kg, Olsen-P 27.1 mg/kg, NH₄OAc-K 156.2 mg/kg, and KMnO₄-N 98.3 mg/kg.

In this tea field, we selected 16 tea plants with uniform growth vigor as the test plant materials. Subsequently, rhizosphere soils and fine root samples within the perimeter of tree canopy at 5–15 cm depth were collected. Representative soils and root samples covering four trees per block were collected as a composite sample with each sample replicated four times. Following soil sample collection, the collected soil samples were stored at -80°C after removing root segments and screens.

The 1-cm-long fine root segment was cleaned with 10% (w/v) KOH solution and stained with 0.05% (w/v) trypan blue in lactoglycerol (Phillips and Hayman 1970). The mycorrhizal structure in roots was microscopically observed, and the root mycorrhizal colonization was calculated using the formulae: Root mycorrhizal colonization (%) = Mycorrhizal colonized root length / Total observed root length × 100.

The total genomic DNA from rhizosphere soils and roots was extracted using the DNA purification ELISA kits. The DNA concentration and quality were checked by a NanoDrop Spectrophotometer. Subsequently, the qualified DNA was diluted to 10 ng/μL and stored at -80°C for downstream use.

According to Geel *et al.* (2014), four specific AMF primers were selected: AML1 (F): 5'-ATCAACTTTTCGATGGTAGGATAGA-3'; AML2 (R): 5'-GAACCCAAACACTTTGGTTTCC-3'; AMV4.5NF (F): 5'-AAGCTCGTAGTTGAATTTTCG-3'; AMDGR (R): 5'-CCCAACTATCCCTATTAATCAT-3'. The 18S genes were amplified using the above primer with 12 nt unique barcode. A 25 μL PCR mixture contained 1× PCR buffer, 1.5 mM MgCl₂, 0.4 μM deoxynucleoside triphosphate, 1.0 μM primers, 0.5 U of KOD-Plus-Neo (TOYOBO), and 10 ng template DNA. The PCR amplification program consisted of initial denaturation at 94°C for 1 min, followed by 30 cycles (denaturation at 94°C for 20 s, annealing at 50°C for 30 s, and elongation at 72°C for 30 s), and a final extension

at 72°C for 5 min. Three replicates of PCR reactions for each sample were combined together. The PCR products mixed with 1/6 volume of 6X loading buffer were loaded on 2% agarose gel for detection. Samples with bright main strip between 200–400bp were chosen. The electrophoresis band was purified using the OMEGA Gel Extraction Kit. PCR products from different samples were pooled with equal molar amount.

Sequencing libraries were generated using the TruSeq DNA PCR-Free Sample Prep Kit following manufacturer's recommendations and index codes were added. The library quality was assessed on the Qubit@2.0 Fluorometer (Thermo Scientific) and the Agilent Bioanalyzer 2100 system. At last, the library was applied to the paired-end sequencing (2×250 bp) with the Illumina Hiseq at the Rhonin Biosciences Co., Ltd.

The sequences were analyzed according to the Usearch (<http://drive5.com/uparse/>) and the QIIME pipeline (Caporaso *et al.* 2010). Paired-end reads from the original DNA fragments were merged using the FLASH (Magoč and Salzberg 2011). Whereafter, the sequences were assigned to each sample according to the unique barcode. Later, the sequences were clustered into operational taxonomic units (OTUs) at 97% similarity level as the identity threshold using the UPARSE algorithms (Edgar 2013). Taxonomy was assigned using the MAARJAM database (<http://maarjam.botany.ut.ee/>) and the BLAST method. All data analyses were performed using R6 (<https://www.R-project.org>). Rarefaction curves were generated based on the alpha-diversity metrics. The clustering analysis was carried out with the unweighted/Pair group method using arithmetic mean.

RESULTS AND DISCUSSION

Root mycorrhizal colonization

The extent of mycorrhizal colonization is a pre-requisite to measure the dependence of the crop and its subsequent effect on growth response (Lü *et al.* 2018). The tea plants were colonized by native AMF species, showing the typical arbuscular mycorrhizal structure (Fig 1). Root mycorrhizal colonization in tea plants was 32.71±3.50%. Earlier studies conducted by Balasuriya *et al.* (1991) reported such mycorrhizal colonization in tea crops in Sri Lanka, which represented arbuscular mycorrhizal plants.

AMF-diversity

Based on rRNA genes, OTUs were clustered to evaluate fungal and bacterial species (Wu *et al.* 2017b). In the tea field, we found 163 and 177 OTUs in rhizosphere soils and roots from the SSU rRNA clone library, respectively. Out of them, as many 111 OTUs overlapped considering both rhizosphere soils and roots, suggesting the high similarity of AMF diversity between rhizosphere AMF-diversity and root derived AMF-diversity. On the other hand, a significantly higher OTU number was observed in roots than rhizosphere soils. Wu *et al.* (2017b) earlier reported similar findings in

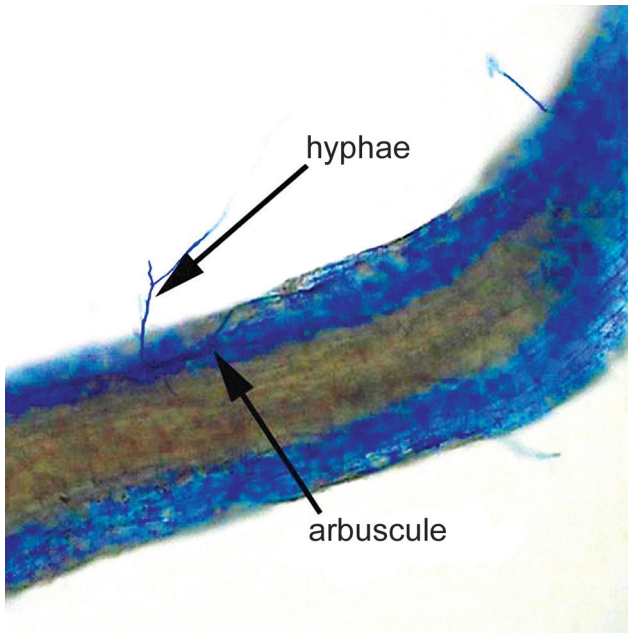


Fig 1 Mycorrhizal structure as seen in the roots of *Camellia sinensis* cv. Yichang Dayecha.

citrus plants which suggested that roots inhabited much higher AMF-diversity than rhizosphere, considering the dependence of soils.

Rarefaction curves were used to compare the species diversity in an ecosystem (Yang *et al.* 2017). The present study showed that the curves approached the asymptote, when the number of the clones rose to 120 (Fig 2). These observations suggested that each sample in rhizosphere soils and roots had highly similar AMF communities at different levels of their organizational structure. At 97% similarity level, as Shannon value, a high alpha diversity in AMF species was shown in roots than rhizosphere soils, implying that roots contained far more number of AMF species than rhizosphere soils. It will be highly interesting to track the source of such diversity within roots than rhizosphere, duly supported by ecological niches of different AMF species.

AMF community at genus and species levels

The AMF community at the genus level (Fig 3) indicated that only *Glomus* was predominantly present in tea roots, while both, *Claroideoglomus* and *Glomus* were observed within tea rhizosphere soils. On the other hand,

Claroideoglomus was observed in the sample of AMF_S1 and AMF_S3, indicating a variant distribution of this genus in tea rhizosphere soils. Such results further implied that in tea, *Glomus* was the most predominant genus amongst different AMF-based communities. Singh *et al.* (2008) earlier reported the *Glomus* as most predominant genus in under natural and cultivated tea crops.

Glomus Glo20 was observed in all the rhizosphere soil and root samples, with the highest relative abundance in rhizosphere soils (>75%) and roots with better (>25%) read abundance (Fig 4). In rhizosphere soils, the top five AMF clones consisted of: *Glomus Glo20*, *Glomus viscosum*, *Glomus Liu2012b_Phylo_17*, *Glomus Wirsel_OTU13*, and *Glomus Glo7* (Fig 5). While, in roots, the top five AMF clones were: *Glomus Glo20*, *Glomus Wirsel_OTU16*, *Glomus Glo7*, *Glomus acnaGlo2*, and *Glomus Glo_C* (Fig 5). The AMF genus, *Glomus Glo20* was relatively more frequent soils than in

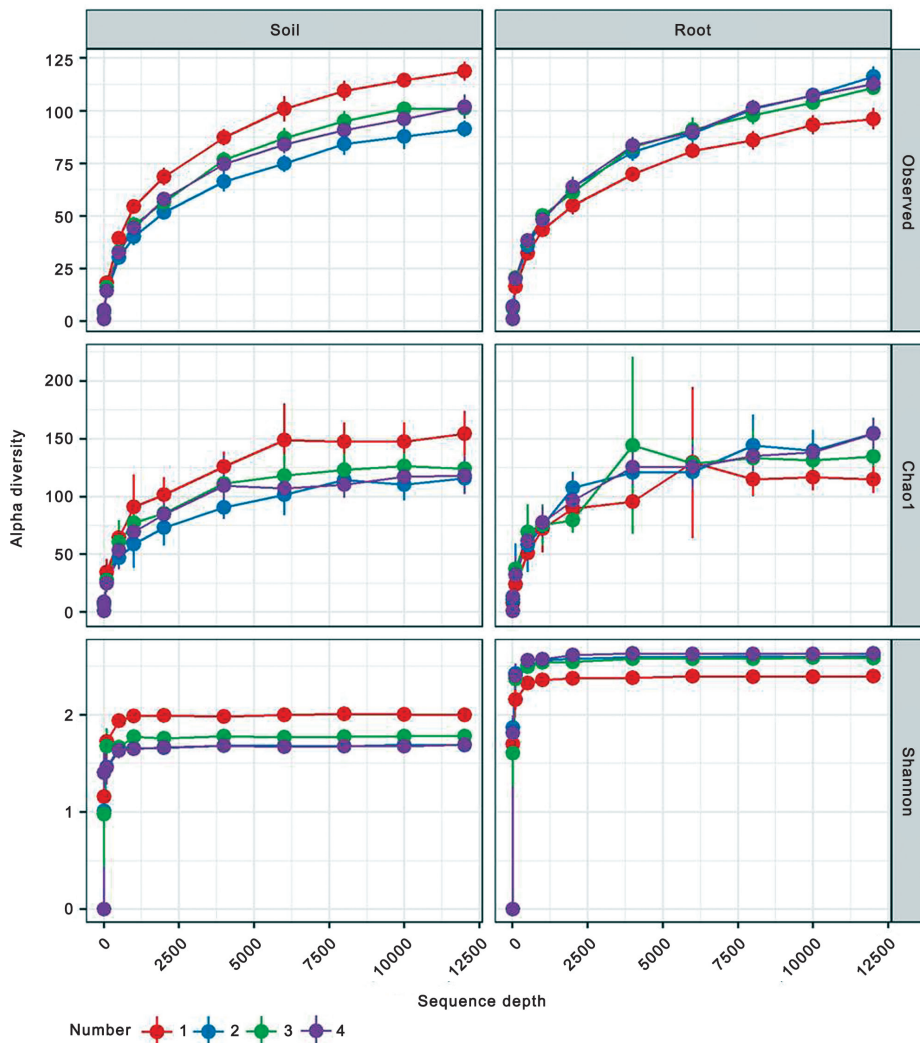


Fig 2 Rarefaction curve of OTUs based on alpha diversity of sequence depth in the root and rhizosphere soil under *Camellia sinensis* cv. Yichang Dayecha.

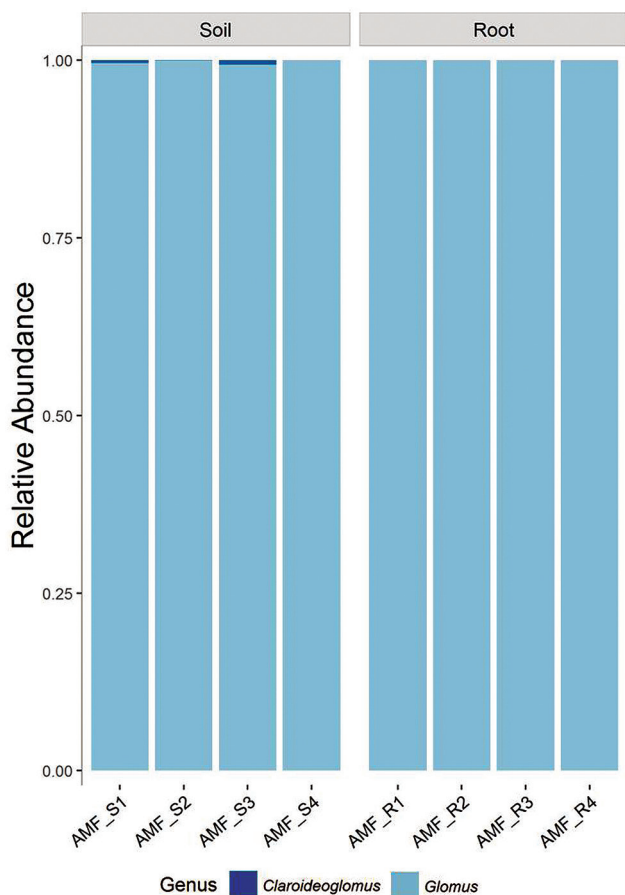


Fig 3 Relative abundance of AMF genus levels in plant and rhizosphere soil under *Camellia sinensis* cv. Yichang Dayecha. AMF_R1 to AMF_R4 s and AMF_S1 to AMF_S4 represent four samples each from plant roots and rhizosphere soil, respectively.

rhizosphere roots, while *Glomus Glo7* was relatively higher in roots than rhizosphere soils. These observations indicated a special inhabited habituation for two contrasting *Glomus* sp. in tea crops, which were earlier observed in citrus rhizosphere as well (Wu *et al.* 2017b).

The clustering analysis further showed that all the four rhizosphere soil samples and four root samples were grouped with the high taxonomic similarity with the database sequences (Fig 6), indicating good group diversity in AMF of tea crops.

In short, tea crops under natural habitat were, hence, colonized by native AMF species, showing a relatively lower rate of colonization in the roots. The SSU rRNA showed 163 and 177 OTUs in rhizosphere soils and roots, respectively. Roots possessed considerably higher OTU number than rhizosphere soils, suggesting that roots inhabited higher AMF species than rhizosphere soils. Such results provide a strong perspective at molecular levels to utilize AMF-diversity of tea mycorrhizosphere in developing an AMF-consortium.

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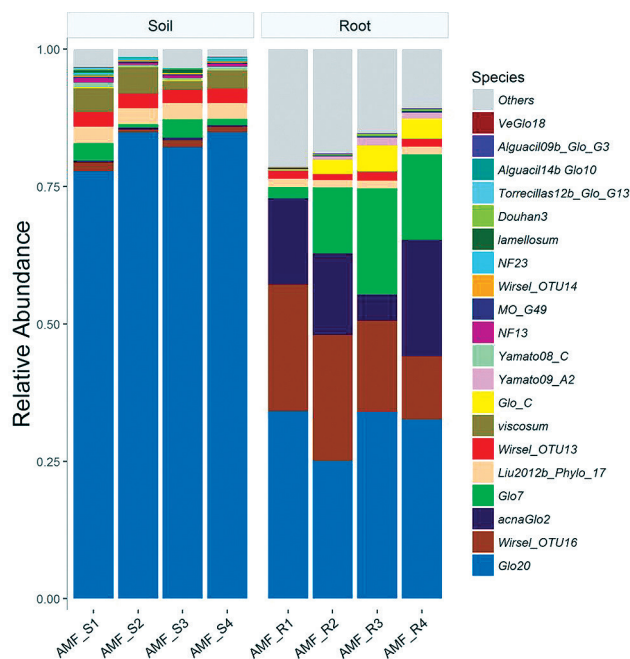


Fig 4 Relative abundance of AMF species levels in plant and rhizosphere soil under *Camellia sinensis* cv. Yichang Dayecha. AMF_R1 to AMF_R4 and AMF_S1 to AMF_S4 represent four samples each from plant roots and rhizosphere soil, respectively.

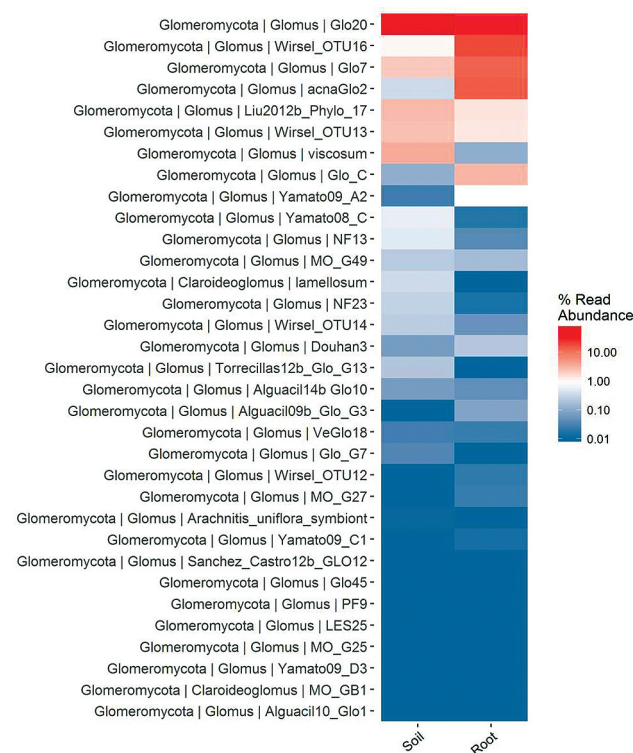


Fig 5 Read abundance of the top 50 species levels of AMF clones in the root and rhizosphere soil under *Camellia sinensis* cv. Yichang Dayecha.

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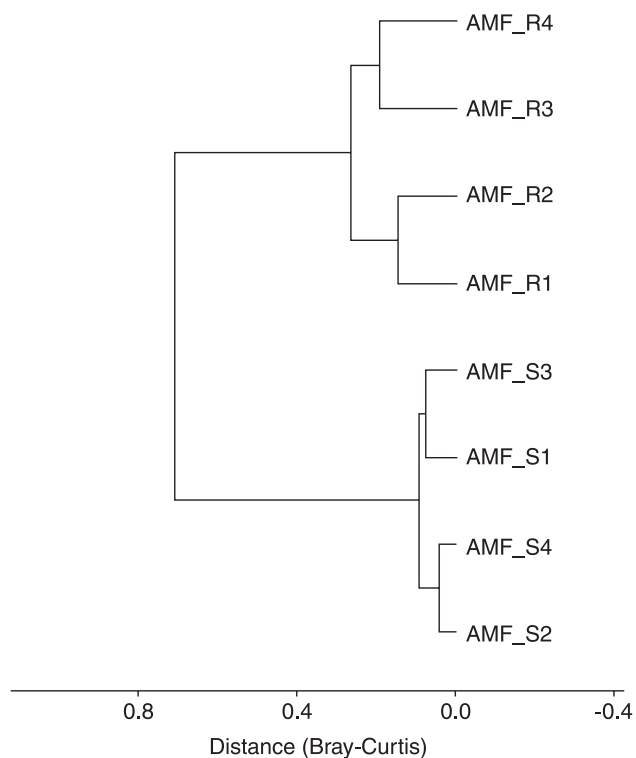


Fig 6 Neighbor-joining tree in terms of SSU rRNA sequences in the root and rhizosphere soil under *Camellia sinensis* cv. Yichang Dayecha. AMF_R1 to AMF_R4 and AMF_S1 to AMF_S4 represent four samples each from plant roots and rhizosphere soil, respectively.

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