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Physico-chemical analysis of rhizospheric soil, mycorrhizal association and root colonization of Mulberry (*Morus alba* L) plants grown in Kalimpong hills

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Abstract
Morus alba L, known as white mulberry, is widely cultivated in the hills and its leaves are the major source of food for the silkworms (*Bombyx mori* L.) employed in the commercial production of silk. The physico-chemical properties of the soil where this plant is cultivated were analysed. The soils analysed had medium to high nitrogen content, but low to medium phosphorus and potash. Since mycorrhizal fungi can help in nutrient mobilization, AMF associated with the rhizosphere of the mulberry tree cultivated in RSRS, Kalimpong were extensively studied in relation to their population in soil, root colonization as well as their diversity. Predominant species of *Glomus* and few species of *Gigaspora* was determined. Histo-pathological studies of host roots showed the presence of vesicles and different kind of hyphal network. AMF spores were tried to be identified up to species level with the help of standard keys. The result indicates that various species of AMF have established successful colonization with the host plant that will further support and help the plant for better growth and development.

Key words: AM Fungi, *Morus alba*, *Glomus* sp, *Gigaspora* sp.

Mulberry (*Morus* spp) is an economically and traditionally very important deciduous plant for the development of sericulture industry. Mulberry plants belong to the family Moraceae and are successfully grown under tropical to temperate climatic conditions in various part of the country. Mulberry leaves are basic food material for silkworm *Bombyx mori* L. The nutritious leaves are the most important growth regulating factors for these silkworms, because, being monophagous insect, they derive almost all the essential nutrients from the mulberry leaves for their survival. Hence, good quality of nutritious mulberry leaves should be fed in abundant quantity for quality silkworm seed and cocoon production.

It is a well known fact that, sericulture is an agro based industry and soil management is a formidable challenge to ensure the productivity and profitability for sustainable sericulture. The yield and quality of mulberry leaves are directly or indirectly affected by "how the soil is handled". From sericultural point of view, the soil characteristics may be referred to as the ability of the soil to produce quality sericulture host plants in a sustainable manner. On the other hand, soil characteristics can be defined as the quality or capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation (Karlen et al., 1997).

Arbuscular Mycorrhizal (AM) fungi are ubiquitous in terrestrial ecosystems and form mutualistic relationship with more than 80 per cent of major group of vascular plants. Root colonization with AMF is a dynamic process, which is influenced by several edaphic factors such as nutrient status of soil, seasons, arbuscular mycorrhizal (AM) strains, soil temperature, soil pH, host cultivar susceptibility to AM colonization and feeder root condition. Mycorrhiza forms critical link between the plant and soil structure and make a large direct contribution to plant growth, plant protection, survival, disease suppression and soil fertility and quality through contribution of soil organic matter, especially phosphorus that is mobilized by the fungus. (Chakraborty et al, 2012).

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Association of AM Fungi with different host plants have been observed by various authors (Allay *et al*, 2012; Chakraborty *et al*, 2013; De *et al*, 2013; Subhashini, 2013) where they have clearly shown that such kind of mutualistic symbiosis occuring in the roots and associated rhizosphere enhances the growth and health status of host plants. Hence this study was carried out to screen the naturally occuring AM fungi with the mulberry plants at the RSRS, Kalimpong so that these can be further utilized for sustainable agriculture for improvement of sericulture.

Materials and Methods

Site characteristics and analysis of soil samples:

The rhizospheric soil samples were collected from two locations of the mulberry field of Regional Sericultural Research Station (RSRS), Kalimpong. The mulberry (*Morus alba* L) variety $BC_2$59 was chosen for the collection of the rhizospheric soil samples for microbial isolation because this variety is known for high yield with nutritious leaves and more popular in the whole hilly region of Darjeeling and Sikkim. The soil samples for morpho-physico-chemical analysis were collected from the entire field, dried, sieved and analyzed by adopting the standard procedure (Black 1985; Jackson 1979).

Isolation of AMF spores from soil

Arbuscular mycorrhizal fungal spores were screened from the rhizospheric soil of the mulberry plant following the Wet sieving and decanting method (Gerdemann and Nicholson,1963). Approximately 250g of soil was suspended in 1litre or more of water. Heavier particls were allowed to settle for few seconds and the liquid was decanted through sieves of various size with stainless steel mesh (60, 85, 100, 150,175 and 200μm), fine enough to remove the larger particles of organic matter but coarse enough for the desired particles to pass through. The suspension that passed though this seive was saved and stirred to resuspend all particle. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different petri plates according to their size and colour. Moreover for further observations or purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982), spores and minimal amount of organic particles were further purified by suspending sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 meshes and rinsed with distilled water to remove sucrose residue. With the help of a simple microscope (20X) parasitized spores, plant debris etc. were separated and clean spores were observed in water (Spain, 1992) and stained with Poly Vinyl Lacto Glycerate (PVLG) and studied microscopically. For further use the spores were stored in Ringer's Solution (8.6g NaCl, 0.3g KCl, 0.33g CaCl-2 in 1 lit. of boiled distilled water) at 4°C or in sterile distilled water.

Spore samples were separated according to their morphology, size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachments etc. for the purpose of identification. The spores were identified up to species level with the help of standard keys (Walker 1992).

Spore count

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

Histo-pathological analysis

The root colonization of Mulberry plants grown in field condition was studied according to the method outlined by Philips and Hayman (1970) for histopathological studies. Roots were cut into 1 cm or smaller pieces and washed in tap water. It was boiled in 2% KOH in hot water bath for one hour. The KOH was decanted and the roots washed with water for 2-3 times. 1% HCl was added and kept for 30 minutes. After decanting the HCl the sample was washed thrice in tap water and cotton blue,

lactic acid and glycerol was added in the ratio 1:1:1 to stain the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxillary cells, and boiled in water bath for 1 hour. The excess stain was decanted and sample placed in 50% glycerol for destaining. The roots were then crushed under pressure in slide and covered with coverslips for microscopic observation. The hyphal structures were viewed under dissecting stereomicroscope under 20X and 40X magnification.

Root colonization

Percentage of root colonization with AMF was determined by counting all the infected and uninfected segments of root tissues and the percentage of infection was calculated as follows AMF infection (%) = [infected root segments/total fragments of roots taken] X 100

Results

Meteorological and geographical analysis

Kalimpong, a block of Darjeeling district (W.B.) is a hilly range of Sub-Himalayan Mountains and a sericulture hub for production of bivoltine silkworm seed cocoons in Eastern and North-eastern India. The Kalimpong hilly area lies between 26°31' to 27°13' N latitudes and 87°59' to 88°53' E longitudes and situated at an altitude of about 3550 feet (1076 m) above MSL. Sandstone, quartzite and mica are the major geologic formation in this area which act as parent materials for the formation of the soils this area. River Teesta and its tributaries are the main water bodies. The climate is sub-tropical type (Sub-Himalayan region) where mean annual temperature 21.0°C with mean summer temperature (May, June and July) 24.6°C and mean winter temperature (December, January & February) 15.2°C (Table 1; Fig. 1 and Fig. 2) respectively. The mean annual precipitation of this area was recorded 1876.3 mm, of which 90.3 percent occurs between June to September. Based on meteorological data, soil moisture regime has been grouped as '*Udic*' and soil temperature regime as '*Thermic*' respectively.

The age of mulberry plantation in RSRS, Kalimpong

Table 1: Meteorological data (2011-2012) of Kalimpong hilly area

Month	Monthly Temperature °C		Mean Monthly Temperature °C	Monthly Rainfall (mm)
	Maximum	Minimum		
January	17.8	8.2	13.0	7.4
February	21.9	11.0	16.5	8.8
March	25.4	14.4	19.9	36.4
April	27.1	16.2	21.7	72.9
May	29.9	19.4	24.6	26.3
June	28.5	21.3	24.9	385.6
July	27.6	21.4	24.5	527.4
August	29.5	21.3	25.4	387.3
September	28.3	20.6	24.4	394.2
October	26.6	17.4	22.0	26.0
November	24.9	12.7	18.8	3.9
December	22.5	10.2	16.3	0.1

(Source: Regional Sericultural Research Station, Kalimpong)

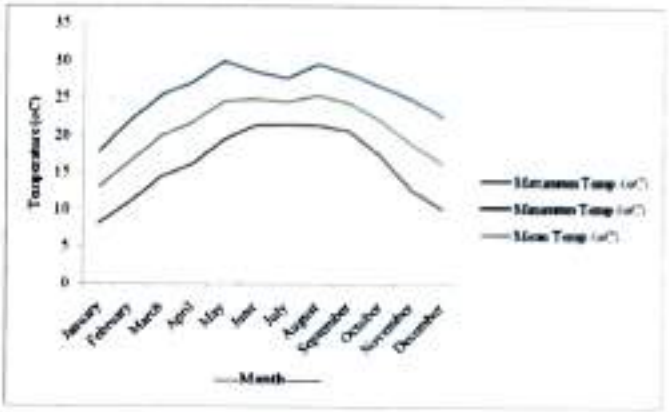

Fig 1: Temperature variations during different months at Kalimpong

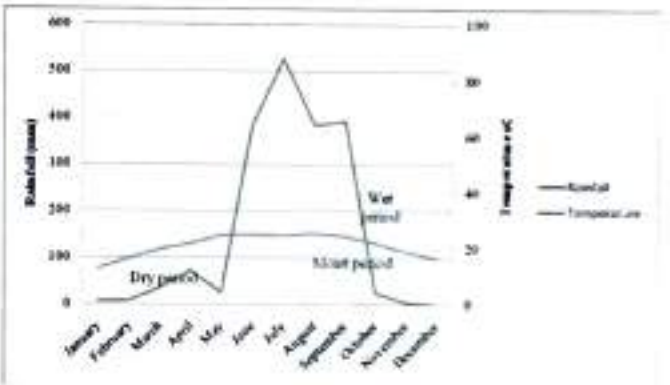

Fig 2: Rainfall and temperature recorded during different months at Kalimpong

research farm (sample 1) is 20 years and above with approximate canopy 70-80%, however, the age of the mulberry plantation in the RSRSA, Kalimpong research farm (sample no. 2) is 10 years and above with approximate canopy 30-40% respectively. The spacing of the plant is 90 X 90 cm and plant nutrients recommended for this area

are NPK (kg ha^{-1}): 150:50:50 and FYM@10 MT ha^{-1} respectively.

Morpho-physico-chemical properties of soils

Morpho-physico-chemical properties in the soils of two plots and sampling sites (1 & 2) of RSRS nad RSRSA, Kalimpong is given in Table 2. Based on the rhizospheric soil samples of few terraces of plot no. 5 analyzed, sand, silt and clay percent in this plot are 73%, 10% and 17% respectively which classified as sandy loam. The soil of the sample 2 was also very similar with sand, silt and clay being 73%, 11% and 16% respectively. Chemical characteristics and nutrient availability e.g. pH (1:2.5), Electrical conductivity (dSm-^{1}), organic carbon (%), available NPK ((kg ha^{-1}) in this research farm are 5.57, 0.10, 0.84, 481.0, 14.1 and 179.2 respectively, while of site 2 was 4.5, 0.10, 0.54, 398.8, 14.6 and 257.6 respectively.

Table 2: Physico-chemical properties of soil sample

Sample No.	Site	Sand	Silt	Clay	Texture	pH (1:2.5)	EC (dSm-^{1})	Organic C (%)	Available Nitrogen (kg ha^{-1})	Available Phosphorus (kg ha^{-1})	Available Potash (kg ha^{-1})
		(%)									
Sample 1	Rhizospheric	73	10	17	Sandy loam	5.57	0.1	0.84	481.0	14.1	179.2
	Range (whole research farm)	64-73	10-16	17-20	Sandy loam to sandy clay loam	5.03 - 6.90	0.06-1.07	0.84-2.34	338.7-722.5	14.1-23.3	112.0-291.2
Sample 2	Rhizospheric	73	11	16	Sandy loam	4.5	0.1	0.54	398.8	14.6	257.6
	Range (whole research farm)	66-73	11-18	16-20	Sandy loam to sandy clay loam	4.20-6.50	0.05-0.40	0.36-1.98	353.7-654.8	9.4-19.0	112.0-313.6

Morpho-physico-chemical properties in the soils of entire research farm are variable. Soils of this research farm are shallow to very deep in depth; dark yellowish brown (10 YR 4/4) to brown (10 YR 5/4 and 6/4) in colour; sandy loam to sandy clay loam texture; single grain to fine, medium, subangular blocky structure; dry semi hard, moist very friable to friable, wet slightly sticky to sticky and wet slightly plastic consistency; very fine to fine, few to many pores and clear to gradual wavy horizon boundary.

Based on terrace wise soil samples analyzed from entire research farm in sample 1, the pH in the soils ranged from 5.03 to 6.90; EC ranged from 0.06 to 1.07 dSm-^{1}; organic carbon ranged from 0.84 to 2.34%; available nitrogen ranged from 338.7 to 722.5 kg ha^{-1}; available phosphorus ranged from 14.1 to 23.3 kg ha^{-1} and available potash ranged from 112.0 to 291.2 kg ha^{-1} respectively. In sample 2 the pH ranged from 4.20 to 6.50; EC ranged from 0.05 to 0.40 dSm-^{1} organic carbon content ranged from 0.36 to 1.98%; available nitrogen ranged from 353.7 to 654.8 kg ha^{-1} available phosphorus and potash ranged from 9.4 to 19.0 kg ha^{-1} and 112.0 to 313.6 kg ha^{-1} respectively.

Association of AMF in rhizosphere of Mulberry tree

AMF spores were collected from host plant at two different sites (Sample 1 and Sample 2). The morphological features of the isolated spores were critically screened with special reference to their variance in size, wall thickness, shape and wall layers. In both the samples presence of *Glomus constrictum* was abundant followed by *G clarum* and some other *Glomus* sp. Presence of *Gigaspora* sp was only about 1% where mainly *G gigantea* was prominent. Detailed microscopic observations were made, photographs taken, all the essential spore charecters were noted and were compared with the available monographs and other literature (Figure 3) Description of the spore charecters of the prevelant AMF are given in Table 3. Percentage of spores present in both the samples are also shown in figure 4. Histopathological analysis of the root samples revealed the presence of intra and inter-radical hyphae, vesicles and arbuscules.

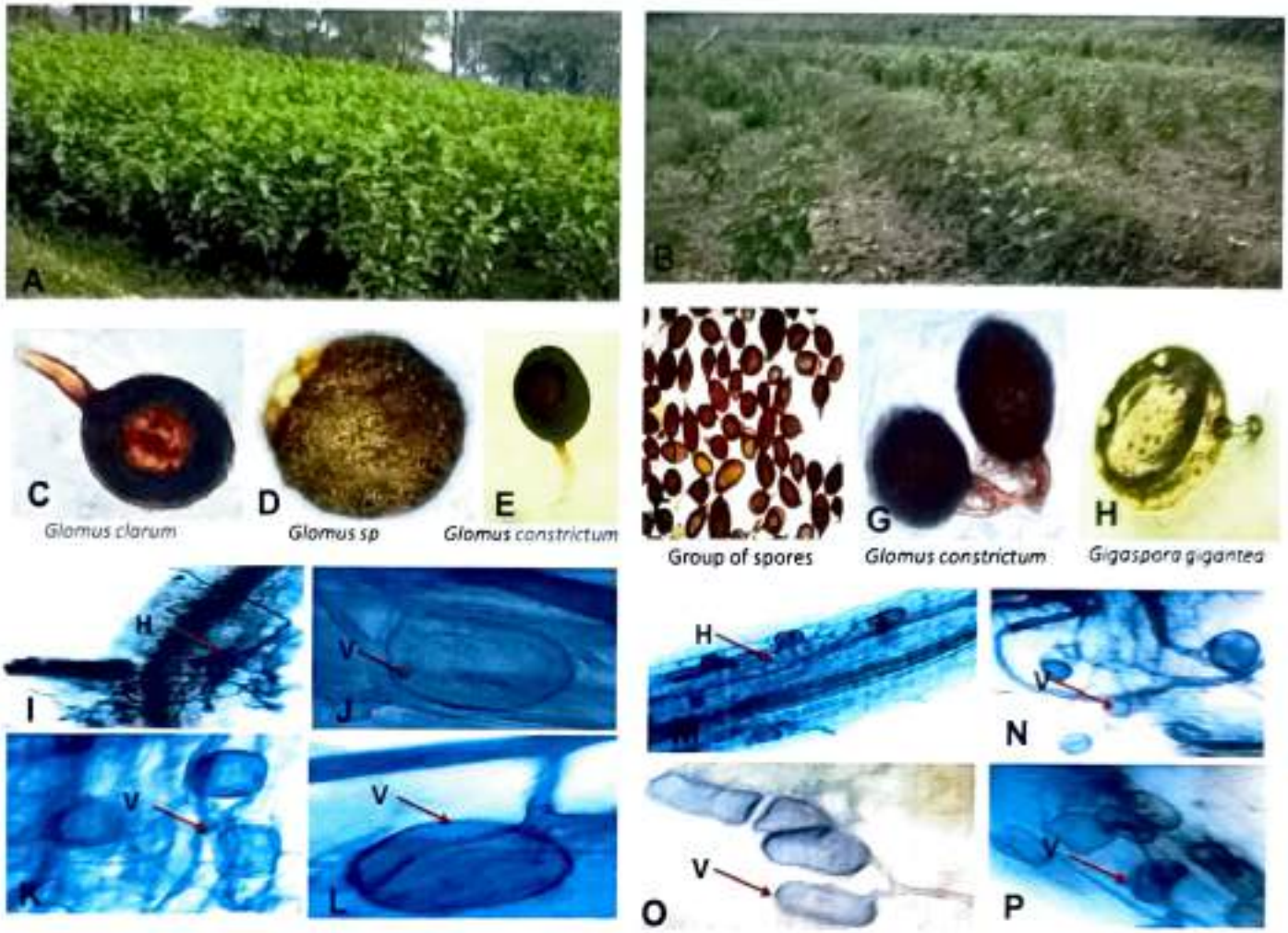

Figure 3: A,B – Morus alba (Variety $BC_2$59) at two different sites, C,D,E – AMF spores isolated from soil at location 1, E,F,G – AMF spores isolated from soil at location 2; I,J,K,L - Histopathology of roots of showing hyphae (H) and vesicles (V) at location 1; M,N,O,P - Histopathology of roots of showing hyphae (H) and vesicles (V) at location 2

This colonization of host root with AMF signifies the establishment of mycorrhizal network in soil and root that helps in symbiotic exchanges between the fungi and the host plant that will increase the immobilization of nutrients and will improve the plant growth.

Discussion

While studying the morpho-physico-chemical properties in the soils of both research farms, it was found that, there was no much textural variation in this soil. However, the variation in soil depth as reported above was due to nature of terrace, rocky phase, sloppy land developed on hill side and soil erosion etc. The light texture was due to sandstone, quartzite and other light textured secondary rocks acted as parent material for the formation of this soil. Poor soil structure and low consistency was

Table 3: Microscopic characters of AMF spores associated with Mulberry roots

Genus and Species	Colour	Shape	Spore Layer	Spore size (µm)	Other description
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 clarum	Hyaline to pale yellow	Globose to subglobose sometimes ovoid	3	90-100 x 140-180	One subtending hyphae, straight to curved, hyaline to pale yellow wall and thick at the spore base.
Gigaspora. Gigantea	Greenish yellow	Globose to Subglobose	2	250-270 x 265-370	Formed terminally or laterally on a bulbous sporangenous cell

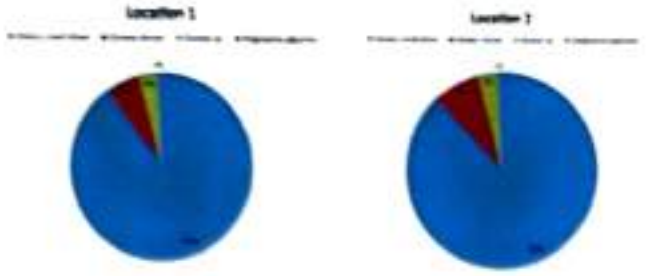

Fig. 4 Percentage of spore population in rhizospheric soil of *Morus alba* from two locations

due to light soil texture.

The variation of colour was due to prevalence of well drained conditions, admixture of organic matter (Ram *et al*., 2013; Rao *et al*., 2008) whereas the variation in soil texture was caused by slope, terrace and translocation or illuviation of clay in lower horizons. (Nayak *et al*., 2002). The variation in soil structure and consistency was due to variation in clay content of pedons (Singh and Agrawal, 2003). While comparing the nutrients availability between both research farms, it was observed that, the pH and EC of the soils of RSRSA farm was quite low and grouped under strong to moderate acidic. Likewise, the OC content in the soils of RSRSA farm was also quite low than RSRS research farm.

Considering the range <272 kg ha^{-1} 'low', 272-544 kg ha^{-1} medium' and >544 kg ha^{-1} high' (Baruah and Barthakur, 1997), the soils of both research farms and farmers' fields have medium to high available nitrogen content, which highly differ from plot to plot and terrace to terrace. In general, soils of Kalimpong hills were recorded as medium to slight high nitrogen content. High nitrogen content might be due to forest leaf litter, application of farm yard manures and seri compost, which helps to improve the physical, chemical and microbial properties of soils.

Next to nitrogen, phosphorus is a very important essential nutrient for plant growth and is found in every living plant cell. It is involved in several key plant functions, including energy transfer, photosynthesis, transformation of sugars and starches, nutrient movement within the plant and transfer of genetic characteristics from one generation to the next. Considering the range <22.5 kg ha^{-1} 'low', 22.5-56.0 kg ha^{-1} medium' and >56.0 kg ha^{-1} high' (Baruah and Barthakur, 1997), the soils of both research farms have low to medium available phosphorus content, which significantly varied from plot to plot and terrace to terrace. The availability of phosphorus were significantly correlate with soil pH, slope and size of terraces, hence, soils of RSRSA found highly deficient of phosphorus followed by RSRS farm and farmers' field.

Potash is third essential macronutrients. Like nitrogen, potash is absorbed by plants in significantly larger quantities than any other nutrient. It accumulates mainly in vacuole and cytoplasm without forming organic matter in cells and play very important role in the plant metabolism, resistance to lodging, frost, winter-hardiness and prevention from the disease and pests etc. Considering the range <136.0 kg ha^{-1} 'low', 136.0-338.0 kg ha^{-1} medium' and >338.0 kg ha^{-1} high' (Baruah and Barthakur, 1997), the soils of both the research farms and farmers' fields have low to medium available potash content, which significantly varied from plot to plot and terrace to terrace.

Predominant AMF species of another sericulturally important plant, *Persea bombycina*, was studied by Chakraborty et al. (2013), where presence of *Glomus* sp was dominant, followed by *Scutellospora*, *Acaulospora* and *Gigaspora* sp. Histopathology of the roots also revealed the presence of hyphae, vesicles and arbuscules showing successful colonization of AM fungi within the roots of the host plants. De *et al* (2013) studied the predominant existence of various populations of *Glomus* and *Gigaspora* sp in *Thuja orientalis* (L). Histopathological studies of host roots showed various types of hyphal network and arbuscules which indicated that various spp. of AMF have established their colonization in host root tissue that signified beneficial symbiotic exchanges between fungi and plant. AMF in commercially available form (Phosphofert) have been utilised as a biofertiliser as a part of integrated soil nutrient managementof mulberry under temperate climate (Rathore *et al*., 2011). Efficacy of native bioinoculants, viz. AM fungi and Azotobacter, separately as well as in combination was evaluated for enhancing biomass productivity of *Morus alba*

where combined imoculation gave best results with respect to growth, yield and microbial populations. (Sharma et al., 2005). This suggests that the association of AM fungi with the plant roots improves the growth of Mulberry.

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