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Root colonization of mandarin plants grown in orchards of Darjeeling hills and plains with arbuscular mycorrhizal fungi and their effects on plant growth

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Abstract
Citrus reticulata is an ancient commercial crop being cultivated in Darjeeling-Sikkim hills. Many diseases are prevalent in mandarin plants, out of them, bacterial & fungal diseases are mostly dangerous. Arbuscular Mycorrhizal Fungi were screened from rhizosphere of mandarin plants from the four different regions using wet sieving and decanting method. Microscopical observation revealed the presence of different genus of AM fungi present in the root as hyphae, spores and sporocarp. *Glomus mosseae, G. fasciculatum, G. aggregatum, G. badium, G. constrictum, G. versiforme, Gigaspora gigantea, G. margarita, Acaulospora capsicula, A. bireticulata, Sclerocystis* and *Scutellospora rubra* were found to be dominant in all the soil samples of mandarin. Species of *Glomus* were found to be high in both hilly and foothill regions. *Glomus mosseae* and *G. fasciculatum* were selected for mass multiplication in maize plant in pots. Histopathological study of root showed the presence of vesicles and arbuscules. AMF infection and total number of spores per 100 gram of soil were recorded. Scanning Electron Microscopy (SEM) of AMF spores of mandarin revealed clear morphology, spore wall characters and hyphal attachment of spores. Total phosphate content of the soil, soil analysis and enzyme activities in roots and leaves of mandarin plant from the different regions were studied. Three major defense enzymes peroxidase, chitinase and β-1.3- glucanase showed enhanced activities and the total phosphate content also decreased in soil with respect to control. Present study evaluates the effect of AMF in plant growth and phosphate solubilization.

Keywords: *Citrus reticulata, Glomus mosseae, G. fasciculatum*

Mandarin orange (*Citrus reticulata* Blanco) the loose jacket orange which is a principal cash crop of India is cultivated to an alleviation of 1500 mm in Darjeeling – Sikkim hills. It belongs to the family Rutaceae under the order Sapindales. Mandarin is grown in the tropical / sub -tropical regions 35° N to 35° S of equator. There are four natural and one hybrid cultivars of mandarin in India grown in five different belts as a dominant cash crop. The total production is reckoned at about 760 thousand tonnes per year. Loose jacketed orange such as Nagpur Santra, Coorg orange, Kamala orange of Manipur, Khasi orange of Assam, Sikkim orange of Darjeeling and Kinnow of Punjab belong to the category of Mandarins in India.

Mandarin is widely consumed as fresh fruit and also used for producing canned segments, juice- concentrate, squash, beverages, jams as well as marmalades. The peel of Mandarin is the source of essential oils which are used in the cosmetic and pharmaceutical industries (Frazier and Westhote, 1978). It can also serve as a basic material for the production of cattle feed, candies and alcohol.

However, mandarin orange is susceptible to various pest and diseases which results to decline in production and productivity. The intensity sometimes is so severe that thousands of hectare cultivated areas are declined every year which is commonly referred as citrus decline or citrus dieback. The commons citrus diseases are growing, tristeza, cranker, foot/root rot, wilting etc. Root rot is an alarming problem of Darjeeling mandarin and one of its important causal organism is *Fusarium* spp.

AMF are an important group of soil–borne microorganisms that contribute sustainability to the establishment, productivity and longevity of natural or man-made ecosystems by the virtue of forming a symbiotic association with most terrestrial plants by forming an extensive network of external hyphae functioning as plant rootlets by spreading into a vast area underground and absorb nitrogen, phosphorus, potassium, calcium, sulfur, ferric, manganese, copper and zinc from the soil and then translocate these nutrients to the plants with those roots they are associated (Gerdemann, 1975). The symbiotic associations of AMF with most terrestrial plans are well documented but there are only few reports of symbiotic association between mandarin plant and AMF. The extrametrical fungal hyphae can extend several centimeters into the soil and absorb large amounts of nutrients for the host root (Khan *et al.*, 2000).

These extraradical hyphal networks and their hyphae help in improving the texture of the soil as they contain and release glomalin, which is a putative glycoprotein,

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assayed from soil. Glomalin is a Glomalin-related soil protein (GRSP) that is correlated with aggregate water stability (Wright and Upadhyaya 1998, Rillig 2004; Rillig and Mummey 2006). Improved soil structure increases water infiltration and can reduce soil erosion (Tisdall and Oades 1982).Efforts are being undertaken to develop a bio formulations which can minimize the disease occurrence.

Considering the importance of association of AMF with mandarin orange the present investigation was made to assess the AMF population from three different locations of Darjeeling hills (Kalimpong, Mirik, Bijanbari). For the assessment of AMF population in plains, rhizosphere soil was collected from mandarin plants being grown in experimental garden of Immuno-Phytopathology Laboratory, Department of Botany, NBU.

Materials and method

Isolation of AMF spores

Arbuscular mycorrhizal fungal spores were screened from those soil samples of Mandarin rhizosphere by the wet sieving and decanting method (Gerdeman & Nicholson, 1963). Soil samples (100gm each of the representative root zone) were collected, suspended in water (1 l) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50µm) arranged one below the other. The spores were picked by the help of fine bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one liter of boiled distilled water) at 4°C for further study and observation. It is evident from various studies that each plant has multiple AM fungi population. The soils of the collected samples were further analyzed to know the chemical composition of the soil, viz. moisture content, pH, amount of carbon, N_2 etc.

Plant material

Citrus reticulata seedlings were obtained from orchards of Kalimpong, Mirik, Bijanbari and experimental field of Immuno-phytopathology Laboratory of North Bengal University, Siliguri. They were maintained in 12" earthen pots with sterilized soil.

Identification of AMF spores

Spore samples were separated according to their morphology, size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified up to species level with the help of standard keys (Walker 1981; Schneck and Perez 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layers, position and abundance), hyphal branching patterns, the diameter, structure (especially near entry points) and the staining intensity of hyphae.

Spore count

Rhizosphere soil (100g) was taken and suspended in 250 ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores were then counted and spore percentage of different genera was obtained.

Histo-pathological analysis

The root specimen were taken from field and washed with tap water. The root were cut into pieces, after washing treated with 10%KOH added, kept in water bath for 1h, then 1% HCL was added to neutralize the alkalinity. The root pieces were then washed with water (after 30 min) and staining was done by simmering the roots in cotton blue: lactophenol(1:4) for 3-4min with mild heating. Degree of contrast between fungal tissues and back ground plant cells was obtained according to the duration of storage of tissues. 1% HCl was added to acidify the tissues, as most histological stains are acidic. A little amendment in this process is noteworthy because it has been noticed that extraradical spore bearing hyphae and other extraradical fungal tissues with root segments are destroyed or dissolved when it is boiled in hot water bath at 90°C twice with 2% KOH followed by 0.05 cotton blue and lacto glycerol for staining the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells etc. The total staining process can be done without heating but keeping the root fragments in 1-2% KOH for 24-48 hours in a Petri dish and another 12 to 18 hours in cotton blue and lactoglycerol with minimum movements of the samples yields remarkable result. In this method the spore bearing hyphal structures, auxiliary cells etc. are clearly visible and percent colonization can be determined with better accuracy. After preparing the roots the hyphal structures were viewed under dissecting stereomicroscope under 20X and 40X magnification. Percent root colonization was estimated by using slide method as described by Giovannetti and Mosse (1980).

Mass multiplication of AM spores

Glomus mosseae and *G. fasciculatum* were selected from among the mass of other AM fungi with the help of fine tweezers under dissecting microscope. The spores were washed several times with distilled water and Chloramin T to remove adhered debris. They were then inoculated in roots of 7-10 days old maize seedlings which were grown in petri plates. After inoculation they were transferred to black plastic pots (12 inch) having autoclaved soil to discard the presence of other fungal propagules. After 45 days the presence of spores of *G. mosseae* and *G. fasciculatum* were confirmed.

Artificial inoculation of mandarin roots

Healthy spores of *G. mosseae* and *G. fasciculatum* were collected from the maize plants and rinsed with sterile distilled water. Filter paper were cut into small circles of 5 mm diameter and about 5-6 spores were transferred to the filter paper. The paper was then adhered to the roots

Table 1: Physico-chemical factors and AM infection (%) in rhizosphere soil samples of mandarin

Factors	Kalim-pong	Mirik	Bijan-bari	Foot-hills
Soil type:	Clay	Sandy clay	Clay	Clay
Sand(%):	48	54	42	46
Silt(%):	10	04	16	14
Clay(%):	42	42	42	40
pH:	6.01	4.81	4.31	5.07
Moisture(%):	21.64	19.27	11.62	20.95
P_2O_5 ppm:	23.94	34.82	20.67	31.35
K_2O ppm:	31.34	197.67	84.89	48.97
Organic C(%):	0.48	1.11	1.31	0.75
Nitrogen(%):	0.05	0.11	0.14	0.08
Colonization (%):	98	79	86	84

of 1 month old seedlings of mandarin plants with the help of tweezers.

Extraction and quantification of soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 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 (Mehlich 1984). Ammonium molybdate-ascorbic acid method was followed for quantitative estimation of phosphate as described by Knudsen and Beegle (1988).

Assay of enzyme activities

Leaves and roots of mandarin seedlings grown in treated or control potted soil collected from different regions were used for all biochemical analyses. Samples were collected for assay 1 month after inoculation.

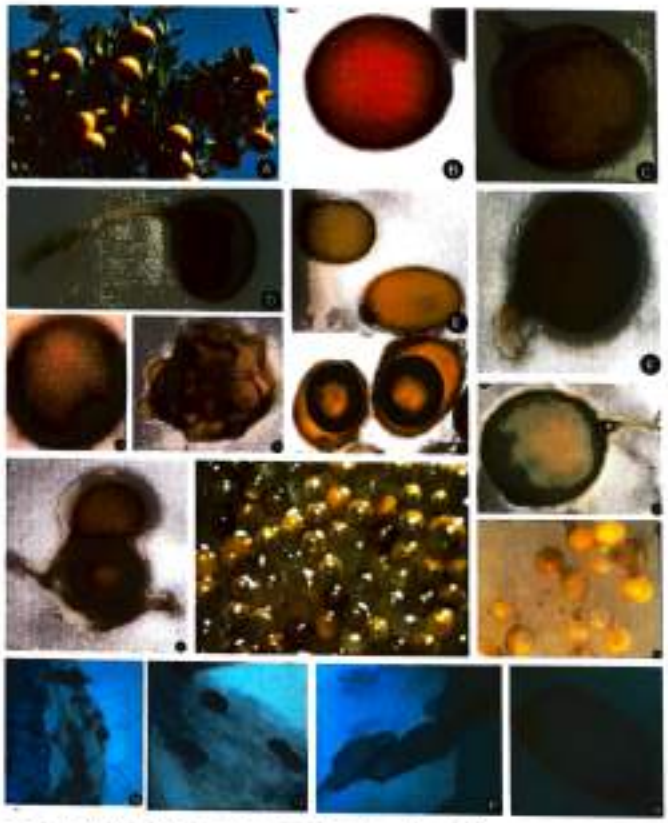

Figure 1 A: Fruit bearing mandarin plant; B: *Acaulospora capsicula*, C: *Glomus mosseae*, D: *G. mosseae* with long hyphal attachment, E: *G. badium*, F: *G. constrictum*, G: *Acaulospora bireticulata*, H: Sporocarp of *Glomus*, I: *Scutellospora rubra*, J: *Gigaspora margarica*, K: *Glomus fasciculata*, L: Mass multiplied spores of *Gigaspora giganta*, M: Enlarged view of the same, N: Mycorrhizal hyphae in root tissue, O-Q: Vesicle, flattened vesicles and single enlarged view of vesicle

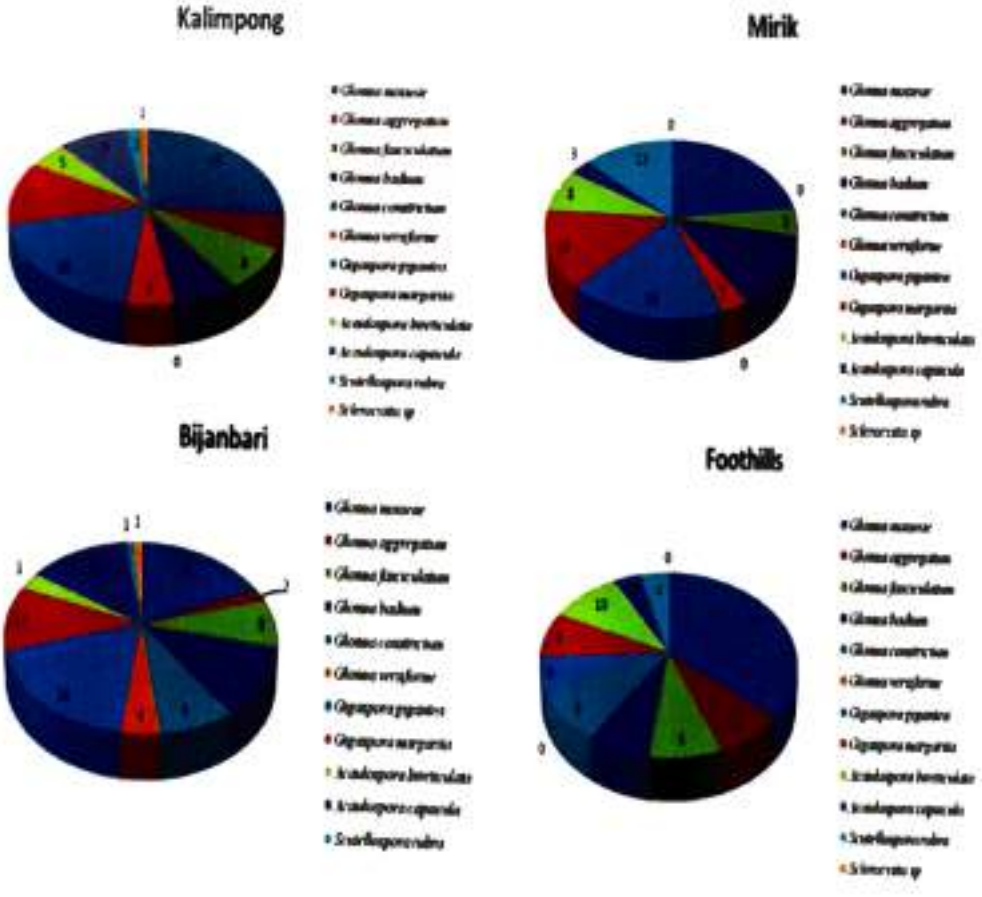

Figure 2: Percentage population of dominant AMF spores in mandarin soil

Table 2: Microscopic characters of AMF Spores associated with mandarin roots

Genus & species	Colour	Shape	Spore layer	Spore size (µm)	Other descriptions
Glomus fasciculatum	Pale yellow to bright brown	Globose to subglobose	3	70-120	Spore layer continuous
Glomus mosseae	Brown to orange -brown	Globose to subglobose	3	200	Hyphae are double layered
Glomus aggregatum	Pale yellow	Globose to oval	1-2	200-1800 x 200-1400	Sporocarps formed in loose clusters
Glomus badium	Reddish brown to dark brown to black	Globose, subglobose to ovoid	3	51-90 x 75-120	Subtending hypha of each spore is usually very short
Glomus constrictum	Brownish orange to dark brown	Globose to subglobose, sometimes ovoid	2	110-130 x 150-160	Subtending hyphae straight or curved, usually markedly constricted at the spore base
Glomus versiforme	Orange to red brown	Globose to subglobose, sometimes ovoid	2	60-160	Sporocarps are irregular, they arise from a basal pad of pale grayish yellow, loose mycelium with a few interspersed spores
Gigaspora gigantea	Greenish yellow	Globose to subglobose	2	250-270 x 265-370	Formed terminally or laterally on a bulbous sporogenous cell
Gigaspora margarita	Yellowish white to sunflower yellow	Globose to subglobose	2	300-340 x 360-380	Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell
Acaulospora capsicula	Orange red to capsicum red	Globose to subglobose	3	220-310 x 290-440	Sporiferous saccule pale yellow to brownish yellow which usually falls off when spores mature
Acaulospora bireticulata	Brownish	Globose	3	280-410	Surface ornamentation is prominent. Spores are borne laterally from the neck of a sporiferous saccule.
Scutellospora rubra	Dark orange-brown to red-brown	Globose to subglobose	3	140-220	Germinal walls are formed completely separate from the spore wall
Sclerocystis	Brown to blackish brown	Globose to subglobose		300-600 x 400-700	Chlamydospores arranged side by side in a single layer radially arranged on a central plexus of hyphae

Table 3: Soil phosphate (µg/g tissue) content in rhizosphere of mandarin plants after application of microorganisms

	Kalimpong	Mirik	Bijanbari	Foothills
Control	47.19± 0.625	49.13 ± 0.0144	48.39 ± 0.387	47.12 ± 0.071
Glomus mosseae	33.67 ± 0.287	32.45 ± 0.262	31.99 ±0.41	33.06 ±0.461
G. fasciculatum	31.75 ± 0.2165	31.64 ± 0.086	32.40 ± 0.318	32.03 ± 0.14
G. mosseae + G. fasciculatum	30.38 ± 0.198	30.23 ± 0.296	29.85 ± 0.13	29.58 ± 0.29±

Peroxidase (POX, EC1.11.1.7.). Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity expressed as the increase in Δ A 465/g tissue/ min.

Chitinase (CHT, EC 3.2.1.14). Chitinase was extracted and assayed following the method of Boller and Mauch (1988). The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

β- 1,3- glucanase (β-GLU, EC 3.2.1.38). β-1,3-glucanase was extracted and assayed from the samples following the method of Pan *et al* (1991). The amount of glucose liberated was determined spectrophotometrically using a standard curve. Activity was expressed as µg glucose released /min/g tissue.

Table 4: Activities of β-1, 3 glucanase in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Glucanase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	212	113	200	105	198	123	215	118
Glomus mosseae	313	189	323	156	309	176	320	168
Glomus fasciculatum	252	179	263	163	261	175	250	173
G. mosseae + *G.fasciculatum*	302	191	346	170	304	169	321	198

Table 5: Peroxidase activity in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Peroxidase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	89	29	78	30	88	36	76	32
Glomus mosseae	110	64	102	61	120	71	116	72
G. fasciculatum	101	40	105	45	106	42	98	50
G. mosseae + *G.fasciculatum*	135	68	129	70	131	72	140	73

Results and discussion

The benefits and wide host range of AM fungi has led to it being used as a bioinoculant to improve plant nutrition and growth. This study focussed on the use of AM fungi, its application and the quantification of the increased defence related enzymes responsible for disease resistance and subsequently improving plant health status. Arbuscular Mycorrhizal fungi from the selected places of hills (Kalimpong, Mirik, Bijanbari) and foothills were screened from the rhizosphere of mandarin plant. On observation it was found that *Glomus mosseae* dominated the AM population in all the soil samples followed by *G. fasciculatum*. Results are presented in Fig 2. Percentage of AM spores determined from different regions showed maximum of different *Glomus* sp., followed by *Gigaspora* sp., *Acaulospora* and *Scutellospora*. Histopathological study revealed the presence of vesicles and arbuscules in the root segments determining the fact that infection of the AM spores has taken place (Fig 1). Organisms of AMF have a bimodal pattern of differentiation (Morton, 1990). The vegetative thallus consists of arbuscules, intraradical vesicles (shared only by species in the suborder Glomineae), extraradical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extraradical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham et al., 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids and are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991).The different types of spores which were observed in the rhizosphere of mandarin soil have been identified and described as shown in Table 2. Spore colour, shape and size are the most prominent factors in their identification. The hyphae of each genus also differs in their morphology and number of wall layers.

Scanning Electron Microscopy (SEM) (Fig 3) of the spores revealed the spore wall morphology. *Glomus mosseae* has a rough outer surface. The outer layer is sloughed. Spores of *G. fasciculatum* are found in aggregates of 2-6. The surface is rough and shape is subglobose. The wall of *Gigaspora gigantea* is smooth walled with the typical bulbous suspensor. The polygonal reticulum on the spore wall surface is characteristic of *Acaulospora reticulata*.

Total phosphate content of soil had decreased due to application indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 3). Dual application of *G. mosseae* and *G. fasciculatum* was more effective in solubilising the insoluble phosphate present in the soil than when applied singly. This study aimed at improving the understanding of AM fungal interactions in the rhizosphere using a field trial which was to an extent successful. Application of *G. mosseae* in the rhizosphere of citrus plants led to an increase in the growth of seedlings in terms of increase in height and number of leaves. Joint inoculation with both the microorganisms (*G. mosseae* and *T. hamatum*) gave most significant results (Allay and Chakraborty, 2010). *B. pumilus* along with *G. mosseae* could improve seedling growth in terms of height and leaf number and also helped in solubilising phosphate, suggesting a synergistic effect (Chakraborty *et al*, 2011). Fresh shoot

Table 6: Chitinase activity in leaves and roots of mandarin following application of *G. mosseae* and *G.fasciculatum*

Chitinase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	88	80	86	75	89	77	92	81
Glomus mosseae	106	94	105	93	98	99	110	101
G. fasciculatum	89	83	93	91	101	82	102	85
G. mosseae + *G. fasciculatum*	133	97	124	95	126	98	145	100

biomass was also found to be increased when tomato plants were treated with *Glomus mosseae, Acaulospora laevis* and *Trichoderma harzianum* (Tanwar *et al*, 2010).

The pH of soil varied from 4.5 to 6. The pH of Kalimpong soil is around 6.0 which is ideal for citrus plants (Table 1). Not much difference was seen in the availability of carbon and nitrogen respectively in the 4 different regions. The moisture content of Kalimpong soil is also the highest. Citrus trees prefer draining sandy loam soil hence the amount of sand in all the soil was high.

Activities of 3 defense enzymes- chitinase, 1,3 - β glucanase and peroxidase were assayed in leaves and roots of mandarin seedlings subjected to various treatments- i.e., *G. mosseae*, *G. fasciculatum* and *G. mosseae* + *G. fasciculatum*. Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced due to the various treatments (Table 4, 5 & 6). The most significant was in dual application of AMF in all the cases. In an earlier study, when mandarin seedlings were pre-treated either with *B. pumilus* or *G. mosseae* prior to challenge inoculation with the pathogen (*Fusarium oxysporum*), activities of all three defense enzymes increased significantly (Chakraborty *et al*, 2011).

The overall results of the present study have shown that *G. mosseae* and *G. fasciculatum* can promote growth of mandarin plants. However, *G. mosseae* was found to be the best colonizer as well as responsible for induced accumulation of defense enzymes in the host plant.

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