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Effect of indigenous arbuscular - mycorrhiza (*Glomus* spp) on apple (*Malus domestica*) seedlings grown in replant disease soil

PRAVEEN MEHTA[1] and NARENDER K BHARAT[2]

Dr Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh 173 230

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ABSTRACT

Poor growth and short life of newly planted apple trees at old apple site is known as apple (*Malus domestica* Borkh) replant problem. It is very difficult to be managed due to its complex etiology. To find out a sustainable management of this problem, effect of indigenous arbuscular-mycorrhizal fungi (AMF) was observed on growth of apple seedlings grown in apple replant diseased (ARD) soil. Out of five AMF isolates collected from apple orchards in Himachal Pradesh, one isolate AMFS-2 (*Glomus fasciculatum*) was selected on the basis of its colonization and growth promotion in inoculated apple seedlings. This AMF isolate was then used to see its effect on growth of apple seedlings grown in ARD soil collected from replant affected apple orchards. The AMF inoculation resulted significant increase in growth parameters, viz. plant height, stem diameter, internodal length, leaf area, shoot/ root fresh and dry weight of inoculated seedlings as compared to uninoculated seedlings grown in ARD soil in pot cultures. The effect of AMF inoculation on seedling growth was more when these were grown in ARD or virgin soil fumigated with formaldehyde. The phosphorus content of AMF inoculated seedlings were significantly more than uninoculated ones. The population of fungi, bacteria and actinomycetes was also less in the rhizosphere of mycorrhizal inoculated apple seedlings grown in ARD soil.

Key words: Apple, Arbuscular mycorrhiza, *Glomus* spp, Replant problem

Himachal Pradesh is known as 'Apple state' of the country. Apple (*Malus domestica* Borkh) cultivation in the state has been increased many fold since last few decades and the area under apple cultivation has reached up to 99564 hectares with annual production of 280 105 metric tonnes (Anonymous. 2009). The area under apple cultivation has increased, but the productivity has not increased accordingly. One of the reasons for low productivity is the age of orchards as most of the orchards in the state have outlived their economic bearing life. The Department of Horticulture of State Government is helping the farmers in introducing newly improved and highly productive varieties. But because of limitation of cultivated land in hilly areas growers are compelled to plant new seedlings on the old apple site. This practice makes seedling plants vulnerable to replant problem, i.e. poor growth and short life of apple trees planted at sites where apple tree grew before. The apple replant problem is wide spread throughout the world and is known to be caused by various abiotic and biotic stresses (Utkhede and Smith 1994). These include harmful microorganisms (biotic) and nutritional deficiencies or excesses, soil pH, phytotoxins etc. (abiotic). Due to its complex etiology, replant problem is very difficult to manage. The only practice adopted worldwide for its management is soil fumigation with chemical fumigants. But soil fumigation with chemicals has failed to provide a sustainable growth of new seedlings as it also destroyed beneficial soil microflora including arbuscular-mycorrhizal fungi (AMF) besides other environmental issues. The AM fungi live in association with the roots of many plant species including apple and benefit the host plants by increasing growth, nutrient uptake and defence mechanisms (Bharat and Bhardwaj 2001). The distributions of AM fungi are known to various climatic zones and their ability to colonize apple roots is well established (Sharma and Sharma 2006, Bharat and Bhardwaj 2002). The present study was planned to find out a potent indigenous AM fungal isolate and see its effect on the growth of apple seedlings grown in apple replant disease (ARD) soil.

MATERIALS AND METHODS

The study was conducted in the Department of Mycology and Plant Pathology, Dr Y S Parmar University of Horticulture and forestry, Nauni, Solan, Himachal Pradesh during 2008-

[1] Horticulture Extension Officer (e mail: praveenmehta2008@gmail.com), Directorate of Horticulture, Naubahar, Shimla, Himachal Pradesh 171 002; [2] Scientist (e mail: nbharat07@rediffmail.com), Department of Mycology and Plant Pathology

10. Soil samples were collected from apple orchards located in wet temperate region of Himachal Pradesh (2000 to 3000 m above msl). The AMF spores were extracted by wet sieving and decanting technique of Gerdemann and Necolson (1963). Morphologically similar types of AMF spores in each sample occurring in high frequency were picked up through auto pipette under stereoscopic binocular microscope and kept in sodium azide (0.05%) solution separately. Five different isolates of AM Fungi namely AMFS-1(*Glomus macrocarpum*), AMFS-2 (*Glomus fasciculatum*), AMFS-3 (*Glomus mosseae*), AMFS-4 (*Gigaspora albida)* and AMFS-5 (*Acaulospora bireticulata)* were isolated on the basis of their frequent occurrence in the apple orchards at different locations. The identification of the species was done with the help of synoptic keys (Mukerji 1996, Trappe 1982, Hall and Fish 1979). For multiplication of cultures of AMF isolates, the spores were mixed in upper layer of sterilized soil in glass funnels, separately. Thereafter, surface sterilized (5% NaOHCl for five minutes) seeds of *Trigonella* sp. were sown. These seeds were allowed to grow for 40 days in inoculated funnel. Seedlings were watered with sterilized water as and when required. For mass multiplication of the AMF cultures, roots of inoculated *Trigonella* plants grown in funnel for 40 days were chopped up along with soil and the culture was then mixed @ 5% w/w in the upper two inch layer of sterilized soil : sand mixture (1:3) contained in earthen pots (10´ × 10´). The pots were seeded with Guinea grass (*Panicum maximum*). The seeded pots were kept under polyhouse and were watered as and when required with sterilized water (Kapoor *et al.* 2008). The grass was allowed to grow for three months.

Apple seedling stock was raised after stratifying the crab apple seed in a refrigerator for 60-80 days at 2-5°C. Stratified seeds were sown in plastic pots containing 3 kg sand: soil: FYM (1:2:1) mixture which was thoroughly sterilized for 2 hr at 15 lbs/inch^2 in an autoclave. Culture of each AMF isolate which contained chopped roots of Guinea grass mixed with rhizosphere soil along with chlamydospores was mixed (200 g/pot) in upper layer of soil before sowing of apple seeds in separate pots. Five to seven seeds per pot were sown and only two plants per pot were allowed to grow for 90 days under glass house conditions. These plants were watered with sterilized water as and when required. Observations on growth parameters (height, stem diameter, internodal length, shoot/ root fresh and dry weight) of the inoculated and uninoculated seedlings were taken as per the standard methods.

Apple replant disease (ARD) soil samples were collected from replant affected orchards along with virgin soil samples (of non-apple plantation site) from five different locations in wet temperate region of Himachal Pradesh. Soil samples collected from different locations (20 kg/location) were mixed to prepare a composite sample of ARD as well as virgin soil saparately. The experimental soil was then examined for its chemical characteristics such as pH and EC (Jackson 1967), Organic carbon (Piper 1966), available N (Subbiah and Asija 1956), P (Olsan *et al.* 1954) and K (Jackson 1967). The pH of virgin soil was found neutral, however, ARD soil had lower pH. EC value was in safe limits. Organic carbon contents were low in both the samples. Available P was 391.2 kg/ha in ARD and 257.6 kg/ha in virgin soil, whereas available K was 421.7 kg/ha in ARD soil and 315.8 kg/ha in virgin soil. The available P and K in both the soil samples were in higher amount while available N was low in virgin soil (263.4 kg/ha) and medium in ARD soil (288.5 kg/ha).

One half each of ARD soil and virgin soil was treated with 5 per cent formaldehyde solution separately and the other respective half's were left untreated. The treated soil was covered with 25 µ transparent polythene sheet in a manner so as to make it air tight for one week. After that the soil was uncovered and raking was done till the soil was free of fumes of formalin.

Well stratified crab apple seeds were sown (5-7 seeds/ pot) in winter months (December to March) in plastic pots containing 8 kg sand: Soil: FYM (1:2:1) mixture thoroughly sterilized for 2 h at 15 lbs/inch^2 in an autoclave. Some of the pots were inoculated at the time of sowing with culture of selected AMF isolate. The seedlings were allowed to grow for one year. Seedlings were uprooted from the nursery stock during second week of February and transplanted in the plastic pots (30 cm) containing 3 kg of soil. Pots were filled with soil having different treatments and two seedlings were transplanted in each pot.

The ARD and virgin soil collected from various locations were subjected to different treatments. The treatments were: 1: ARD soil, 2: virgin soil, 3: ARD soil + AMF inoculation, 4: virgin soil+ AMF inoculation, 5: ARD soil+ formaldehyde fumigation, 6: virgin soil+ formaldehyde fumigation, 7: ARD soil+ formaldehyde fumigation + AMF inoculation, 8: virgin soil+ formaldehyde fumigation + AMF inoculation. Each treatment was replicated five times.

Plant growth parameters such as seedling height (cm), stem diameter (mm), internodal length (cm), leaf area (cm^2), shoot root fresh and dry weight (g) were recorded after 90 days of transplanting. The data on seedling height, stem diameter were recorded at the time of transplanting and after 90 days of transplanting and in this way the increase in these parameters was calculated.

For estimation of NPK, collection, cleaning, grinding and storage of leaf samples were carried out as per the procedures of Kenworthy (1984) and Chapman (1964). Samples were digested for the estimation of N as described by Jackson (1967) and determined by micro-Kjeldhal's method (AOAC 1980). P was determined by Vandomolydo-phosphoric acid yellow colour method (Koeing and Johnson 1942) and K by EC12 Atomic Absorption Spectrophotometer Model-4129 (Jackson 1967) after digestion of sample as described by Piper (1966). The data thus generated were

expressed in percentage on dry weight basis.

Colonization of apple roots by AMF was assessed after 90 days. Seedlings were gently uprooted and the tertiary roots were cut in small pieces (1 cm). The root pieces were stained following differential staining method of Phillips and Hayman (1970). A set of stained root pieces was observed under microscope for the presence of mycelium, vesicles and arbuscules of the AMF and percent root colonization was observed by gridline intersect method of Giovannetti and Mosse (1980).

The spore population of AMF in soil was determined after extraction from soil by wet sieving and decanting technique of Gerdemann and Nicolson (1963). 50 g soil was suspended in 200 ml water in a flask, stirred vigorously and the heavier particles were allowed to settle down for few seconds. The suspension was then decanted through a series of sieves (300, 250, 106 and 45 μ) arranged one over another in a descending order. The residue left in each sieve was thoroughly washed under tap water and whatever residue left in the sieve was collected in a beaker and the final volume was made to 20 ml. 2 ml of the suspension was transferred to the counting plate and examined under stereoscopic binocular microscope and the number of spores per 50 g soil basis were thus calculated.

Colony forming units (cfu) per g of fungi, bacteria, and actinomycetes were observed in the rhizosphere soil of apple seedlings grown under different treatments by using dilution plate technique (Atlas 1995). The media used for this study were peptone dextrose rose Bengal agar medium (for fungi), nutrient agar medium (for bacteria) and starch ammonium agar medium (for actinomycetes).

The data generated under present study were analyzed statistically as per the method described by Gomez and Gomez (1976). The level of significance used for 'F' and 'T' was compared at 0.05.

RESULTS AND DISCUSSION

Evaluation of different isolates of indigenous arbuscular-mycorrhizal fungi

Under present study five isolates of indigenous arbuscular-mycorrhizal fungi (AMF), collected from apple orchard soil and multiplied on Guinea grass were evaluated for their effect on growth of apple seedlings. Perusal of the data presented in Table 1 indicated that all the AMF isolates increased the growth parameters of inoculated apple seedlings over control. However, maximum increase in growth parameters, viz. height, stem diameter, internodal length, leaf area, shoot/root fresh and dry weight was observed in the seedlings inoculated with isolate AMFS-2. The percentage of root colonization was also higher with this isolate as compared to others. Several workers have also reported increased growth of fruit plant seedlings including apple upon inoculation with AM fungi especially *Glomus* spp. (Hashmi *et al.* 2010, Krishna *et al.* 2006, Covey *et al.* 1981). The extra-materical mycelia of AM fungi serve to enhance the flux of phosphorus (P) into the host plant by its extension beyond the zones of depletion immediately around the roots. This increased uptake of P is considered as the main reason behind increased growth of mycorrhizal plants than non-mycorrhizal plants (Wilcox 1991).

Effect of AMF inoculation on growth of apple seedlings grown in apple replant disease soil

The data of evaluation studies indicated the superiority of isolate AMFS-2 amongst all the five isolates of AM fungi in colonizing the roots and increasing growth of apple seedlings. This isolate was then selected to see its effect on growth of apple seedlings grown in ARD soil collected from affected orchards. The data in Table 2 indicated that the growth parameters of apple seedlings, viz. height, stem diameter, internodal length, leaf area, shoot/ root fresh and dry weight increased significantly upon inoculation with indigenous AMF as compared to un-inoculated seedlings grown in ARD as well as virgin soil. The increase in height of AMF inoculated apple seedlings grown in ARD soil was 14.07 cm as compared to 12.89 cm in non-mycorrhizal seedlings after 90 days of transplanting. Similar trend in other growth parameters of seedlings was observed upon mycorrhizal inoculation. Mycorrhizal inoculated seedlings when grown in fumigated ARD soil also showed enhanced growth as compared to un-inoculated seedlings grown in fumigated ARD soil. The increase in height and stem diameter of mycorrhizal inoculated apple seedlings grown in fumigated ARD soil was 23.90 cm and 1.92 mm as compared to 19.60 cm and 1.75 mm, respectively in non-mycorrhizal seedlings grown in fumigated ARD soil. Utkhede and Smith (2000) also observed increased growth of apple seedlings in ARD soil upon mycorrhizal inoculation and this increased growth provided by mycorrhizal inoculation in ARD soil was attributed to be due to increased absorption of phosphorus and other nutrients by the inoculated seedlings. In the present study also, a significantly higher percentage of P content in mycorrhizal inoculated seedlings was observed as compared to uninoculated ones (Table 3). Mosse (1973) has already reported that AM fungi benefited host plant primarily by increased uptake of P, Zn, Cu, Fe and K. These nutrients not only increase the growth but also make the host plants strong enough to tolerate the attack of various pathogens. Increased concentration of P might be the reason for better growth of mycorrhizal inoculated apple seedlings grown in ARD soil. Moreover, mycorrhizal associations are known to impart resistance to host plants against various soil-borne pathogens (Azcon-Aguilar and Barea 1996) which might be another reason behind enhanced growth of apple seedlings grown in ARD soil. In apple, lower incidence of root rot (*Dematophora necatrix*) and better growth in apple seedlings upon inoculation with indigenous AM fungi was observed by Bharat and Bhardwaj (2001) and Raj and Sharma (2009)

Table 1 Effect of indigenous AMF cultures on apple seedlings growth parameters and percentage of root colonization

AMF isolate	Seedling height (cm)	Stem diameter (mm)	Internodal length (cm)	Leaf area (cm^2)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)	AMF root colonization (%)*
AMFS-1	18.57^{b}	3.04^{ab}	1.57^{ab}	12.26^{ab}	5.30^{a}	2.43^{ab}	2.25^{a}	1.00^{ab}	11.24 (3.49)^{a}
AMFS-2	21.56^{a}	3.33^{a}	1.94^{a}	12.97^{a}	5.75^{a}	3.25^{a}	2.46^{a}	1.25^{a}	13.00 (3.61)^{a}
AMFS-3	19.47^{ab}	3.09^{ab}	1.74^{ab}	12.74^{a}	5.62^{a}	3.00^{a}	2.37^{a}	1.25^{a}	11.48 (3.48)^{a}
AMFS-4	16.04^{c}	3.06^{ab}	1.47^{b}	10.12^{b}	4.67^{ab}	2.62^{a}	1.93^{ab}	1.12^{ab}	9.64 (3.25)^{ab}
AMFS-5	14.00^{cd}	2.88^{b}	1.31^{b}	10.08^{bc}	3.71^{b}	1.50^{b}	1.31^{b}	0.62^{b}	9.04 (3.11)^{b}
Control	12.65^{d}	2.42^{c}	1.22^{b}	8.70^{c}	2.92^{b}	1.25^{b}	1.11^{b}	0.31^{b}	0.00 (0.00)^{c}
CD (P=0.05)	1.52	1.25	0.15	2.16	1.43	1.08	0.86	0.54	0.37

Figures in each column are mean value of five replicates Values within a column with different letters are significantly different at P = 0.05, *Figures in parentheses are square root transformed value

Table 2 Effect of AMF inoculation and other treatments on growth parameters of apple seedlings grown in replant disease soil

Treatment	Increase in seedling height (cm)	Increase in stem diameter (mm)	Intrenodal length (cm)	Leaf area (cm^2)	Shoot fresh weight (g)	Root fresh weight (g)	Shoot dry weight (g)	Root dry weight (g)
Apple replant diseased (ARD) soil	12.89^{e}	1.33^{e}	1.39^{b}	15.57^{d}	14.80^{c}	6.25^{e}	8.00^{d}	3.75^{c}
Virgin soil (Non apple soil)	14.85^{d}	1.62^{c}	1.41^{b}	15.50^{d}	14.45^{c}	7.10^{de}	8.80^{cd}	4.50^{b}
ARD soil + AMF inoculation	14.07^{d}	1.45^{d}	1.43^{b}	15.65^{d}	17.30^{b}	8.10^{d}	9.40^{c}	4.95^{b}
Virgin soil + AMF inoculation	19.98^{b}	1.72^{b}	1.44^{ab}	18.56^{c}	15.75^{bc}	8.00^{d}	9.60^{bc}	5.15^{b}
ARD soil + formaldehyde fumigation	19.60^{b}	1.75^{b}	1.43^{b}	20.03^{b}	20.07^{a}	9.50^{c}	11.70^{ab}	5.45^{ab}
Virgin soil + formaldehyde fumigation	18.08^{c}	1.55^{c}	1.44^{ab}	18.56^{c}	19.20^{ab}	9.35^{cd}	10.65^{b}	5.50^{ab}
ARD soil + formaldehyde fumigation + AMF inoculation	23.90^{a}	1.92^{a}	1.51^{a}	20.32^{b}	21.45^{a}	12.55^{a}	12.0^{a}	5.80^{a}
Virgin soil + formaldehyde fumigation + AMF inoculation	24.40^{a}	1.92^{a}	1.50^{a}	24.42^{a}	20.85^{a}	10.85^{b}	12.10^{a}	6.00^{a}
CD (P=0.05)	1.04	0.08	0.07	0.82	3.10	1.33	1.10	0.66

Figures in each column are mean value of five replicates, Values within a column with different letters are significantly different at P = 0.05

under Indian conditions. They attributed the decreased incidence of root rot to the increased growth and disease resistance in mycorrhizal inoculated apple seedlings than non-mycorrhizal ones. Irrespective of mycorrhizal inoculation, the fumigation of ARD soil with formaldehyde also showed increase in growth of apple seedlings as compared to non-fumigated ARD soil (Table 2). Fumigation of ARD soil has also been found to increase height and girth of apple seedlings by Liu and coworkers (2005) and this increased growth was correlated with the destruction of disease causing microflora in treated soil. Soil fumigation is believed to disturb natural equilibrium between pathogen and antagonistic microorganism in soil as it destroyed all microorganism whether pathogenic or beneficial in the treated soil. Due to this reasons sometimes seedlings grown in fumigated ARD soil could not show good growth because of the destruction of beneficial soil microflora like AM fungi. It is further confirmed in the present study as mycorrhizal colonization and spore population was observed negligible in fumigated

Table 3 Effect of AMF inoculation and other treatments on leaf nutrient status of apple seedlings grown in replant disease soil

Treatment	N (%)	P (%)	K (%)
Apple replant diseased (ARD) soil	2.42	0.17^{bc}	1.80^{ab}
Virgin soil (Non apple soil)	2.40	0.16^{c}	1.82^{ab}
ARD soil + AMF inoculation	2.43	0.20^{a}	1.86^{a}
Virgin soil + AMF inoculation	2.43	0.18^{b}	1.85^{ab}
ARD soil + formaldehyde fumigation	2.40	0.13^{d}	1.77^{b}
Virgin soil + formaldehyde fumigation	2.40	0.13^{d}	1.76^{b}
ARD soil + formaldehyde fumigation + AMF inoculation	2.40	0.17^{bc}	1.79^{b}
Virgin soil + formaldehyde fumigation + AMF inoculation	2.41	0.17^{bc}	1.80^{ab}
CD (P = 0.05)	N.S.	0.01	0.06

Figures in each column are mean value of five replicates values within a column with different letters are significantly different at $P = 0.05$

Table 4 Effect of AMF inoculation and other treatments on AMF spore population and root colonization of apple seedlings grown in replant disease soil

Treatment	Number of spores/50g soil	AMF root colonization (%)*
Apple replant diseased (ARD) soil	214[b]	16.36 (4.16)[d]
Virgin soil (Non apple soil)	148[d]	13.46 (3.80)[e]
ARD soil + AMF inoculation	286[a]	25.48 (5.14)[a]
Virgin soil + AMF inoculation	200[c]	21.06 (4.69)[b]
ARD soil + formaldehyde fumigation	4[f]	0.00 (0.00)[f]
Virgin soil + formaldehyde fumigation	3[f]	0.00 (0.00)[f]
ARD soil + formaldehyde fumigation + AMF inoculation	128[e]	17.88 (4.34)[c]
Virgin soil + formaldehyde fumigation + AMF inoculation	116[e]	16.24 (4.15)[d]
CD (P=0.05)	13	0.05

Figures in each column are mean value of five replicates Values within a column with different letters are significantly different at P = 0.05, *Figures in parentheses are square root transformed value

Table 5 Effect AMF inoculation and other treatments on soil microflora associated with apple seedlings grown in replant disease soil

Treatment	Fungi ($\times 10^3$ cfu/ g soil)	Bacteria ($\times 10^4$ cfu/ g soil)	Actinomycetes ($\times 10^3$ cfu/ g soil)
Apple replant diseased (ARD) soil	20.25[a]	94.75[a]	5.0[a]
Virgin soil (Non apple soil)	10.25[c]	66.0[b]	2.75[c]
ARD soil+ AMF inoculation	16.50[b]	66.25[b]	3.75[b]
Virgin soil+ AMF inoculation	6.50[d]	56.25[c]	1.75[d]
ARD soil+ formaldehyde fumigation	5.25[e]	25.75[e]	1.50[d]
Virgin soil+ formaldehyde fumigation	4.0[f]	26.50[e]	1.20[d]
ARD soil + formaldehyde fumigation + AMF inoculation	4.0[f]	30.25[d]	1.25[d]
Virgin soil + formaldehyde fumigation + AMF inoculation	3.50[f]	28.25[de]	1.50[d]
CD (P=0.05)	1.23	3.64	0.82

Figures in each column are mean value of five replicates, Values within a column with different letters are significantly different at P = 0.05

ARD as well as virgin soil while root colonization and spore population were observed maximum in the roots of apple seedlings grown in non-fumigated ARD and virgin soil inoculated with AM fungi (Table 4). Under present study prior fumigation of ARD soil with formaldehyde and plantation of mycorrhizal inoculated seedlings proved as an effective measure to reduce the effect of apple replant problem and get better growth of seedlings. The increased growth of apple and peach seedlings in fumigated soil upon mycorrhizal inoculation was also observed by Bingyne and Shengrui (1998). They were of the opinion that the increase in seedling growth was because of increased activity and response of AM fungi in fumigated soil. And the increased AM fungal response was due to the reduced population of competitive soil microorganisms after fumigation. In present studies also, the population of soil microflora, especially fungi (Table 5) other than AM fungi was minimum in the rhizosphere of mycorrhizal seedlings grown in fumigated ARD as well as virgin soil as compared to non-mycorrhizal seedlings grown in non-fumigated soil. Increased uptake of phosphorus and reduction in the population of harmful microflora in the rhizosphere of apple seedlings inoculated with AM fungi might be the reasons behind the better growth of AMF inoculated seedlings in fumigated and non-fumigated ARD soil.

Therefore, it can be concluded that artificial inoculation of newly planted apple seedlings with potent indigenous AM fungal culture (*Glomus fasciculatum*) in formaldehyde fumigated or non-fumigated soil proved as a good management practice for apple replant problem.

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