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Association of AM fungi in the rhizosphere of *Thuja orientalis* (L)

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Abstract
Thuja orientalis (L) is used as an ornamental plant as well as medicinal plant in India from prehistoric times and instances were found in various manuscripts revealing its medicinal property against abdominal pain, hook worms, arthritis etc. AMF associated with rhizosphere of *Thuja* were extensively studied in relation to their population in soil, root-length colonization, histopathological study as well as their diversification. Predominant existence of various species of *Glomus, Gigaspora* were determined and their spore surface texture was examined using stereo microscope, besides, species of *Acaulospora, Scutellospora* etc. were also documented. Histopathological studies of host roots showed various types of hyphal network and arbuscules. AMF were tried to identify up to species level with the help of standard keys. The result indicated that the various spp. of AMF have established their colonization in host roots and the host plant have a significant role in root tissue colonization.

Key words: AM Fungi, *Gigaspora sp, Glomus sp, Thuja orientalis*

AM fungal associations have been described in ornamental maples (*Acer* sp.) (Brechet and le Tacon, 1984; Spiess *et al.*, 1991), Prunus genera, such as peaches (*Prunus persica* L.) (McGraw and Schenck, 1980) and sand cherry (Prunus ×cistena N.E. Hansen) (Morrison *et al.*, 1993), and have been recovered from roots of flowering dogwood (*Cornus florida* L.) (Sylvia, 1986). In India, only during the last decade the interest aroused on the role of VAM in rehabilitation of disturbed ecosystems (Kumar *et al.* 1999). Earlier studies on the occurrence of Arbuscular mycorrhizal fungi in medicinal plants mostly concentrate on rhizomes (Taber and Trappe 1982; Selvaraj *et al.* 1986). Later, Nasim (1990), Udea *et al.* (1992), Gautam and Sharma (1996), Selvaraj *et al.* (2001), Muthukumar *et al.* (2001), and Panwar and Tarafdar (2006) reported the occurrence of medicinal plants from India. However AMF association with some important medicinal plants of Sagar, Goa was performed (Deepak Vyas, 2007). Beside this AM association in various medicinal plants were extensively done in Indian subcontinent (Muthukumar and Udaiyan, 2001).

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Mycorrhiza form symbiotic associations between most of the terrestrial plants and play an essential role in plant growth, plant protection, survival, disease suppression and soil quality. The hyphal network/arbuscules promotes the bi-directional nutrient movement where soil nutrients and water move to the plant and plant photosynthates flow to the fungal network. AMF are obligate symbionts and improve the uptake of phosphate and other nutrients from the soil which results for drought and disease resistance to the host plant. The main benefit to the host plant in the mycorrhizal symbiosis is the enhanced uptake of immobile soil nutrients, in particular phosphorus (Jakobsen, 1999). Arbuscular mycorrhizal associations increase nitrogen accumulation in plant tissues as a result of the hyphae out competing for mineralized organic soil nitrogen (Ibijbijen *et al.*, 1996). *Thuja orientalis* (L) of cupressaceae family is an evergreen plant and is mostly used as ornamental plants in the gardens besides this plant is also known for its medicinal values where the leaves of these plants are used as antibacterial, antipyretic, antitussive, astringent, diuretic, expectorant and stomach upsets. Their use is said to improve the growth of hair. They are used internally in the treatment of coughs, hemorrhages, bronchitis,

asthma, skin infections, mumps, bacterial dysentery, arthritic pain and premature baldness. The leaves were harvested for use as required and can be used fresh or dried. Some proponents claim that thuja decreases the toxic effects of chemotherapy and radiation therapy. Herbalists prescribe thuja to treat viral and bacterial infections and coughs and other respiratory ailments, including strep throat and respiratory distress related to congestive heart failure. Herbalists also use it as a diuretic to increase urination, and as an astringent to "purify the blood," reduce inflammation, and cleanse the body of "toxins." Thuja is sometimes used with antibiotics to treat bacterial skin infections and herpes sores. It has even been used by some practitioners to induce abortions. Thuja ointment is applied to the skin for ailments such as psoriasis, eczema, vaginal infections, warts, muscle aches, and rheumatism. The association of AM fungi in such type of plants may vary greatly and is of much importance to know the details of the symbiosis. In the present investigation attempts have been made to find out the association of AM fungi in the rhizosphere of *Thuja orientalis* (L), since no such work has yet been reported.

Materials and Methods

Wet sieving and decanting method of Gerdemann and Nicolson, (1963) was used for the isolation of AMF spores. Approximately 250 gm of soil was suspended in 1 liter of water. Heavier particles were allowed to settle for a few minutes and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200 where BS stands for British Standard) are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension was then passed through these sieves resulting in trapping of heavier particles in the upper zone and light and smaller particles are trapped gradually in the sieves arranged in a descending order from fine to finer. The suspended water were allowed to settle for a 10 minutes and then the liquid was decanted again through the sieve and spores were collected by fine brushes and were kept in different petri plates according to their size and colors. Moreover for further observation for the purification of AMF spores sucrose gradient centrifugation method of Daniels and Skipper, 1982 was used. Spores and minimal amount of organic particles were further purified by suspending sieving in the 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) were poured through the sieve of 200 mesh and rinse 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 stained with Melzar's reagent and studied microscopically. For further use the spores were stored either in distilled water or in Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in 1 liter of boiled distilled water) at 4°C.

For the histopathological study, the root specimens were processed according to the method of Phillips and Hayman (1970). The root samples were cut into 1 cm and were cleared by 2% KOH/NaOH for 15 min at 90°C. Then the samples were washed thrice in tap water and kept for 30 min in 2% HCl. The root samples were boiled for 15 min with 0.05 Cotton Blue. Degree of contrast between fungal tissues and back ground plant cells was obtained according to the duration of storage of the tissues. 1% HCl was added to acidify the tissues as most histological stains are acidic.

The method of Phillips and Hayman (1970) which was followed for histopathological studies revealed that due to boiling in water bath twice, the extra radical hyphae, auxiliary cells and other fungal structures are dissociated in the solution resulting a wrong interpretation for the determination of the percent colonization. So this method was slightly amended and instead of boiling, the root samples were kept in 2% KOH for 72 hours and after washing by water and 2% HCl, the root samples were again kept for 72 hours in 0.05 cotton blue and lactoglycerol. The total treatment was done seldom disturbing the root fragments, keeping in petri-plates which retain the extra radical hyphal structures along with auxiliary cells, intact spores, vesicles etc.

Results and Discussion

AMF spores were collected from the host plant of three different sites. Morphological features of isolated AMF spores were critically examined with special reference to variation in size, wall thickness, shape, wall layers viz. germinal wall, coriaceous wall, amorphous wall, beaded wall etc. In every aspect the genus *Glomus* was predominant followed by *Gigaspora, Acaulospora, Scutellospora and Sclerocystis*. Fungal species identification were carried out using valid and standard keys of Schenck and Perez (1990) and Trappe (1982)

Percent 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: AMF infection (%) = (infected / total fragments taken (no.) = 26 (average of five readings) / 30 (Total no. root fragments taken) x 100 = 0.86 x 100 = 86.66%. Simultaneously, analysis of soil samples collected from the rhizosphere of Thuja plants were done in order to find out the physico-chemical properties of soil where association of the AMF prevails. Results have been presented in Table-1.

Table 1. Rhizosphere soil analysis of *Thuja orientalis* (L)

Soil type	Sandy-clay	P_2O_5(ppm)	38.88
Sand(%)	55	K_2O(ppm)	96.46
Silt(%)	02	Organic Carbon(%)	1.29
Clay(%)	41	Nitrogen(%)	0.18
pH	5.01	Spore No./100gm of soil	84
Moisture(%)	13.68	Colonization(%)	86.66

Association of AMF in rhizosphere of *Thuja orientalis*

Thorough investigation on the association of AMF in the rhizosphere of Thuja plants were done considering twenty different soil samples collected from various location of North Bengal. Population percentage of dominant mycorrhizal fungi e.g. *Glomus, Gigaspora, Acaulospora* and *Scutellospora* has been determined and presented in Figure 1.

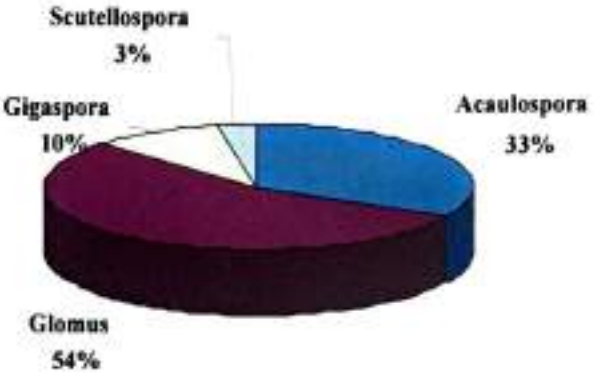

Figure 1. Population percentage of AMF spores in *Thuja orientalis*

Detail microscopic observations were made, photographs taken, all the essential spore characters were noted and compared with the available monographs and other literature. Description of the spore characters of prevalent AMF are enlisted below.

Acaulospora bireticulata

Spores are globose and brownish in colour, diameter ranges from 280-410µm. Surface ornamentation was prominent. Spore wall consists of three layers. Spores were borne laterally from the neck of a sporiferous saccule (Fig.2. I)

Acaulospora capsicula

Spores were bright red or orange red in colour, globose to subglobose with three distinct layers. Layer 1, forming the spore surface, evanescent, hyaline, 1.5µm thick in average and continuous with the wall of a sporiferous saccule, usually completely sloughed in mature spores. Layer 2 laminate, smooth, orange red to capsicum red 4.2µm thick. Layer 3 laminate, rigid, hyaline, 2.4µm thick, easily separating from layer 2.

Glomus fasciculatum

Colour pale yellow to bright brown with globose to subglobose in shape. Spores were produced directly with one or more subtending hyphae attached to it. Spore wall was continuous. Spore wall consisting of three layers (L1, L2, and L3). Spore size ranges from 70-120µm in diameter (Fig.2 B).

Glomus aggregatum

Spores globose to oval in shape. Size ranges from 40-120µm in diameter, color-pale yellow. Formed

singly or in sporocarps. Spore wall consist of 1-2 layers. Sporocarps are formed in loose clusters, from a single stalk, diameter ranges from 200-1800 x 200-1400µm in size.

Glomus mosseae

Brown to orange-brown in colour, shape, globose to sub-globose with an average diameter of 200µm. Presence of three hyaline layers with subtending hyphae attached. Hyphae was double layered (Fig. 2.A).

Glomus drummondii

Spores occur singly in the soil; develops from the tip of extra radical hyphae of mycorrhizal roots. Spores were golden yellow, globose to subglobose, average diameter is 70µm in diameter; single subtending hypha attached with the spore. Spore wall consists of three distinct layers (Fig.2 C).

Glomus constrictum

Spores were single in the soil with one subtending hypha, colour brownish orange to dark brown globose to subglobose; 160µm diam in average. Spores consists of one wall containing two layers, most juvenile spores with spore wall layer 1 only. Subtending hypha brownish orange to dark brown; straight or curved; usually markedly constricted at the spore base, sometimes cylindrical, flared to funnel-shaped; composed of two layers continuous with spore wall layers 1 and 2. *Glomus constrictum* is one of the most frequently found arbuscular mycorrhizal fungus in cultivated and uncultivated soils (Fig.2 D).

Glomus clarum

Spores, single in the soil; hyaline to pale yellow, globose to subglobose; 150µm diam; sometimes ovoid; 90-100 x 140-180µm; with one subtending hypha. hyaline to pale yellow straight to curved; wall of subtending hypha hyaline to pale yellow, thick at the spore base; composed of three layers (Fig.2 E).

Glomus aggregatum

Spores formed singly in the soil, in aggregates, in roots, aggregates ranges from 160-1600 x 250-1900µm, without a peridium, with two to over one hundred spores loosely distributed. Colour of spores were pastel yellow to yellowish brown; mostly globose to subglobose; rarely pyriform to irregular; usually with a single subtending hypha, rarely with two (Fig.2 G).

Gigaspora gigantea

Spores single in the soil; formed terminally or laterally on a bulbous sporogenous cell; greenish yellow (globose to subglobose; 300µm diam; sometimes ovoid; 250 x 270µm. Subcellular structure of spores consists of a spore wall with two layers and one germinal wall (Fig.2 H).

Gigaspora margarita

Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell. Spores yellowish white to sunflower yellow; globose to subglobose; 357µm diam; sometimes ovoid; 320 x 370µm. Sporogenous cell orange to brownish yellow. Structure of sporogenous cell composed of two layers. Layer 1 hyaline, 1.7µm thick approximately. Continuous with spore wall layer 1. Layer 2 orange to brownish yellow, 5.6µm thick, continuous with spore wall layer 2.

Scutellospora pellucida

Spores single in the soil; formed terminally on a bulbous subtending hypha; hyaline to yolk yellow; globose to subglobose; 195µm diam; sometimes ovoid; 130-155 x 160-235µm.

Scutellospora rubra

Spores colour: dark orange-brown to red-brown at maturity, immature spores are white to cream with a rose tint under a dissecting microscope. Shape: globose to subglobose. Size 180µm in average (Fig.2 H).

Besides, frequency of identified AMF in three locations were also critically analysed. The results have been computed and presented in Table- 2.

The major biological characteristic of AMF is their obligate biotrophic nature. This means that each of their life cycle steps requires the association with a living plant. As with most of the filamentous fungi, AMF propagation can occur either by spores differentiation and germination or by mycelium extension through soil and roots. Spores are differentiated by budding intercalary or apically on

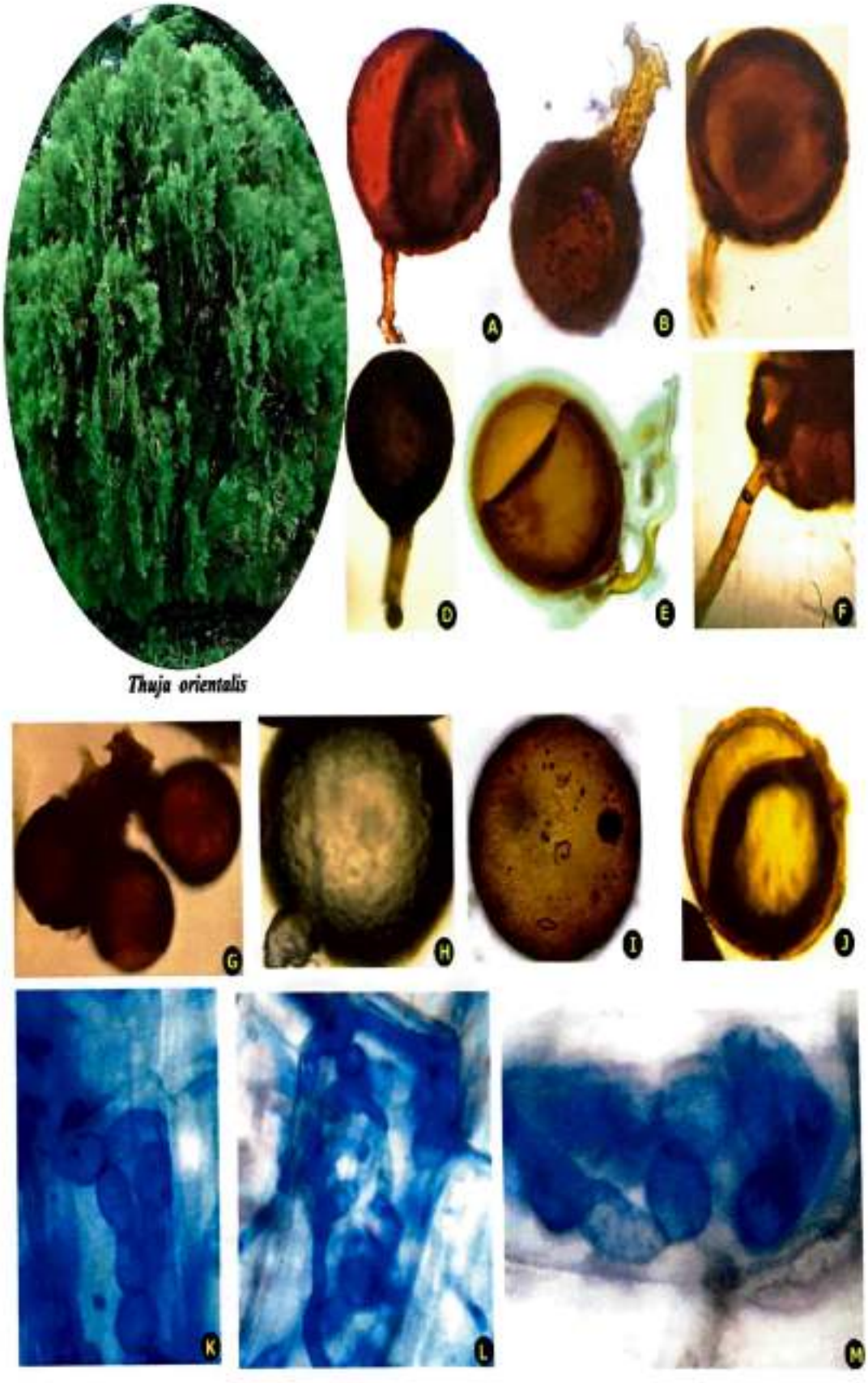

Figure 2. A - M. Spores and Histopathology. A. *Glomus mosseae*; B. *Glomus fasciculatum*; C. *Glomus drummondii*; D. *Glomus constrictum*; E. *Glomus clarum*; F. Sporocarp of *Glomus* sp. G. *Glomus aggregatum* H. *Gigaspora gigantea* I. *Acaulospora bireticulata* H. *Scutelospora rubra* K.M Different types of arbuscules formed by *Gigaspora* spp.

Table 2. The frequency of AMF spores in the study areas

Name of AMF	LOCATION		
	Darjeeling 27°03'N 88°18'E	Coochbehar 26°22'N 89°29'E	Jalpaiguri 26°32'N 88°46'E
Acaulospora			
A. alpina	+	+	+
A.bireticulata	+	+	+
A.capsicula	-	+	+
A.delicata	-	-	+
Glomus			
G.mosseae	+	+	+
G. fasciculatum	+	+	+
G. aggregatum	-	+	+
G. albidum	-	-	+
G. ambisporum	+	+	+
G. constrictum	+	+	+
G. badium	+	+	+
G. deserticola	+	+	+
G. drummondii	+	+	+
Gigaspora			
Gi. gigantea	+	+	+
Gi. margarita	+	+	+
Gi. albida	-	+	+
Scutellospora			
S. calospora	+	+	+
S. pellucida	+	+	+
S. rubra	-	+	+

hyphae. AMF species identification is based on spore characters, spore wall architecture, and the morphology of subtending hyphae. Some molecular tools to differentiate among AMF species and strains have been developed. Sexual reproduction has not yet been observed for these symbiotic fungi; therefore they are considered asexual. Fungal filaments grow through soil particles and come in contact with young plant roots, the fungal threads its way through root surface, and then grow between and inside cortical cells. The wide dispersal of the fungl network through its filaments gives the plant-root mycorrhiza access to a much larger volume of soil than the root system itself. The establishment of mycorrhizal networks in roots and soil constitute a soil-root fungal continium, which is required for beneficial symbiotic exchanges between fungi and plant. AM mycelium can spread throughout the soil surrounding the root system and increase the ability to explore soil areas, accessing water and nutrients for plant roots. Benefits to plants are improved by water and nutrient uptake, enhanced P transport, drought and disease resistance. Benefits to fungi are the supply of photosynthates to the fungal network located in the cortical cells of the plant and the surrounding soil. All water, nutrients, and photosynthates exchanges occur via the fungal filament network that bridged plant rhizosphere and plant roots. Arbuscules found in the roots of *Thuja* are haustoria-like structures that are formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface. These arbuscules are the exchange site of nutrients. In *Thuja* the arbuscules

formed are highly coiled with swollen trunks and is formed either singly or in clusters and vesicles are absent nearer to the arbuscules which indicate that these arbuscules are formed by *Gigaspora* sp. In some root fragments deep blue in coloured, thin walled, ellipsoidal structures were found in abundant were known as vesicles. Auxiliary cells were formed by short ramifications occurring at one or simultaneously at both sides of extra radical hyphae. High population of AM fungi such as species of *Glomus, Gigaspora, Scutellospora* and *Acaulospora* were obtained from the rhizosphere soil of *Thuja.*

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