Arbuscular Mycorrhiza Improves Leaf Food Quality of Tea Plants

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Abstract

Tea (Camellia sinensis) plants inhabit arbuscular mycorrhizal fungi (AMF) in rhizosphere, whereas it is not clear whether AMF improves leaf food quality of tea plants. A potted study was conducted to determine effects of Claroideoglomus etunicatum, Diversispora spura, and a mixture of the three AMF species on leaf sugar, amino acid, soluble protein, tea polyphenol, catechuic acid, and flavonoid contents of Camellia sinensis ‘Fuding Dabaicha’ seedlings. After 12 weeks of AMF inoculation, mycorrhizal plants recorded significantly higher shoot biomass and total leaf area, whilst the effect was ranked as C. etunicatum > D. spura > mixed-AMF > D. versiformis in the decreasing order. AMF treatments significantly increased leaf total amino acid concentrations, accompanied with up-regulation of amino acid synthetic enzymes genes glutamine synthetase (CsGS), glutamate synthase (CsGOGAT) and glutamate dehydrogenase (CsGDH). Leaf glucose, sucrose, total soluble protein, tea polyphenol, catechuic acid, and flavonoid contents were significantly higher in AMF- than in non-AMF-inoculated plants. In addition, mycorrhizal inoculation notably up-regulated the expression level of leaf 3-hydroxy-3-methylglutaryl coenzyme gene (CsHMGR), ascorbate peroxidase gene (CsAPX), and tea caffeine synthase 1 gene (CsTCSI). These results implied that AMF inoculation had positive effects on leaf food quality partly by means of up-regulation of relevant gene expression in ‘Fuding Dabaicha’ seedlings.

Keywords: soil microorganism; sucrose; symbiotic fungi; tea polyphenol; white tea

Introduction

Tea (Camellia sinensis (L.) O. Kuntze) is an important commercial crop consumed worldwide, primarily as a beverage made from the processed leaves. Tea tree growth is often affected by soil nutrient levels, temperature, pruning, and soil microbes including arbuscular mycorrhizal fungi (AMF) (Burgess and Carr, 1996; Van Lelyveld et al., 1990; Sharma and Kayang, 2017). AMF effects on plant growth have been reported in many plants, including tea plants (Alisaghazrad et al., 2011; Shao et al., 2018). Singh et al. (2008) observed AMF inhabited in tea plant rhizosphere, dominated by Acaulospora, Gigaspora, Glomus and Scutellospora. As reported by Kahneh et al. (2006), inoculation with Glomus etunicatum, G. intraradices and G. versiforme significantly increased plant growth and leaf nutrient levels in tea plants. It seems that AMF has potential capacity to improve plant growth in tea plants.

In addition to growth and nutrients, AMF inoculation also improves food quality of crops (Lingua et al., 2013). As reported by Lingua et al. (2013), AMF inoculation with Glomus sp. markedly increased the concentration of cyaniding 3-glucoside, pelargonidin 3-glucoside, and pelargonidin malonyl glucoside in strawberry fruits. Inoculation with G. mossea in tomato plants had a significantly positive effect on the number of fruits, in company with the increase in amino acid abundance in the fruit, with glutamine and asparagines as the most responsive amino acids (Salvioli et al., 2012). In chili ancho, AMF treatment strongly increased carotene concentrations and xanthophylls concentrations in fruits (Mena-Violante et al., 2006). Until now, the information regarding AMF effect on food quality of tea is relatively scarce.

Food quality of tea is closely associated with relevant enzymes, including glutamine synthetase (GS), glutamate synthase (GOGAT) and glutamate dehydrogenase (GDH),
who take part in amino acid biosynthesis pathway (Lin et al., 2012). The GS, GOGAT, and GDH expression is important in tea quality. Previous studies also reported that AMF could regulate the expression of host plant genes (Cicatelli et al., 2012; Vangeli et al., 2018). Chen et al. (2013) reported that inoculation with Funneliformis mossea enhanced the expression of stress-related marker genes in cucumbers. Earlier studies reported the stimulated effects of AMF on Pi transporter expression in maize (Hui et al., 2013), root tonoplast intrinsic protein gene expression in drought-stressed trifoliate orange (He et al., 2019), auxin-related gene expression in P-stressed trifoliate orange (Liu et al., 2018), and arsenite transporter expression in rice (Chen et al., 2012). However, it is not clear whether inoculation with AMF regulates the expression levels of relative genes in tea plants involved in antioxidant protected systems (mainly ascorbate peroxidase biosynthesis), and tea quality (mainly amino acids, tea caffeine, and terpene metabolisms).

Since many studies showed a positive effect of AMF on food quality of crop plants, we here hypothesize that AMF colonization could improve food quality of tea plants through up-regulation of some potential gene expression. To confirm the hypothesis, *C. sinensis* ‘Fuding Dabaicha’ was selected and inoculated with AMF, and leaf food quality and relevant gene expression were analyzed.

Materials and Methods

**Experimental design**

The experiment was arranged in an absolutely randomized blocked design with five AMF treatments: (1) inoculation with Claroideoglomus etunicatum (W.N. Becker & Gerd.) C. Walker & A. Schüßler, (2) inoculation with Diversispora spurca (C.M. Pfeiff., C. Walker & Bloss) C. Walker & A. Schüßler, (3) inoculation with D. versiformis (P. Karst.) Oehl, G.A. Silva & Sieverd, (4) mixed inoculation with *C. etunicatum*, *D. spurca* and *D. versiformis* (the ratio is 1:1:1), and (5) inoculation with non-AMF (non-AMF). Four replicates were conducted in each treatment and each replicate had one seedling, totaling twenty pots in this experiment.

**Experimental set-up**

Seeds of tea (*C. sinensis* ‘Fuding Dabaicha’) plants were sterilized with 75% of alcohol solutions for 10 min, rinsed with distilled water, and sown into autoclaved (121 °C, 0.11 MPa, 1 h) sands for germination under 28/20 day/night temperature conditions. After approximately one month, a two-leaf-old seedling was transplanted into a pot (18 cm upper diameter × 11 cm height × 15 cm bottom diameter), where 230 g of autoclaved (121 °C, 0.11 MPa, 2 h) soils was supplied. The potted soil was collected from the 20-yr-old tea plantation (31°15′N and 111°05′E) with Yichang Dayecha cultivar, which was located in the Shuiyueshi town, Xingshan, Yichang, China.

The AMF species used here included Claroideoglomus etunicatum, Diversispora spurca, and *D. versiformis*. A mixture of the above three AMF species was also considered as a mycorrhizal treatment. Non-AM fungal treatment received same accounts of autoclaved (121 °C, 0.11 MPa, 1 h) inoculums plus 3 mL filtrate of inoculum to keep similar microbial communities, except for these AMF strains. The monospecific spores of these AMF species were propagated on white clover in the disinfested mixture of soils and sands using pot culture for 12 weeks. Mycorrhizal inoculums included spores, infected root segments, and growth substrates. For AMF inoculations, 1200 spores of mycorrhizal inoculum in each AM fungal species were applied into rhizosphere of a tea seedling in the pot.

The AMF- and non-AMF-colonized plants were grown in a greenhouse of Yangtze University campus from April 16 to July 9, 2017, where photon flux density was 948 μmol/m², with 28/23 °C average day/night temperature and 82% relative air humidity.

**Measurements of root colonization and growth performance**

At harvesting, total leaves were scanned with an EPSON Flat-Scanner (V700, Seiko Epson Corp, Japan) and analyzed with the WINRHIZO 2007d (Regent Instruments Inc., Quebec, Canada) for surface area. Subsequently, the seedlings were divided into shoot and root, and shoot biomass was measured.

Root mycorrhizal colonization was measured based on the method of Phillips and Hayman (1970). Approximately 1-2 cm root segments of each seedling were collected, stained by typan blue, and microscopically observed. The mycorrhizal colonization was calculated by the following formula: AMF colonization (%) = 100 × root length infected by AMF / total root length observed.

**Determinations of leaf food quality parameters**

Sucrose, glucose and fructose concentrations in leaves were determined as described by Wu et al. (2010). Total soluble protein concentrations in leaves were evaluated by the method of Bradford (1976) using bovine serum albumin as the standard. Flavonoid concentrations were determined by Cheng et al. (2004). Total amino acid concentrations in leaves were measured by the ninhydrin method outlined by Tchameni et al. (2012). Tea polyphenol concentrations in leaves were assayed by de La Rosa et al. (2011). Catechic acid concentrations in leaves were determined by the Vanillin method using the catechic acid as the standard (Zhao, 2010).

**Gene expression analysis**

The relative expression of *CsGDH*, *CsGS*, *CsGOGAT*, 3-hydroxy-3-methylglutaryl coenzyme (HMGCR), ascorbate peroxidase (*CsAPX*), and tea caffeine synthase 1 (*CsCYS1*) in leaves was analyzed by real-time quantitative PCR (qRT-PCR). Total RNA was extracted from the leaves of these plants, respectively, using an EASY spin Plus plant RNA kit (RN 38, Aidlab Biotecnologías CO. Ltd., China). RNA samples were reverse-transcribed using the PrimeScript™ RT reagent kit with gDNA eraser (PK02006, Takara Bio. Inc, Japan), qRT-PCR were run on a CFX96 Real Time PCR Detection System (BIO-RAD, USA). These primers for selected genes were designed based on the Genbank (http://www.ncbi.nlm.nih.gov/genbank/) and shown in Table 1. qRT-PCR reactions were carried out in the following compositions: 3.5 μL sterile water, 0.5 μL
cDNA, 5 μL SYBR GREEN PCR Master Mix (Applied Biosystem), 0.5 μL forward prime, and 0.5 μL reverse prime. qRT-PCR determinations were performed on three independent biological samples with three technical replications for each sample were examined. Quantification of the gene expression was done with the 2-ΔΔC_t method (Livak and Schmitgen, 2001) in which the housekeeping gene (GADPH) acted as the control. The measured transcripts were normalized to the relative expression value in non-AM plants.

Statistical analysis
All the data were analyzed with one way ANOVA (SAS 8.1v, SAS Institute, Inc., 2001) to determine significances, and the Duncan’s Multiple Range Test at the 5% level was used to compare significant differences between treatments. The Pearson’s correlation coefficients were done using the Proc Corr’s procedure (SAS 8.1v).

Results
Root mycorrhizal colonization and leaf growth
The mycorrhizal colonization was not found in non-AMF treatment, but in AMF-inoculated seedlings. The significantly higher root colonization among treatments was ranked as C. etunicatum > mixed-AMF > D. spurca > D. versiformis at the decreasing order, indicating a favor of ‘Fuding Dabaicha’ colonization by colonization of C. etunicatum.

In this work, compared to the non-AMF-inoculation seedling, total leaf area and shoot biomass were significantly increased by 60.0% and 107.0% after inoculated with C. etunicatum, by 52.8% and 81.9% with D. spurca, by 21.7% and 68.4% with mixed-AMF, and by 8.0% and 28.7% with D. versiformis, respectively (Table 2).

Changes in leaf food quality
Leaf sucrose, fructose, and glucose contents were considerably higher in AM than in non-AM seedlings, regardless of AMF treatments, except for fructose contents between D. versiformis and non-AMF treatments (Table 3). Compared with non-AM plants, AM plants exhibited 129.7-625.0% significantly higher leaf sucrose contents, 39.8-590.9% higher leaf fructose contents, and 66.0-117.4% higher leaf glucose contents, respectively.

AMF inoculation conferred significantly positive effects on total amino acid, total soluble protein, tea polyphenol, catechic acid, and flavonoid contents in ‘Fuding Dabaicha’ (Table 3). Compared to non-AMF plants, inoculation with C. etunicatum, D. spurca, D. versiformis and mixed-AMF significantly improved leaf tea polyphenol (69.0-168.5%), total amino acid (0.6-52.5%), catechic acid (178.1-1127.4%), flavonoid (130.2-262.7%) contents of tea plants.

Mycorrhizal tea plants showed 0.6-52.5% higher leaf total amino acid contents and 73.7-199.0% higher total soluble protein contents, compared with non-mycorrhizal plants. It suggests that mycorrhizal symbiosis can stimulate amino acid and soluble protein synthesis in host plants.

Relative expression of leaf quality genes
Compared to the non-AMF seedling, the relative expression of leaf CgGDH, CgGS and CgGOGAT was significantly up-regulated by 1.73, 1.77 and 1.75 times after inoculated with C. etunicatum, by 2.58, 1.82 and 3.63 times with D. spurca, and by 1.27, 1.32 and 1.34 times with mixed-AMF, respectively (Fig. 1).

Mycorrhizal inoculation is notably up-regulated the relative expression of leaf CgHMGR, CgAPX, and CgTCSI (Fig 2a-c). Compared to the non-AMF-inoculated seedling, the relative expression of leaf CgHMGR, CgAPX and CgTCSI was significantly up-regulated by 2.01, 2.01 and 1.89 times after inoculated with C. etunicatum, by 1.52, 1.79 and 1.32 times with D. spurca, and by 1.21, 1.42 and 1.22 times with mixed-AMF, respectively.

Table 1. The specific primers of relevant genes designed for real time quantitative PCR amplification

| Gene name | Accession | Sequence (5'-3') - forward | Sequence (5'-3') - reverse |
|-----------|-----------|-----------------------------|-----------------------------|
| GADPH     | XM_002263109 | TTTGCATCTTGAGGCTTCT | CAGTGGGAAACACGGAAAAC |
| CgHMGR    | K946250   | CTCTTCCTCCTCCTTCCTTCCT | CTTTGGCCTTGTTGATAGT |
| CgAPX     | EU547804  | TTCTATACCTGGGCTGGATGG | AATGGTCACATCCCCTATG |
| CgGDH     | JN602371  | AAGCCTGCAAATCATCCTACTG  | TCCTGTCCCATAGAACCCTTG |
| CgGS      | JN602372  | CCTCAGAAGGAAAGGCTGGAGA | AACATGCGTTGGGCTGAAATC |
| CgGOGAT   | JN602373  | TGCTCAGGAGGCTTCTG  | CATGATGCGAGGCTTGAGGATA |
| CgTCSI    | AB031280  | TTCCGGAGTATGTGATGGGATTA | TGAGTCCTTTGGTGCCCTGA |

Table 2. Effects of AMF inoculation on growth of Camellia sinensis ‘Fuding Dabaicha’ seedlings

| Treatments | Root mycorrhizal colonization (%) | Shoot biomass (g FW/plant) | Total leaf area (cm²) |
|-----------|----------------------------------|---------------------------|-----------------------|
| C. etunicatum | 40.2±1.65a                      | 3.5±0.13a                 | 23.9±1.17a             |
| D. spurca  | 24.9±1.55c                      | 3.1±0.05b                 | 22.8±1.29a             |
| D. versiformis | 15.1±1.06d                   | 2.2±0.11d                 | 16.8±1.23c             |
| Mixed-AMF | 32.7±1.18b                      | 2.8±0.04c                 | 18.2±1.01b             |
| Non-AMF   | 0±0c                            | 1.7±0.09c                 | 14.9±1.17c             |

Note: Data (means ± SD, n = 4) followed by different letters in same column are significantly different at P<0.05.
The variation in mycorrhizal colonization of tea plants was observed in different AMF treatments, which is perhaps due to the AMF specificity and the compatibility between AMF and host plants (van der Heijden et al., 1998). Meanwhile, mixed AMF was less effective than C. etunicatum inoculation alone, indicating competing of different strains about nutrient.

Compared to the non-AMF-inoculation seedling, total leaf area and shoot biomass were significantly increased in AMF-inoculation seedling. The result was in line with the earlier report by Fajardo et al. (2014), who observed that inoculation with Dentiscutata heterogama and Rhizophagus manihotis improved the shoot biomass and total leaf area of Camellia sinensis 'Fuding Dabaicha' seedlings.

Discussion

The variation in mycorrhizal colonization of tea plants was observed in different AMF treatments, which is perhaps due to the AMF specificity and the compatibility between AMF and host plants (van der Heijden et al., 1998). Meanwhile, mixed AMF was less effective than C. etunicatum inoculation alone, indicating competing of different strains about nutrient.

Table 3. Effects of AMF-inoculation on leaf quality parameters content (mg/plant DW) of Camellia sinensis 'Fuding Dabaicha' seedlings

| Treatments     | Sucrose  | Fructose | Glucose  | Tea polyphenol | Total amino acid | Catechuic acid | Flavonoid | Total soluble protein |
|----------------|----------|----------|----------|----------------|------------------|----------------|-----------|----------------------|
| C. etunicatum  | 369.0±4.8a | 162.8±5.2a | 179.8±13.3a | 8081±117a       | 1899.0±63.5b     | 644±31.1a     | 23.76±0.66a | 57.2±3±4.91a         |
| D. spurca      | 238.8±7.6c | 94.0±5.1c  | 174.1±13.3a | 730±166b        | 1986.2±41.1a     | 276.9±22.6c   | 15.08±0.65d | 62.8±6.12a           |
| D. versiformis | 116.9±4.5d | 33.0±1.3d  | 137.3±3.2b  | 5088±261d       | 1310.8±41.6d     | 146.0±10.2d   | 19.88±0.26c | 36.50±3.42b          |
| Mixed-AMF      | 258.0±6.4b | 111.0±12.7b| 184.4±7.1a  | 7037±215c       | 1696.0±27.6c     | 475.5±26.0b   | 21.85±0.36b | 42.0±2.13b           |
| Non-AMF        | 50.9±1.6c  | 23.6±0.4d  | 82.7±5.8c  | 3010±41e         | 1302.7±46.8d     | 52.5±4.2e     | 6.55±0.20c | 21.0±2.61c           |

Note: Data (means ± SD, n = 4) followed by different letters in same column are significantly different at P < 0.05.

Table 4. Correlation coefficients between leaf food quality parameter contents and root mycorrhizal colonization in Camellia sinensis 'Fuding Dabaicha' seedlings

| Mycorrhizal colonization (%) | Tea polyphenol | Total amino acid | Catechuic acid | Flavonoid | Total soluble protein | Sucrose | Fructose | Glucose |
|-----------------------------|----------------|-----------------|----------------|----------|----------------------|---------|----------|---------|
|                             | 0.96**         | 0.79**          | 0.95**         | 0.87**   | 0.77**               |         |          | 0.94**  | 0.93**  |

Note: ** P < 0.01.

Fig. 1. Effects of AMF inoculation on relative expression of leaf CsGDH, CsGS, and CsGOGAT in Camellia sinensis 'Fuding Dabaicha' seedlings. Data (means ± SD, n = 3) are significantly different (P < 0.05) followed by different letters above the bars.

Fig. 2. Effects of AMF inoculation on relative expression of leaf CsHMGR (a) , CsAPX (b) and CsTCS1 (c) in Camellia sinensis 'Fuding Dabaicha' seedlings. Data (means ± SD, n = 3) are significantly different (P < 0.05) followed by different letters above the bars.
The shoot biomass and total leaf area in AM plants is often related with the improvement of nutrient acquisitions by extraradical hyphae (Camenzind and Rillig, 2013; Zou et al., 2015). Interestingly, there was an opposite result regarding root mycorrhizal colonization and plant biomass and total leaf area with mixed-AMF treatment versus D. spurca, indicating that the effect of single AMF was disturbed by other AMF species.

The change of leaf sugar (i.e., sucrose, fructose and glucose) is of great importance for tea plant growth and food quality (Ravnskov et al., 2003; Hartmann and Trumbole, 2016). In this work, mycorrhization plant had higher leaf sucrose, fructose, and glucose contents. A similar result was reported by Wu et al. (2015) in trifoliate orange for sucrose and glucose contents. Moreover, mycorrhizal colonization of tea plant roots was significantly positively correlated with leaf sucrose, fructose, and glucose contents. Such sugar increase under mycorrhization might be due to both improved plant nutrition and mycorrhiza-requested carbon (Cely et al., 2016).

Food quality of tea is associated with some biochemical substances imparting liquor, flavor, and aroma characteristic (Singh et al., 2010). In this work, inoculation AMF significantly improved the content of tea polyphenols, catechic acid, flavonoid, amino acids, and soluble proteins. Tea polyphenols, catechic acid, and flavonoid have potent antioxidant activities, which play protective roles against many diseases (Kerio et al., 2013). Tea polyphenol is the main secondary metabolism in tea, among which catechin accounts for about 70% of the total tea polyphenol (Xia et al., 2009). Zhao et al. (2014) also discovered that inoculation with G. mosseae resulted in an increase in the total polyphenol of Camellia sinensis. In our work, AMF inoculation significantly increased the content of catechic acid regardless of the application of AMF species. The flavonoid has been suggested as AM signaling compounds, but also is induced by AM symbiosis (Larose et al., 2002). In a study by Zubek et al. (2015), inoculation with Rhizophagus irregularis, Funneliformis mosseae and an inoculum composed of both isolates increased root flavonoid contents of Viola tricolor. It concludes that AMF heavily accelerate the accumulation of secondary metabolites in tea plants. The relevant mechanisms are still studied in future works by means of metabolomics.

Amino acids and soluble proteins are important factors in tea food quality. Tchameni et al. (2012) inoculated Gignaspora margarita and G. mosseae on cocoa (Theobroma cacao) plants and showed that the amino acids of leaves were significantly increased in mycorrhizal plants. Tomato roots inoculated with G. intraradices had higher contents of total amino acid in root exudates (Zubek et al., 2015). Inoculation with a mixture of Rhizogomus intraradices and Funneliformis mosseae increased leaf soluble protein contents in lettuce (Baslam et al., 2013; Sanmartin et al., 2014). Moreover, root mycorrhizal colonization was significantly positively correlated with tea polyphenol, total amino acid, catechic acid and total solution protein. In fact, the germinating spores of AMF can use nitrogen sources for the de novo synthesis of amino acids (Gachomo et al., 2009), which could increase protein synthesis.

GS, GOGAT, and GDH expression is related with amino acid concentrations (Lin et al., 2012). In the present study, mycorrhizal inoculation notably up-regulated the expression level of leaf CsGDH, CsGS and CsGOGAT. This is not in line with the result reported by Jacob et al. (2014). Meanwhile, Lin et al. (2012) reported that the expression of GS was negatively related to the contents of theanine, lysine and lactamine, the expression of GOGAT was negatively related to the contents of theanine, and the expression of GDH was positively related to the contents of theanine in tea plants. Therefore, the research on change of amino acid components needs further experiments. However, higher expression of CsGDH, CsGS and CsGOGAT potentially accelerated amino acid accumulation of tea plants, as reported in this study. Therefore, it concludes that AMF upregulated CsGDH, CsGS and CsGOGAT expression levels, to regulate amino acid biosynthesis.

HMGR is the first rate-limiting enzyme in the mevalonate pathway (MVA) of plants, which has an important impact on the metabolism of plant terpenes (Li et al., 2014). TCS1 is the main enzyme in caffeine biosynthesis of tea plants, which catalyzed the N-3 and N-1 to form methylation (Mizuno et al., 2003; Jin et al., 2016). In the present study, mycorrhizal inoculation significantly up-regulated the relative expression of leaf CsHMGR, and GSTCS1. It concludes that AM symbiosis upregulated CsHMGR and GSTCS1 to accelerate terpene and caffeine accumulation, although terpene and caffeine were not detected in this study.

APX is the main enzyme to effectively remove reactive oxygen species in plants, which improves plant resistance (Sarowar et al., 2005). Under adversity stress, plants usually upregulate the expression levels of one or more antioxidant enzyme genes, to enhance plant resistance (Hu et al., 2012; Zhang et al., 2017). The changes in leaf CsAPX expression of tea plants under mycorrhization are in line with the result reported by Liu et al. (2011) in Cucumis sativus plants colonized by F. mosseae under low temperature conditions. Therefore, it could be speculated that AMF induces the expression of CsAPX gene to regulate the adverse resistance of plants.

Conclusions

Claroideoglomus etunicatum, Diversispora sparc, D. versiformis and a mixture of the three AMF could positively improve leaf food quality (sugars, total amino acid, total soluble protein, tea polyphenol, catechic acid, and flavonoid) by means of upregulating relative expression of CsGDH, CsGS, CsGOGAT, CsHMGR, CsGOGAT and CsAPX in the ‘Fuding Dabaicha’ seedlings. It supported the above hypothesis, namely, AMF colonization could improve food quality of tea plants through up-regulation of some potential gene expression. It seems to apply AMF as a biological agent to increase food quality of tea plants.

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