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Original Research Article

Diversity of AM Fungi Population in Tea plantation of Sikkim and Exploring its Friendly Association with the Plant

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Abstract: Tea rhizosphere is abode for several micro-biota - a few beneficial and some antagonistic, among these Arbuscular Mycorrhizal (AM) fungi is a most common beneficial microorganism. Tea plantation, Temi Tea Estate Sikkim, was explored for Arbuscular Mycorrhizal fungal association to know their diversity and also to incorporate their use in agrosystems. AM spore was isolated and identified, further screening of the root for VAM infection were also carried out and percent of infections is 70- 80%. The species found were mostly *Glomus* and is found to be most dominant species followed by *Gigaspora*, *Acaulospora* and *Scutellospora*. *Glomus mosseae* was selected for further study in relation to its effects on growth and development of plants. Inoculation of *Glomus mosseae* found to effective in the growth of the plants and to some extend disease control. Different growth parameters of plant like phosphate utilization by tea plants followed by some biochemical changes like phenol, protein, enzymes and catechins following treatments were determined and results were found to be positive for sustainable maintenance of tea plant health.

Keywords: *Glomus mosseae,* Tea, Catechin, Charcoal Stump Rot.

Introduction

Tea (*Camellia sinensis* (L.) O. Kuntze) is the most important plantation crop of north east India. Tea (China type) was introduced in North East India in 1836. Tea grown in Sikkim is famous by its brand name 'Temi Tea' and it's becoming a major base for production and supply of organically produced tea in the region.

The overall interactions between tea roots, microbes and environmental conditions prevailing in the tea rhizosphere seem to favor the growth of microbes. Since very limited and isolated efforts were made for tapping of microbial diversity, identification, evaluation and preserving them for different applications, thus the selection and inoculation of specific microbial strains or by simply promoting naturally existing microbes hold great promise in sustainable agricultural systems.

The diversity of AMF (Arbuscular mycorrhizal fungi) has significant ecological consequence because of their potential to promote plant growth. They represent a widespread mutualistic association between soil-borne fungi of phylum Glomeromycota and most land plants (1). In this kind of mutual association, fungus assists the plant mainly by improving the supply of water and mineral nutrients, especially phosphate and in return, the obligate biotrophic AM fungus is provided by the plant with assimilates accrued from photosynthesis. The fungus exhibit endosymbiosis in which they inhibit the root cortical cells and forms Arbuscules within the cell and Vesicles outside their host cells which led to their name (2). Thus, the composition and dynamics of populations of AMF have a marked impact on the structure and diversity of the associated plant communities, in agricultural ecosystems (3,4,5).

So its exploration and diversity has an important role to play in our society for successful management of the mycorrhizosphere, and will certainly create healthier, more resilient agrosystems.

Among the various diseases in tea, charcoal stump rot disease is one of the primary root diseases of tea and its incidence has been reported from northeast India tea plantation (6). Therefore the present study was undertaken, to explore the diversity of AM fungi and selection of dominant species to study their effects on growth and development of the tea plants and further explore their possibilities to use as biofertilizer and also for the suppression of charcoal stump rot disease of tea.

Materials and Methods

Sampling area: Soil samples were collected from the rhizosphere of tea bushes at 5-15 cm depth, from the Temi tea estate,

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South Sikkim and kept in sterile autoclavable polythene bags, and were brought to the laboratory and preserved at refrigeration temperature of 4ºC till further use.

Isolation and Identification of AM spores: About 1kg of rhizosphere soil sample were taken for isolation of AM spore which was done by wet sieving and decanting technique (7), the spores were filtered in a line grid marked filter paper (8). The spore population (no. of spores/ 5gm soil sample) was identified following the key manual and the monograph (9, 10). Clean AMF spores were separated using a simple microscope (20x), stained with Melzer's reagent, microscopical observations were made and photographs were taken.

Analyses of soil samples: The soil samples collected were given for analysis in the Soil Testing laboratory, Institute of Plantation Science and Management, North Bengal University. Moisture content, pH for soil type, soil texture, carbon and nitrogen ratio, K and P available etc. were determined. Results have been presented in Table 1.

Screening of root for VAM infection: The AM fungi do not cause obvious morphological changes of roots; however, they produce arbuscules and, in many cases, vesicles in roots. To observe AM structures within the roots, tea plants were dug out with the help of auger manually, washed with tap water gently to free them from soil particles and stored in FAA (formalin aceto alcohol) prior to staining. For staining, a root segment of 1cm each was put into the test tube and were boiled in 10% KOH solution for 15-20 minutes in a water bath (sometimes even 60 minutes for hard roots) washed in tap water, and stained with chlorazol black E (11,12). For confirmation of infection, the presence of intracellular hyphae, vesicles and arbuscules or both characteristics were taken into consideration. Percent root colonization was determined by following the method (13) and the result has been presented in Table 1.

VAM root staining: For observation of VAM mycelia, roots were cut in small pieces (1cm) and then boiled in 10% NaoH for 2 hours in a water bath, washed in distilled water, 2% HCl was added to neutralize (half hour) and again washed with water. Staining was done with cotton blue: lactophenol (1:4) for 3-4 minutes with mild washing. Kept in 50% glycerol for some time and removed and made slide for observation.

Table 1: Soil characters of tea rhizosphere and colonization of AM fungi in Temi Tea Estate, Sikkim.

Parameters	Temi Tea Garden (Sikkim)
Soil type	Sandy-Clay
Sand (%)	55
Silt (%)	7
Clay (%)	43
pH	4.43
Moisture (%)	22.08
P_2O_5 (ppm)	40.26
K_2O (ppm)	123.65
Organic Carbon (%)	1.27
Nitrogen (%)	0.11
Spore No. / gm of soil	115
% Colonization	85

Mass multiplication of AM fungi: The AM fungi are obligate biotrophs and depend entirely on the plant to provide them with carbon. When not in association with a plant, AM fungi exist in the soil as resting spores. So these fungi can grow only in the presence of a living plant, which means that their propagation requires the use of pot cultures, either in greenhouse or in a growth chambers. For mass multiplication, AM fungi isolated from rhizosphere were inoculated in *Sorghum bicolor*, *Cynodon dactylon*, and *Zea mays* in pots taking sterile soil.

Plant material: Different tea varieties of *Camellia sinensis* were selected from the tea Germplasm Bank maintained in the premises of Immuno-phytopathology laboratory, Department of Botany, NBU, Siliguri.

Fungal pathogen: Tea root pathogen *Ustulina zonata* was obtained from Tocklai Tea Research Institute, Jorhat Assam.

Preparation of inoculums and inoculation: The inoculum of pathogen *U. zonata* was grown in sand maize meal (sterilized sand: water: maize meal ratio of (9: 1.5: 1, w:w:v) medium, method (14).figure 1. Nursery grown tea seedling were inoculated by adding 100g of *U. zonata* inocula and ensured that inocula were attached to healthy tea roots.

Disease assessment: The rhizosphere of tea plants pre- treated with the antagonists or without treatment was inoculated with pathogen. In pre- treated plants pathogen inoculation was done 3 days after application of antagonist. The inoculated plants were examined at an interval of 15 days upto a period of 45 days for pathogenicity test. Disease intensity was assessed and calculated by using 0-6 scale (15). The disease infections observation were recorded in a continuous 0-6 scale, where 0= no symptoms: 1=plants look sick and root surface started roughening in patches: 2= leaves start withering or looks yellow, light black patches with rough surface appear on roots; 3= defoliation starts with random yellowing, 50% roots become inky black with random patches leaves; 4= shoot tips also start withering followed by random defoliation and 60-70% root affected as it turns black; 5= total defoliation, 70-85% blackening of roots leaves 6= total defoliation with drying of shoots, 85-100%, blackening and drying of roots.

Extraction and HPLC analysis of Catechin: Extraction method was based on following the protocol of (16) with slight modification.

Extraction and quantification of soil phosphate: Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H 2SO4, 0.05N HCL) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No.2 filter paper (17). Quantitative estimation of phosphate was done following ammonium molybdate- ascorbic acid method (18).

Extraction and estimation of phenol: Phenols were extracted from the fresh tea leaves and roots. Folin Ciocalteau's reagent estimated total phenol content, O-dihydroxy phenol was also estimated (19).

Extraction and assay of enzyme: Extraction and assay of ß-1, 3-glucanase (EC.3.2.1.39) was done (20); extraction and assay and assay of chitinase (EC. 3.2.39) was done by following the method described (21) with modifications; Extraction and assay of phenylalanine ammonia lyase (EC.4.3.1.5) and peroxidase was done by following the method described by (22) with modifications; Extraction and assay of polyphenol oxidase (EC.1.1.14.18.1) from the tea leaf tissues was done following the method described (23) with modifications.

Results

Soil and root samples collected from tea plants growing at various locations of Temi Tea Estate, Sikkim were used for determining the association of Vesicular Arbuscular Mycorrhiza, Extracted spores from each soil samples were divided into groups of the same characteristics for identification under microscope. The semi permanent slides were prepared using PVL (polyvinyl alcohol + lactic acid + Glycerol), which affected certain spore characteristics, such as wall characteristics and spore color, diameter, attachment and hyphal wall thickness, etc. The photographic illustrations and population of AM fungal spores and their associations with tea root system obtained from the Temi Tea Estate, Sikkim has been presented in figure 1 and Table 2.

Table 2: Population of AM fungi in the rhizosphere of tea collected from different blocks of Temi Tea Garden.

Genus & species	% of AM spores from different blocks			
	Block 1	Block 2	Block 3	Block 4
Glomus aggregatum	44	23	48	82
G. mosseae	47	22	18	86
G. fasciculatum	45	30	38	82
G. drummondii	-	-	-	12
G. clarum	05	08	08	10
G. microaggregatum	13	12	10	18
G. versiforme	-	-	-	06
Gigaspora decipiens	60	57	48	03
G. gigantea	75	78	82	14
G. margarita	65	72	70	34
G. rosea	25	20	-	08
Acaulospora bireticulata	15	18	16	24
A. spinosa	08	12	10	22
A. denticulata	10	12	10	10
A. capsicula	02	-	01	04
Scutellospora rubra	04	02	04	05
S. pellucida	03	02	04	02

Maximum root colonization of tea roots with AM fungi revealed the presence of *Glomus mosseae* and *Glomus fasciculatum*. The total number of AM fungal species isolated was altogether 17, belonging to four genera, *Glomus, Gigaspora, Acaulospora, and Scultellospora*.

Assessment Growth Parameters: Among the population of AM Fungi *Glomus Mosseae* is one of the largest genera found and was selected for mass multiplication followed application in tea roots for assessment of different growth parameters like number of leaves, branches and height of the tea plants. Result revealed the positive impact following inoculation with the fungus. When the plants were inoculated with *G. mosseae* the growth of the plants increased markedly and Percentage increase in leaf number was also evident. Figure 1.

Soil sample from rhizosphere of the two varieties were collected after 60 days treatment and phosphorous content in soil was analyzed for the amount of phosphorous depletion on mobilization after treatment with, *G. mosseae*. Phosphorous content in soil has been lowered in the treated block due to excess phosphorous mobilization and presented in (Table 3).

Table 3: Total phosphorous in the rhizosphere soil of tea plants subjected to treatments.

Variety	Treatments	Conc. (mg/lit)
TV-18	Control	0.302
	G.mosseae	0.256
TV-26	Control	0.354
	G.mosseae	0.232

Phosphorous solubilization following the treatment was also calculated (Table 4). The result revealed that soil P content had decreased due to application of AMF indicating that the plant could have taken up the phosphorus, which had been solulubilized by AMF.

Table 4: Phosphate utilization by tea plants following treatment with *G.mosseae*.

Treatment	Soil phosphate (µg/g tissue)
Control	49.37^{a} ± 1.18
G. mosseae	31.25^{b} ± 3.60

An average of 3 replicates; ± = standard Error; Difference between values significant at P= 0.01 where superscript is different; not significant where superscript is same.

Finally, field application of AM fungus (*Glomus mosseae*) was made in one selected plot of Tea Germplasm Bank. Tea plants were treated with both AMF after pruning of the tea leaves. Excellent leaf growth was prominent in the field of those plants treated with AMF. Figure 1.

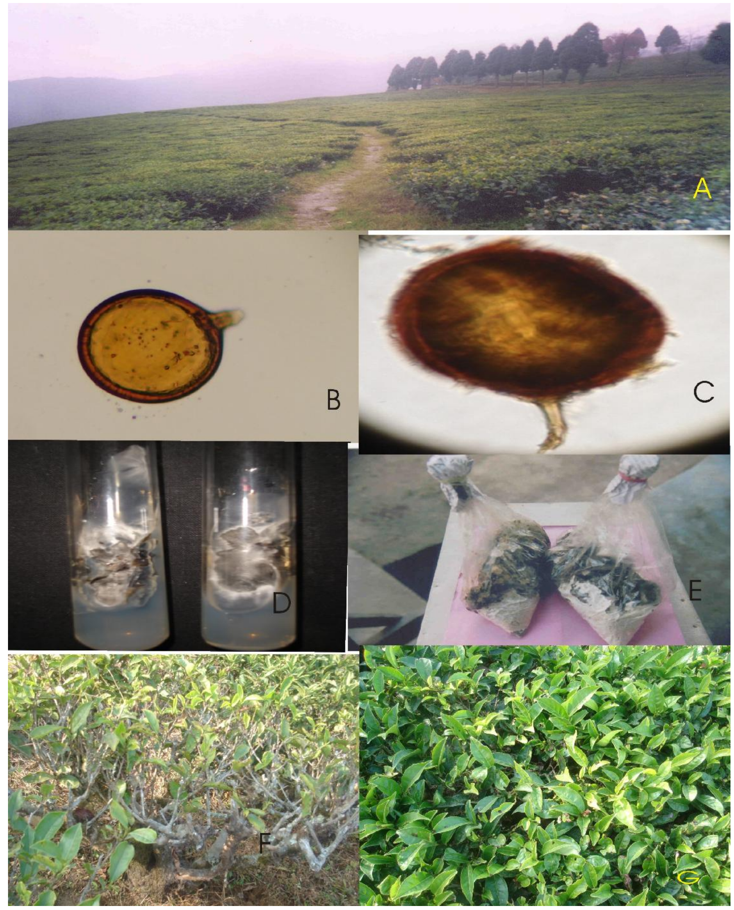

Figure 1: Plantation site of Temi Tea Garden (A), *Glomus mosseae* under bright field (B & C), *Ustulina zonata* in culture tube (D) & in sand maize meal media (E), untreated (F) & treated (G) tea plants

Disease assessment following treatment: Tea plants (TV-18 and T- 17) inoculated with *G. mosseae* subsequently inoculated with *U. zonata* to assess disease development. Disease intensity was assessed as root rot index. It was observed that treatments reduced the disease incidence in relation to untreated inoculated control.

Biochemical changes in tea plants following treatment: An increase in phenol content following treatment was observed (Table 5). Then the plants were inoculated with *G. mosseae* and following this treatment the plants were challenge inoculated with *U. zonata*, the level of defense enzymes like glucanase and chitinase increased markedly. The results have been presented in Figure 2.

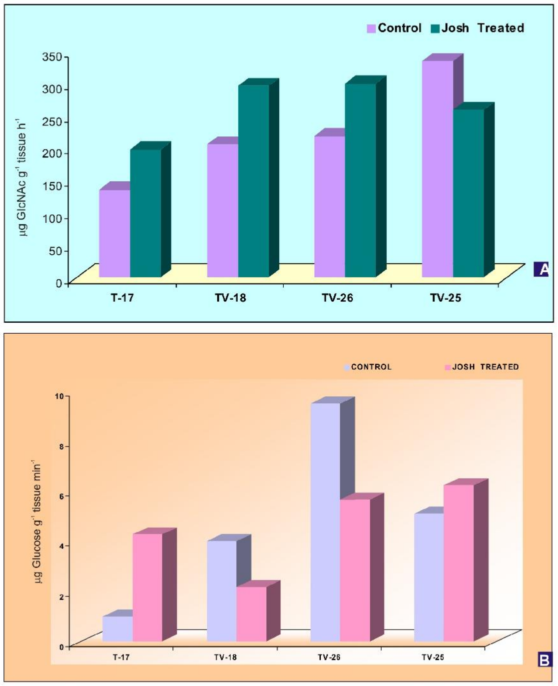

Fig.2 (A & B): Chitinase (A)and beta-1,3,glucanase (B) activities in tea roots following treatment with glomusmosseae

Table 5: Phenol contents in tea leaves of different varieties following fungal (*G.mosseae*) and pathogen application in the rhizosphere

Varieties	Treatment	Phenolcontent(mg/gtissue) Total	O-dihydroxy
T-17	Contro	30.2±3.2	8.2±1.4
	U. zonata	34.4 ± 2.2	8.1± 0.9
	U. zonata + *G.mosseae*	38.5± 2.5	9.5±0.9
TV-18	Control	28.2±1.2	7.5±1.4
	U. zonata	30.8±2.9	8.1±1.4
	U. zonata + *G.mosseae*	35.9±2.5	9.6± 0.9

Catechins: Catechins derived from leaves of plants whose rhizosphere was inoculated with *G. mosseae* were analysed in HPLC. In *G. Mosseae* inoculated tea leaves, peak intensity is high and one very prominent peak can be observed with respect to control. (Figure 3, 4).

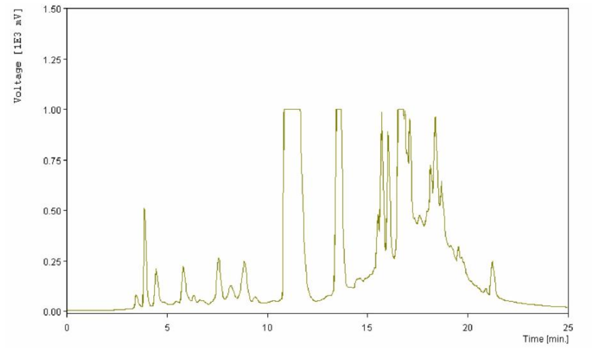

Fig.3: HPLC profile of catechins extracted from tea leaves of untreated (control) plants

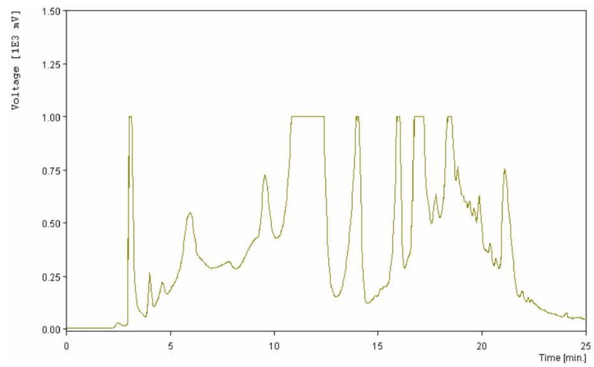

Fig.4: HPLC profile of catechins extracted from tea leaves from *Glomus mosseae* inoculated plants

Discussion

Since soil microorganisms play an important role in nutrient cycling and can reduce diseases, a new approach is to manage the system by increasing the soil biodiversity. Keeping the above in mind, the present study was undertaken in order to isolate and select AM fungi from tea rhizosphere for the benefit of plants itself. Through appropriate management of mycorrhizae in agriculture, it is also possible to maintain soil quality and sustainability while protecting the environment over the long term and reducing cost of production.

In the present study among the population of AM spores *Glomus* species was one of the largest genera found. So the *Glomus mosseae* selected for further experimental purposes. The isolated *G. mosseae* were tested for their effect on growth, development and to disease suppression in tea plants. Results show positive effects after treatments. Improvements in phosphorus acquisition have significant impact on plants growth and health (24). The most recognized AMF potential to mobilize plant nutrients; especially phosphorous is one among the many functional attributes that qualify them to be the plant growth promoting microorganisms par excellence.

Soil samples from rhizosphere of treated tea plants were collected after 60 days and phosphorous content in soil was analyzed for the amount of phosphorous depletion on mobilization after treatments. Phosphorous content in soil has been lowered in the treated block due to excess phosphorous mobilization or utilization by those treated plants.

The potential of the selected AM fungi in plant growth promotion and disease suppression, in vivo experiments were carried out on tea plants. Different varieties of tea plants were selected starting from young seedlings in nursery to two years old. Significant promotion of growth was obtained in seedlings, as well as in two-year-old plants after applications with *G. mosseae*. Results thus revealed that plant growth is promoted by the fungus in tea plants could be the result of improved uptake of inorganic nutrients. They could to some extent reduce charcoal stump root rot intensity caused by *U. zonata*. In a similar study impact of AMF on the reduction of soil borne diseases has mainly been evaluated in studies on soil fungal pathogens such as *Phytopthora, Aphanomyces, Fusarium and Verticillium* (25). The work on different antagonistic microflora, viz. *Trichoderma viride, Trichoderma harzianum, Gliocladium virens, Bacillus subtilis and Pseudomonas fluorescens* have been evaluated against *Ustulina zonata*, (26) causing charcoal stump rot disease of tea.

The major components analyzed in tea leaves in the present study were defense enzymes, polyphenolics, and catechins. In the tested varieties defense related enzymes viz. chitinase (CHT), β-1, 3-glucanase (GLU), peroxidase (PO), phenylalanine ammonia layse (PAL), as well as phenolic compounds increased significantly, especially in the presence of the pathogen. The induction of PO activity by pathogens and methyl jasmonate and existence of multiple molecular forms of peroxidase in tea has also been reported (27). Phenols are also known to play definite roles in a plant defense (28). Alteration of phenol metabolism following fungal infection has been observed in many diseases and phenolics have been implicated in the defense reaction in several instances (29). The involvement of phenol in the defense strategies of tea plants against foliar fungal pathogen (*Bipolaris carbonum, Pestalotiopsis theae*, *Glomerella cingulata*) has been described (30).

Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosynthesis, such as PAL are involved in defense reaction in plants. Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolics compounds have been associated with resistance responses in various host plants, it may be suggested that the activity of PAL could be useful indicators of the activation of defense enzymes.

The quality of tea is judged by its polyphenolic compounds flavanoid, as catechins are major flavor flavanoid compounds of tea, which are plants secondary metabolites. This means they are not essential for the growth of the plant, but is important for its good health and their quantitative changes with respect to different isomeric forms were analyzed by HPLC. It was observed that the treatment with fungus induced some new isomeric forms, which are believed to be enhanced during the plant growth promotion.

Conclusion

The overall results of the present study have shown that AM fungi, *Glomus mosseae* isolated from tea rhizosphere, could be promoted for induction of plant growth promotion and disease reduction to some extent in tea plantation without the use of additional chemical inputs.

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