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Growth and quality parameters of tea (*Camellia sinensis*) mediated by arbuscular mycorrhizal fungi

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Abstract

Arbuscular mycorrhizal fungi (AMF) inoculation not only increases the growth but also improves the quality of many commercial plants. Tea (*Camellia sinensis*) plants were grown on different growth medium (with and without AMF inoculation) and the chemical properties of the leaves were assayed and compared. The growth media were sterilized soil with AMF, sterilized soil, natural soil inoculated with AMF, natural soil, and natural soil in natural condition with AMF. The highest root colonization (23 %) was found in tea plants grown on natural soil with AMF, whereas no colonization was found in the sterilized soil treatment. The highest level of leaf chlorophyll-a (2.74±0.06 μg.mL^{-1}), chlorophyll-b (1.77±0.03 μg.mL^{-1}) and carotenoid (0.35±0.01 μg.mL^{-1}) contents were found in tea plants grown on natural soil under natural condition with AMF. The highest polyphenol concentration (64.46 mg.L^{-1}) was found in natural soil inoculated with AMF whereas the lowest (38.09 mg.L^{-1}) was recorded in sterilized soil. The highest contents of tannin (30.34 mg.mL^{-1}) and reducing sugar (46.61 mg.L^{-1}) were recorded in plants grown on natural soil under natural condition with AMF and the lowest values (21.22 mg.mL^{-1}, 33.16 mg.L^{-1}, respectively) in sterilized soil treatment. Though antioxidant properties (% scavenging effect) did not differed due to treatments, the highest IAA (Indole-3-acetic acid) concentration (3.16 μg.mL^{-1}) was recorded in tea plants grown on natural soil under natural condition with AMF. The study concludes that AMF inoculation improves the quality of tea leaves.

Introduction

Tea (*Camellia sinensis*) plants grow well on acidic soils. These soils show low values of pH, high Al concentration, and negligible availability of nutrients. Low quantity of P is one of the major limiting factors for the productivity of tea plants due to low natural content and high P fixation capacity of acidic soil. Plants grown on acidic soils often encounter relatively severe mineral stress; such effects include toxic (Al and Mn) to deficiency symptoms (Singh *et al.* 2010). Excess Al is especially damaging to root growth and overall development for plants growing in acidic soils (Miyasaka *et al.* 1991). AMF-root symbiosis may help alleviate some of the problems

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that the plants encounter when grown in acidic soils (Li *et al.* 1991) including Al and/or Mn toxicity.

Mycorrhizal fungi functions in promoting plant growth across floodplain chronosequences were described by Sarkar *et al.* (2015b). But their functions in promoting plant growth are unknown across hilly region having low nutrients content. AMF can provide nutrient to plants in those regions. Response to mycorrhizal inoculation is linked with the level of soil fertility and it is well know that P is the most influential element in AMF development and efficiency (Sarkar *et al.* 2016). In P-deficient soils, the yields of agricultural and field crops under field and greenhouse conditions were observed to be highly dependent on their mycorrhizal state (Sarkar *et al.* 2015a). There is a diversification of arbuscular mycorrhizal fungi in tea plants in Uttarakhand state, India (Gupta and Sharma 2010). We found seasonal colonization of arbuscular mycorrhiza fungi in the roots of *Camellia sinensis* (tea) in different tea gardens of India (Sharma *et al.* 2013).

AMF increases supply of plant nutrients by obtaining sources of nutrients which would usually not be accessible to plants (Tarafdar and Marschner 1994). Some ericoid fungi have the ability to break down phenolic compounds in soils that can interfere with absorption of nutrients (Bending and Read 1997). Mycorrhizas can cause changes in root architecture, vascular tissue, etc. to develop in size (Hetrick *et al.* 1994). Such alliances between fungus-plants boost plant growth and enhance root production. Arbuscular mycorrhiza (AM) plays a vital role on enhancing the plant growth and yield by increasing the supply of phosphorus to the host plant. The mycorrhizal beneficiaries can include increased yield, accumulation of nutrients and/or reproductive success (Lewis and Koide 1990). AMF have a great effect on growth and nutrients assimilation of coarse and low nutrient soil (Sarkar *et al.* 2015a). Fungal hyphae can play a significant role in nutrient cycling through the acquisition of nutrients from saprophytic fungi (Lindahl *et al.* 2007). Microbial action in the rhizosphere is a main consideration that decides the accessibility of supplements to plants and has an essential impact on plant wellbeing and efficiency.

The impact of AMF inoculation on the growth and development of many crops and forest species are well documented. However, reports on the effects of AMF on growth and quality of tea are very scanty. The goal of this study was to evaluate the role of arbuscular mycorrhizal fungi in the growth and quality of tea.

Experimental

Soil and plant propagate collection

Soil sample was collected from 0.61 m depth from the top of a tillah (small mountain) located at the Shahjalal University of Science and Technology (SUST) campus (24° 55’ 31.89”N and 91° 50’ 12.34”E) of Bangladesh. Plant propagates of tea variety (BT2) was collected from Malnicherra Tea Estate, Sylhet. Commercial bio-fertilized having spore of arbuscular mycorrhizal fungus named ‘Serakinkon’ was bought from Central Glass Company, Tokyo, Japan. Soils collected from different points on tillah were composited to make a homogenous soil pool and the required amount of soil form the composite bulk was used for the experiment. The properties of the collected soils were homogenous and there were no significant differences between the individual soil samples and the composite soil in terms of soil particle size small stone like, pH, percentage of organic-matter content, total carbon (TC), total nitrogen (TN), total phosphorus (TP), available P concentrations, sulphur (S), potassium (K). The soil used for experiment had an average pH of 4.09 (highly acidic), organic-matter content 0.67 % (very low), available P 2.93 $\mu g.g^{-1}$ soil, TN 0.039 % (very low), and sulphur 12.83 $\mu g.g^{-1}$. Soil that was used had low nutrient compare to optimum nutritional level having very low N, P, K, S and organic matter (OM) contents, and highly acidic.

Experimental procedure and design

The experiment was conducted in the form of pot culture in the experimental tea garden at Shahjalal University of Science and Technology (SUST) campus. The experiment consisted of 5 treatments viz., (i) sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; (ii) sterilized soil (SS) in pot condition; (iii) natural soil inoculated with

mycorrhiza (NS+AM) in pot condition; (iv) natural soil (NS) in pot condition; and (v) natural soil cultivated in ground soil inoculated with mycorrhiza (NSS+AM).

The pots were placed in experimental tea garden and each treatment had three replications (repeated in space). Each pot had diameter of 3.66 m and contained 6 kg of soil. For sterilizing, soil was autoclaved twice at 121 °C for 30 min to eliminate any indigenous AMF. We used a commercial inoculant, Serakinkon powder, which consists of 50 *Gigaspora margarita* Becker & Hall spores per gram powder to inoculate the soils of the respective treatments (Higo *et al.* 2010). Twenty grams of serakinkon powder/kg soil was evenly mixed with soil before using in a pot. Pots were watered regularly by tap water. After 105 day's tea leaf are harvested.

Determination AMF colonization in roots

Roots were collected from plants and washing was done by tap water. The roots were cut into 1 cm in size and boiled in 2.5 % (w/v) potassium hydroxide at 90 °C for 30 min followed by thoroughly washing with tap water. The root samples were acidified with 1 % (v/v) HCl for 24 h and then bleached in 10 % (v/v) hydrogen peroxide for 60 min. The bleached roots were boiled in 0.5 % (w/v) aniline blue at 90 °C for 30 min. Finally, the roots were de-stained in glycerol and observed under microscope. The total number of colonization counted were divided by the number of view fields investigated (Newman *et al.* 1981; Plenchette *et al.* 1983; Dodd and Jeffries 1986).

Estimation of chlorophyll and carotenoids

Chlorophyll-*a*, Chlorophyll-*b* and carotenoids contents of tea leaves were calculated using the method stated by Wellburn (1994). Tea leaves were weighed and grounded with a mortar and paste with a sufficient quantity of prechilled methanol (100 %). Finely ground sample was filtered and made up to 50 mL in a standard volumetric measuring flask using methanol and further diluted five times with methanol. Absorbance of the diluted methanolic extract was read at 470, 653 and 666 nm using a UV-Visible spectrophotometer (Model-T60U, PG instruments limited, UK) against methanol as blank.

Determination of IAA

Leaves and roots of were cut off (1 g) using a scalpel, frozen immediately in a refrigerator and lyophilized. After homogenization, the lyophilized sample was subjected to cool extraction at –20 °C using 7.5 mL of Beileski modified methanol/water/acetic acid mixture (75:20:5, v/v) for 12 h. The solvent used allows the enzymatic degradation of phytohormones to be blocked without extracting large quantities of lipids. Solid was separated by centrifugation for 15 min at 8,000 rpm and extracted second time for 30 min. One mL of Supernatants mixed with one drop of orthophosphoric acid and 2 mL of Salkowski's reagent (50 mL, 35 % (v/v) perchloric acid and 1 mL, 0.5 M Ferric chloride). We measured IAA content by spectrophotometric method at 530 nm (Lwin *et al.* 2012).

Estimation of polyphenols and reducing sugars

Tea leaves comprising two leaves and an apical bud (approx. 1 g) were ground well with addition of 100 % ethanol. The contents were filtered and the filtrate was made up to 50 mL with 100 % ethyl alcohol. The alcoholic extract was used for estimation of polyphenols and reducing sugars.

An aliquot of 1 mL alcoholic extract was taken in a volumetric flask and diluted to 50 mL with distilled water. Two milliliters of diluted extract was mixed with 4 mL of 1:1 Folin-Ciocalteu reagent, 1 mL water and 2 mL of 35 % (w/v) sodium carbonate. The contents were further mixed with distilled water to make 10 mL solution, and the mixture was vigorously shaken and allowed to stand still for 30 min. Using UV-Visible spectrophotometer, the absorbance of the formed blue color was taken at 700 nm against the blank reagent. The quantity of polyphenols present in tea leaves was measured using the standard calibration curve derived from known gallic acid concentrations (10 to 50 $mg.L^{-1}$), and the results were expressed as the equivalent percentage of gallic acid (Dev-Choudhury and Goswami 1983).

Reducing sugar content was estimated following the methods suggested by Hedge *et al.* (1962). An aliquot of 2 mL of the alcoholic extract was diluted to 10 mL with distilled water. The diluent (1 mL) was taken in a test tube and incubated with 4 mL of ice cold acidified anthrone reagent (0.2 %, w/v in concentrated sulphuric acid). The contents were then incubated for 8 min in a boiling water bath and cooled down under running water to ambient temperature. The absorbance of the green color formed was taken in a UV-visible spectrophotometer at 630 nm against the reagent blank. Percent reducing sugars (dextrose equivalents) present in tea samples was computed using the standard calibration curve where known concentrations of (+) Dextrose were used to derive calibration curve.

Determination of total tannin content

The tannins were determined using the Folin-Ciocalteu phenol reagent as reported by Amorim *et al.* (2008). The sample extract (0.2 mL) was mixed with 8.3 mL of distilled water and 0.5 mL of Folin-Ciocalteu phenol reagent, held for 5 min at ambient temperature. After that 1 mL of sodium carbonate solution of 35 % was introduced. The mixture was well stirred, left for 20 min at ambient temperature and absorbance was taken at 725 nm. Instead of sample, the blank was prepared with distilled water.

Determination of antioxidant properties

Fresh and infested tea leaves were collected and dried in a drier for 6 h at 35 °C and powdered. The leaf powders were extracted using 100 % methanol with continuous swirling for 1 h at room temperature. Extracts were filtered with Whatman filter paper while methanol was evaporated *via* rotary evaporator and finally, 50 $mg.mL^{-1}$ concentration was made.

The scavenging effects of samples for 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical were monitored according to the method of the previous report by Yen and Chen (1995). Briefly, samples of different concentrations (50 $mg.mL^{-1}$, 10 $mg.mL^{-1}$, 2 $mg.mL^{-1}$, 0.4 $mg.mL^{-1}$ and 0.08 $mg.mL^{-1}$) were taken and the absorbance of each concentration of test sample was measured at 517 nm. 2.0 mL aliquot of test sample (in methanol) was added to 2.0 mL of 0.16 mM DPPH methanolic solution. The mixture was vortexed for 1 min and then left to stand at room temperature for 30 min in the dark, and its absorbance was read at 517 nm.

The ability to scavenge the DPPH radical was calculated using the following equation (Eq. 1):

$$cavenging\ effect\ (\%) = \left[1 - \frac{\text{Sample} - \text{Sample Blank}}{\text{Control treatment}}\right] \times 100 \quad (1)$$

Statistical analysis

The results were analyzed statistically using the SPSS (version 16.0) program (IBM, USA) to determine the average value and standard deviation of the mean. Differences with *p*-values < 0.05 were considered statistically significant.

Results

AMF colonization in roots

Among 5 treatments, colonization (%) was maximum in natural soil inoculated with mycorrhiza (23 %). In sterilized soil (SS), no mycorrhizal colonization was found while 13 % colonization was found in SS+AM treatments. Colonization was less than 5 % in natural soil (NS) while it was 7.5 % in natural soil inoculated with AM (Table 1).

Table 1. AMF colonization in tea root under different treatments.

Treatment†	Colonization [%]
SS+AM	13.0
SS	0.0
NS+AM	23.0
NS	< 5.0
NSS+AM	7.5

†Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM).

Chlorophyll and carotenoids contents in leaves

Photosynthetic pigments, such as chlorophyll and

Table 2. Chlorophyll and carotenoids contents in tea leaves under different treatments.

Treatments†	Chlorophyll a [µg.mL^{-1}]	Chlorophyll b [µg.mL^{-1}]	Carotenoids [µg.mL^{-1}]
SS+AM	1.53±0.01	1.36±0.02	0.21±0.01
SS	0.85±0.05	0.68±0.03	0.12±0.06
NS+AM	1.30±0.05	1.29±0.02	0.10±0.01
NS	0.63±0.01	0.71±0.01	0.04±0.00
NSS+AM	2.74±0.06	1.77±0.03	0.35±0.01

†Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM).

carotenoids, of different treatment were evaluated (Table 2). The highest amount of chlorophyll *a* and *b* was found in leaves of tea plant growing under treatment NSS+AM, while the lowest amount of the these pigments were estimated in tea plants grown under NS and SS treatments, respectively. The highest value of carotenoid content was found in tea plants growing under treatment NSS+AM, however, plants growing under natural condition (NS) produced the lowest carotenoid content in leaves.

IAA concentration

AMF treatment had significant effect on IAA concentrations in tissues of tea plant (Fig. 1).
The highest amount of IAA was found in treatment NS+AM (3.16 µg.mL^{-1}) and the lowest amount was found in treatment SS (1.23 µg.mL^{-1}) (Fig. 1).

Polyphenol content in leaves

Polyphenol contents in leaves of tea grown under different treatment were determined (Fig. 2). It was significantly affected by AMF treatment. The highest concentration of polyphenol was found on NS+AM (64.46 mg.L^{-1}) followed by NSS+AM (59.32 mg.L^{-1}). The lowest concentration of the same was found in SS (38.09 mg.L^{-1}) (Fig. 2).

Tannin content in leaves

Tannin concentration in tea leaf was significantly affected by AMF treatment (Fig. 3). The highest concentration of tannin found in NSS+AM

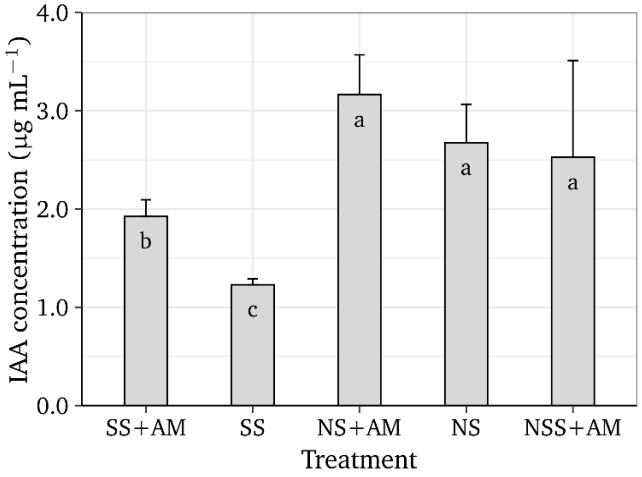

Fig. 1. IAA concentrations in tissues of tea plant under different treatments. Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM). Error bars indicate ± SD of the mean.

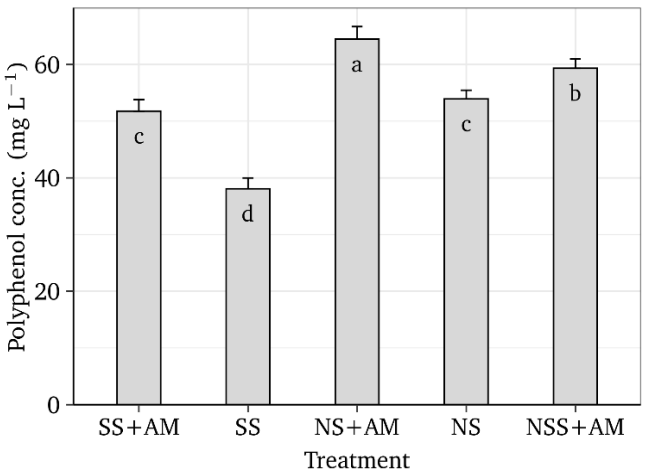

Fig. 2. Polyphenol concentrations in tea leaves grown under different treatments. Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM). Error bars indicate ± SD of the mean.

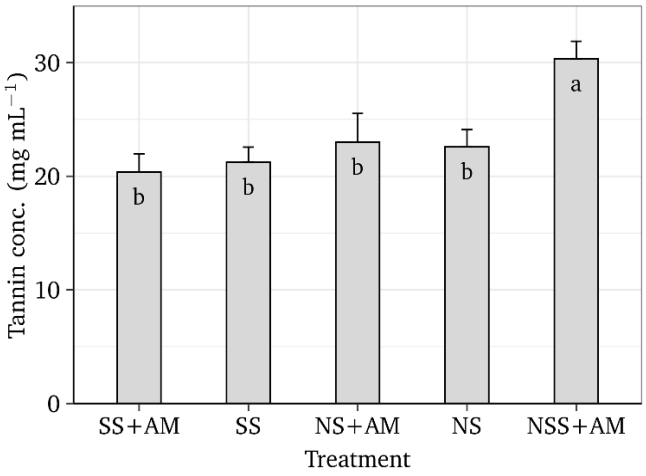

Fig. 3. Tannin concentrations in tea leaves grown under different treatments. Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM). Error bars indicate ± SD of the mean.

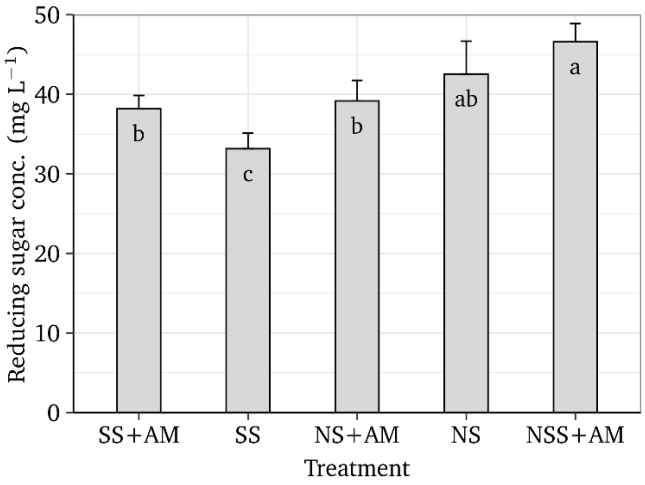

Fig. 4. Reducing sugar concentrations in tea leaves grown under different treatments. Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM). Error bars indicate ± SD of the mean.

(30.34±0.51 mg.mL^{-1}) and the lowest concentration was found in SS+AM (21.22±0.54 mg.mL^{-1}).

Reducing sugars in leaves

Amount of reducing sugars in examined tea leaves ranged from 33.16 ppm to 46.61 ppm. It was significantly affected by AMF inoculation. We got the highest reducing sugar level in NS+AM (46.61±0.77 mg.L^{-1}). Lowest amount among was found in SS (33.16±0.65 mg.L^{-1}) (Fig. 4).

Antioxidant properties of leaves

The scavenging effect of tea leaves on DPPH radicals varied with different treatments ranged from 89.04 % to 94.97 % in 0.2 mg.mL^{-1}; 95.33 % to 97.75 % in 0.4 mg.mL^{-1} and 98.91 % to 99.78 % in 0.6 mg.mL^{-1} (Table 3). Maximum amount was found in NS+AM treatment in most of the cases though minimum amount was varied.

Discussion

Effects of AMF on colonization and growth influencing parameters

Mycorrhizal colonization differs from 0 to 90 % in different plants (Khan 1978) and Mycorrhizal colony exists in soil naturally (Liu *et al.* 2016).
In the present experiment, mycorrhizal colonization increased in tea plants with the inoculation of AM fungi in pot condition but the colonization level

Table 3. Antioxidant properties of tea leaves grown under different treatments.

Treatments†	Scavenging effect [%] of different concentrations of tea leaf ‡		
	0.2 mg.mL^{-1}	0.4 mg.mL^{-1}	0.6 mg.mL^{-1}
SS+AM	89.04±1.29	95.33±2.21	98.91±1.78
SS	94.94±1.35	97.75±0.05	98.69±0.08
NS+AM	94.97±0.71	97.75±0.19	98.87±0.02
NS	88.91±4.95	96.30±0.11	98.06±0.07
NSS+AM	93.14±2.11	96.78±0.06	99.78±1.21

†Sterilized soil inoculated with mycorrhiza (SS+AM) in pot condition; sterilized soil (SS) in pot condition; natural soil inoculated with mycorrhiza (NS+AM) in pot condition; natural soil (NS) in pot condition, and natural soil under natural condition inoculated with mycorrhiza (NSS+AM); scavenging effect on ‡1,1-diphenyl-2-picrylhydrazyl (DPPH) radical.

was comparatively low from expected level in natural condition. One of the possible cause was insufficient time of plant growth. To get average 60 – 80 % of colonization it requires six to eight month in natural condition (Sharma *et al.* 2013). But in artificial application mycorrhizal colony varies within 23 – 28 % (Sarkar *et al.* 2015a) and response can be found in 90 – 120 days after plantation in pot condition though it depends on plants, nutritional status, types of mycorrhiza etc.

The present experimental result shows that tea plants inoculated with AM fungi had better growth. In chlorophyll test, the concentration of chlorophyll was higher in treatment NSS+AM as it was inoculated with mycorrhiza. At the same time, indigenous AM fungi strains were existed which also performed in colony development. Previous study documented the same result (Sarkar *et al.* 2015a).

In addition, mycorrhiza uptake nutrients such as phosphorus, sulphur, nitrogen from soil so the concentration of chlorophyll in AMF inoculation was greater than uninoculated plant while phosphorus is responsible for change in chlorophyll concentration in leaves (Zuccarini 2007).

IAA is an important parameter in leaves and roots that indicates the growth hormone auxin present in leaves and roots. Higher amount of IAA indicates high rate of growth in plant. Among the treatments, we found that NS+AM had higher amount of IAA. Both AMF commercial and indigenous helps to achieve higher amount of auxin. Natural soil (NS) and natural soil in soil condition (NSS+AM) also show interesting results in this way. But SS shows lower result that indicates poor growth rate. This results are in agreement with previous works where AMF was reported to change IAA concentration in tissue and affect growth and quality of tea leaves (Kornberg and Krebs 1957).

Effect of AMF on tea quality parameter

Polyphenol content was increased with the inoculation of AMF and the response was higher in pot condition. Depending on the application of AMF, the concentration of polyphenol changes as AMF uptake nitrogen, phosphorus like nutrient from soil (Chabeli *et al.* 2008) which can support the present experimental results. Tannin is important parameter for color development of tea. Tannin content in NSS+AM treatment showed the highest value where sterilized sample showed lower result as initially present mycorrhiza and other important essential microorganism were killed by sterilization. We know that AMF uptakes organic nitrogen from soil (Whiteside *et al.* 2012) that is responsible for change in tannin concentration in leaves (Hofland-Zijlstra and Berendse 2009). In the case of reducing sugar, the natural soil treatment inoculated with mycorrhiza showed higher rate of reducing sugar content. The amount of reducing sugar changed by the effect of mycorrhizal application or non-application. The AMF applied treatment contained much amount of reducing sugar compare to other treatments (Singh *et al.* 2010). But for antioxidant content, only a little differences shown by inoculated or non-inoculated treatments.

Conclusion

The study was carried out to evaluate the effect of AMF on quality parameters of tea (*Camellia sinensis*). Tea plants with AMF application had higher root colonization than the non-inoculated plants. The AMF colonization significantly and positively affected most of the quality parameters of the tea leaves. Polyphenol content and antioxidant properties of tea leaves can be increased by applying arbuscular mycorrhizal fungi. AMF application also increased the leaf pigment content. These results imply that application of AMF will be much effective for tea cultivation and the load of chemical fertilizers can be reduced by its application.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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