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ENDOPHYTIC AND SYMBIOTIC MYCOTROPHY IN *EQUISETUM ARVENSE* L.: A MEDICINAL SPORE-DISPERSING VASCULAR SPOROPHYTE

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ABSTRACT

Arbuscular mycorrhizae (AM) are one of the most widespread and common type of symbiotic associations. *Equisetum arvense* L. is an important medicinal species of seedless vascular plants used in *Khasi Hills* under Nongkhyllem Reserve Forest, Nongpoh, Meghalaya, India. The mycorrhizal status of this species is meager and scarce in literature. In this study, typical AM structures were observed in sporophytes. The percentage of root length colonized by AM fungi was ranged from 80-90%. The morphological AM colonization pattern was typical Paris-type in rhizome part whereas in young roots (rhizoids), an Intermediate type of infection (both Paris and Arum types) was present. True vesicles were not seen but typical arbusculate and vesciculate structures, microsclerotia and cynophycean filamentous/trichomes structures were seen associated in the rhizoids of sporophyte. Dark septate endophyte (DSE) infection was only seen in rhizome part of sporophyte rather than rhizoids in which fine endophytes as well as endomycorrhizal infection were present. This study depicts that *Equisetum* rhizoids host abundant and diverse endorhizal fungal associates and also the evolution of *Equisetum arvense* sporophyte from hydric to mesic habitation due to its typical Intermediate type of endophytic and symbiotic association.

INTRODUCTION

The vascular plants are divided artificially into two major groups, the seedless (or spore-dispersing) vascular plants and the seed plants. There are four major Phyla of spore-dispersing vascular plants: Psilotales, Lycophyta, Equisetales and Pterophyta. The first three Phyla, often referred to as the "fern allies" are not prevalent organisms in our ecosystems today although they are well represented in the fossil record. All of the vascular plants have a dominant sporophyte generation, and a reduced, often, dependent gametophyte stage. *Equisetum* is the sole surviving genus of Class-Equisetopsida (Sphenopsida). *Equisetum arvense* Linn. (Family–Equisetaceae, Order-Equisetales) is commonly known as Horsetail. In Khasi hills, Meghalaya, it is known as '*Teengthei'* in local dialect. The name *Equisetum* is derived from the Latin roots *equus*, meaning "horse," and *seta*, meaning "bristle". Horsetail is a non-flowering spore dispersing vascular plant found throughout Europe, Asia, the Middle East, and North America. The plant is a perennial with hollow stems and shoots that look like Asparagus species. As the plant dries,

silica crystals that form in the stems and branches look like feathery tails and give the plant a scratching effect that accounts for its name 'scouring rush' and its historic use in polishing of metals, particularly pewter**.** It was used traditionally by *Khasi* people to stop bleeding, heal ulcers and wounds and treat injury problems. Horsetail contains silicon, which plays a role in strengthening bone. For that reason, it is sometimes suggested as a treatment for osteoporosis. It is also used as a diuretic, and as an ingredient in some cosmetics. Horsetail is used as a diuretic; has haemostatic properties; also considered useful in dropsy, gravel and kidney affection; the ash of the plant is useful in acidity of the stomach and in dyspepsia (Sandhu *et al*., 2010). It is also a 'metallophyte' and indicates the presence of gold and silica mineral in the soil (Nemec *et al*., 1936).

Seed less vascular plants including Lycophytes and Monilophytes (Equisetales) are of ancient origin and occupy a very important position in the origin and evolution of vascular plants (Remy *et al*., 1994). Mycotrophic and symbiotic status of Lycophytes and Monilophytes is meager and not much known (Dhillion, 1993; Zhao, 2000; Zhang *et al*., 2004). There are some research papers in which such association was found lacking or absent due to evolution of sporophyte (Treu *et al*., 1996; Schmidt and Oberwinkler, 1993; Berch and Kendrick, 1982; Malloch *et al*., 1980; Read *et al*., 2000). A review of the literature also reveals that work on *Equisetum* roots is sparse (Bierhorst,1958; Hauke, 1978). But Nasim et al.,1987; who concluded that *Equisetum* hosted abundant AM fungi (but claimed to have found AM structures in above-ground and in dead plant parts only), some researchers like Dhillion (1993); Koske et al. (1985); reported moderate to little endorhizal colonization in Horsetail. Although, Muthukumar and Udaiyan (2000); have studied the occurrence of AM interactions in some pteridophytes on Western Ghats of India, but very little or almost no work was found in North-East Indian Pteridophytes particularly in *Khasi Hills* under Nongkhyllem Reserve Forest, Nongpoh, Meghalaya, India.

So the aim of the present study was to analyze the occurrence and abundance of mycorrhizal and other endophyts in sporophytes of *E. arvense* growing naturally in *Khasi Hills* under Nongkhyllem Reserve Forest, Meghalaya, India for symbiotic mycotrophy.

MATERIALS AND METHODS

Study Area

This study was carried out in Nongkhyllem Reserve Forest, located near Nongpoh, Meghalaya, India ($25^0 5'$-$26^0 10'$ N latitude, $89^0 47'$-$92^0 47'$ E longitude). Nongpoh is the district- headquarter of Ri-Bhoi district of Meghalaya and it is situated on Guwahati-Shillong National Highway (NH-40). Nongkhyllem Reserve Forest, Nongpoh is having an area of 96 km^2 excluding the 29 km^2 area of Nongkhyllem Wildlife Sanctuary. Due to the diverse climatic and topographic conditions, Nongkhyllem Reserve Forest supports a vast floral diversity, including a large variety bryophytes, pteridophytes, epiphytes, succulent plants shrubs and trees.

Soil sample collection

Rhizospheric soil samples from the roots/rhizomes were collected from road-side stream and moist and damp bench during August-September, 2010 by digging out a small amount of soil close to plant roots up to the depth of 15-30 cm and these samples were kept in sterilized polythene bags at 5-10ºC for further processing in the laboratory for mycorrhizal quantification and root colonization.

Isolation of spores

Isolation of VAM spores was done by wet sieving and decanting (Gerdemann and

Nicolson, 1963) technique. Approximately 100gm of soil were suspended in 1 liter or more of water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of different sizes in the order 150µm, 120µm, 90µm, 63µm, 45µm which removes large particles of organic matter, but course enough to allow the desired spores to pass through. The seivings retained on different sieves were collected on different Petri dishes then the trapped spores were transferred to Whatman filter paper no. 1 by repeating washing with water. The spores were picked by hydrodermic needles under stereo- binocular microscope.

Mycorrhizal quantification

For quantitative estimation of VAM spores, Gaur and Adholeya modified method (1994) was used. The filter paper was divided into many small sectors by marking with a ball pen. The total number of spores was counted by adding the number of spores present in each sector under stereo-binocular microscope.

Identification of VAM fungi

For identification of VAM spores the following criteria were used like conventional morphological character i.e. color, size, shape wall structure, surface, ornamentation of spores, nature and size of subtending hyphae, bulbous suspensor, the number and arrangement of the spores in the sporocarp. These VAM spores were identified by using the keys of Schenck and Perez (1990); Morton and Benny (1990), and Mukerji (1996).

Clearing and staining of root segments

Rhizospheric soil samples were stained according to Phillips and Hayman (1970) method. Roots were first cleared with 10% (w/v) KOH at 98^0C for one hour. After clearing, roots were bleached with a 5% H_2O_2 (v/v) solution for 10 minutes. Equisetum roots were darker, so they were bleached in this solution for 40 minutes. All samples were acidified with 1% (v/v) HCl for 5 minutes and stained with 0.05% (w/v)Trypan Blue in acidic glycerol by heating them at 98^0C for 15 minutes. The stained roots were stored in acidic glycerol for further study.

Analyses of the root samples

Ten stained root pieces approximately 1 cm long were mounted on a slide in Glycerol and were examined with a light trinocular microscope (Labovision, BIOXL). A total of three replicates were made. Typical structures that indicated the presence of mycorrhizas or other root associated fungi, cynophycean filamentous like structures were documented and microphotographed with light trinocular microscope attached digital camera (Canon A3100IS) and Image-ZoomBrowser Ex analysis software for Windows. The mycorrhizal type present in each sample was designated according to Harley and Smith (1983) classification. The criteria used in this study for the determination of AM was the presence of vesicles, arbuscules and mycelium at least in one individual root segment and the occurrence of the rest of typical AM structures in the samples (intra or intercellular hyphae, vesicles, coils and arbuscules) and were classified into Arum or Paris-type (Smith and Smith, 1997). The percentage of root length colonized by AM fungi was estimated according to the magnified line-intersect method described by McGonigle *et al*. (1990).

RESULTS AND DISCUSSION

In this study, *Equisetum arvense* roots were examined for endomycorhizal association. Results pertaining to diversity of AM fungi and physico-chemical traits of rhizospheric soil are reported in Table 1. The pH, temperature and E conductivity of rhizospheric soil were (6.44± 0.0), (25.5 ±0.0), (15.5± 0.0) respectively. The spore quantification of sample was 20spores/10gm of soil and *Glomus rubeforme*, *Glomus albidum* and *Glomus* species were

present in the rhizosphere (Table-1, Plate-1A & H-I).

Percent colonization and other endorhizal structures associated with *E. arvense* L. are shown in Table-2. It was seen that the rhizome is having cent percent total colonization, (Hyphal infection 100%) but mostly the Paris-Type of AM colonization was observed. Arbusculate infection and dark septate endophyte infection (DSE) were observed only on a few segments of rhizome. Whereas Microsclerotium (MS), Microsporangiate like aggregation (MSLA), Cynophycean filamentous like structures (CFLS), Fine endophyte infection (FE) and Peloton like hyphal coils (PLC) were seen in rhizoids which were absent in rhizome part of sporophyte. Hyphal infection was 95% in rhizoids but mostly Intermediate type (IT) i.e. both Arum-type and Paris-type of infections were observed in them. The arbusculate infection was present in 60% of segments whereas vesiculate like infection was observed only in few segments (30%) but true vesicles were not seen in all the segments. More interestingly, the Cynophycean filamentous/trichomes like structures (CFLS) were found in few young rhizoids segments of the sporophyte which indicates that sporophyte may takes nitrogen from cynophycean members which live in symbiotic association with rhizoids (Table-2, Plate- 1B-G).

Arbuscular mycorrhizas (AM), as the most widespread type of mycorrhizas, form symbiotic interactions with the roots of 80% of all terrestrial plant species (Read, 1999). Sporophytes of Equicetaceae have been regarded as non-mycorrhizal by several authors as discussed in introduction part of this paper, however, the results in this study and some previous reports as cited earlier indicate that plants may be extensively colonized by AM fungi. *Equisetum bogotense* collected from Valdivian temperate forest of Patagonia were not obligate mycorrhizal species, but they develop AM under certain conditions, probably related to the habitat and the substrate where they were growing (Fernadez *et al*., 2008). In spite of the fact that Paris-type is the most common type of AM among the seedless vascular plants (Smith and Smith, 1997; Zhang *et al*., 2004). *Equisetum* is commonly associated with damp or wet soils and due to this reason Berch and Kendrick (1982) and Koske *et al*. (1985) partially attributed their findings of relative paucity of mycorrhizal association. This work represents the record of AM fungi in monilophytes (*Equisetum arvensis*) of Nongkhyllem Reserve Forest Nongpoh, Meghalaya and constitutes a tread in the study of the importance of AM fungi, endophytes and other microbes in the rhizosphere of this sporophyte.

Equisetum plants produce extensive, perennial rhizomatous systems with potentially 80% of their biomass being subterranean. *Equisetum* species can store abundant starch below ground (Hauke, 1978) and for this reason *Equisetum* was cultivated for its tubers in the 1920s in upland Bolivia and Peru (Berry 1924). Interactions between *Equisetum* roots and soil mycoflora may well provide important food sources for burrowing animals and soil invertebrates. Mycorrhizal associations have been considered necessary pre-requisites for plants to colonize terrestrial environments (Pirozynski and Malloch, 1975). In addition to AM, another type of root colonizing fungi called Dark Septate Fungal (DSF), infection has been also reported within the rhizoids of Equisetum sporophyte collected from the said site. Similar Dark Septate Fungal infection in different seedless vascular plants were also reported earlier (Cooper, 1976; Berch and Kendrick, 1982; Dhillion, 1993; Jumpponen and Trappe, 1998). Dark Septate Fungi are defined by Jumpponen (2001) as conidial or sterile fungi

that colonize living plant roots without causing any apparent negative effects. The ecology, taxonomic affinities and host range of these DSF are largely unknown (Peterson *et al*., 2004). Including DSF in mycorrhizal studies would definitely yield valuable information about the importance and diversity of these root colonizers (Jumpponen and Trappe, 1998).

The occurrence of Microsclerotia (MS), Microsporangiate like aggregation (MSLA), Cynophycean filamentous like structures (CFLS), Fine endophyte infection (FE) and Peloton like hyphal coils (PLC) in the rhizosphere is especially remarkable, because some structures like Microsporangiate like aggregation, Cynophycean filamentous like structures, Fine endophytic infection and Peloton like hyphal coils have not been previously cited in the literature. As it is becoming important to report in this paper to support the idea of Winter and Friedman (2007) that some seedless vascular plants are capable of forming plant-fungal associations with a diversity of fungal lineages. This information would be very useful to elucidate the nature and ecological importance of these inadequately and un-known root colonizing endophytes (Jumpponen and Trappe, 1998; Jumpponen, 2001; Peterson *et al*., 2004). This result is in agreement with the finding of Boullard (1957). He suggested that the mycorrhizal symbiosis within seedless vascular plant group ranges from obligate to facultative or non-mycorrhizal. The results also corroborate with the results obtained by Zhao (2000), who studied the mycorrhizal status of 256 species of Lycophytes and Monilophytes and found out that only 16% of them were facultative mycorrhizal.

CONCLUSION

Through this study, it is concluded that *Equisetum arvense* is an important medicinal spore-dispersing vascular sporophyte used by *Khasi* tribe in Khasi Hills of Nongkhyllem Reserve Forest, Nongpoh, Meghalaya, India and further depicts that its rhizoids host abundant and diverse endophytic fungal associates that relates the evolution of *Equisetum arvense* sporophyte from hydric to mesic habitation due to its typical Intermediate type of endophytic and symbiotic association. Also, its endorhizal symbiotic associations may play a significant role in nutrient cycling in the ecosystems and have a broad ecological relevance.

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Table 1. Diversity of AM fungi and physico-chemical traits of rhizospheric soil of *E. arvense* L.

Plant name	*p*H of soil	Temperature of rhizosphere	E conductivity	SQ/10gm soil	Endomycorrhizal Diversity
Equisetum arvense L.	6.54 ± 0.0	25.5 ± 0	15.5 ± 0	20	GR GA, GS

SQ Spore quantification
GB- *Glomus boreale* (Thaxter) Trappe & Gerd.
GA- *Glomus albidum* Walker & Rhodes.
GS- *Glomus* species Tulasne & Tulasne.

Table 2. Percent colonization and other endorhizal structures associated with *E. arvense* L.

Sporophyte Part	Total colonization (%)	Hyphal Infection (%)	Arbusculate Infection (%)	Vesciculate Infection (%)	MS	MSLA	CFLS	DSE	FE	PLC
Rhizomes	100	100 (PT)	+ (very few segments)	-	-	-	-	+ (few segments)	-	-
Young Roots (Rhizoids)	100	95 (IT)	60	30	+	+	+	-	+	+

MS-Microsclerotium
MSLA-Microsporangiate like aggregation
CFLS- Cynophycean filamentous like structures
DSE- Dark septate endophyte infection
FE- Fine endphyte infection
PLC- Peloton like hyphal coils
PT- Paris type infection
IT-Intermediate type infection

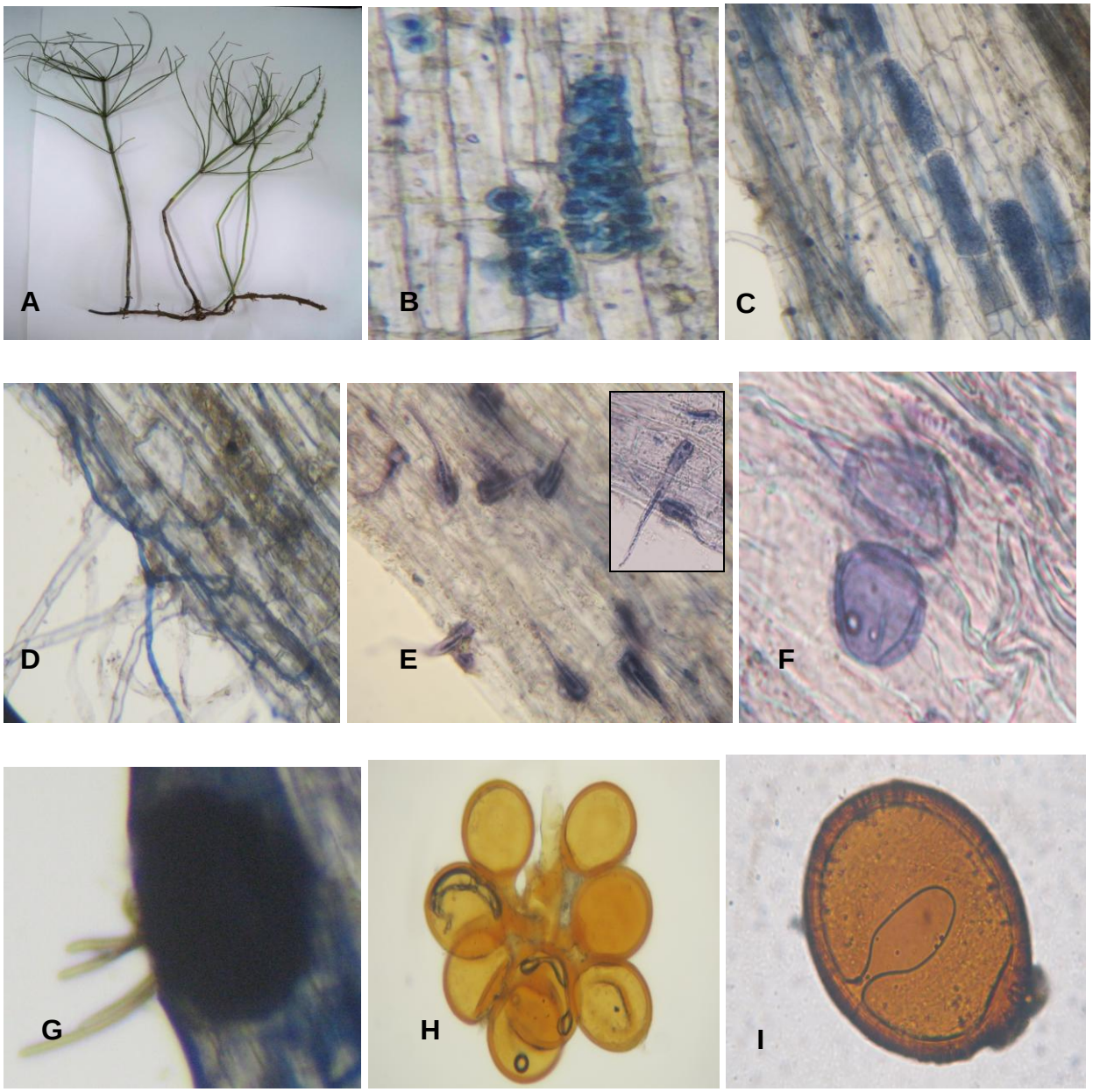

Plate 1. A. *Equisetum* sporophyte, B. Microsporangia, C. Reticulate arbuscules, D. External mycelium entering the cells, E. A colony of Cynophycean filamentous/trichomes structures (in inset: A single cynophycean filament/trichome), F. Akinites, G. Germinating Microsclerotium (with emerging germ tubes), H. *Glomus boreale* (Thaxter) Trappe & Gerd., I. *Glomus* sp.