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# Arbuscular Mycorrhizal Morphology in Sporophyte of *Psilotum nudum*

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ABSTRACT

The mycorrhizal structures of the sporophyte of *Psilotum nudum* were studied by light microscopy. Further, the nature of the fungal associate was characterized through trap-culture technique. The fungal entry into the rhizome was mainly through the rhizoids with occasional direct entry through the epidermis. The fungal colonization resembling the *Paris*-type of mycorrhiza was restricted to the cortex with the rhizome tip and the stele free of any colonization. The transversing hyphae formed coils in the host cells with intracellular vesicles**,** but arbuscules were absent. Single hyphae arising from the coils penetrated the neighbouring cells with a hyphal constriction at the host cell wall region. Intact and collapsed hyphal coils were found in different cells and no recolonization of the host cells was observed. Arbuscular mycorrhizal fungal spores belonging to *Acaulospora scrobiculata*, *Glomus aggregatum* and *Glomus geosporum* were isolated from the trap cultures and also from the soil in which *P. nudum* occurred.

**Key words: Arbuscular mycorrhiza, *Glomus*, *Paris*-type, *Psilotum*, trap culture**

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## Introduction

Psilotales consists of two genera (*Psilotum* and *Tmesipteris*) and approximately ten species. Taxa under *Psilotum* are referred to as "living fossil" by many paleobotanists and plant morphologists. Further a critical analysis of its morphology and shoot organization reveals that despite its simplicity *Psilotum* exhibits the same level of complexity as seed plants (Siegert, 1964). In India two species of *Psilotum* are reported, *i.e*., *Psilotum nudum* (L.) P. Beauv. and *Psilotum complanatum* Sw. According to the survey reports and available information, *P. nudum* has been found in Myanmar, Malaysia and India. In India *P. nudum* is found in the Western Ghats, central India and eastern India (Chauhan et al., 2003).

The achlorophyllous gametophytes of Psilotaceae are consistently associated with aseptate endophytic fungi that form intracellular hyphal coils and masses of irregular swollen vesicles (Holloway, 1939; Davis, 1975; Peterson et al., 1981). Bierhorst (1953) observed two very distinct species of filamentous fungi associated with *P. nudum*. The first of these was a non-septate filamentous fungus *Cladochytridium* with vesiculate hyphae occurring in both gametophytes and sporophytic rhizomes of *P. nudum*. The second was a septate filamentous fungus with uniformly thickened walls occurring exclusively in the gametophytes. In contrast, Zimmerly and Banks (1950) described the endophytic fungus in *Psilotum* gametophytes as being septate. Lawson (1917) suggested that the fungal endophyte in the gametophyte of Psilotaceae is a Phycomycete resembling *Pythium*. Boullard (1979), without identifying the fungus included the *Psilotum* endophyte in the "tolypophagus type" having no vesicles or arbuscules but just pelotons.

An electron microscopic study by Peterson et al. (1981) of the fungus in *Psilotum* gametophytes from green house pots illustrated aseptate hyphae in the rhizoids and coils of hyphae, some terminating in vesicles in the cortical cells. However, Peterson et al., (1981) were unclear of the fungal identity, but drew attention to its similarities with non-arbusculate fungi forming glomeromycotean mycorrhizas. They also suggested that the heterotrophic gametophytes were behaving as a parasite on the fungus, but did not compare these with the host-fungus relationships in the rhizomes of the autotrophic sporophytes.

The absence of arbuscules and the formation of small vesicle-like structures in mycoheterotrophic gametophytes and in some sporophytes of the lower tracheophytes like *Psilotum,* cast some doubt on their fungal affinities. Schmid and Oberwinkler (1993) in their study of *Lycopodium clavatum* L., questioned the glomalean nature of the fungal endophyte, a view echoed for *P. nudum* by Schüßler (2000). In a survey on the Hawaiian pteridophytes, Gemma et al., (1992) found mycorrhizas in *P. nudum*, but not in *P. complanatum*.

Duckett and Lignore (2005) reported intracellular, aseptate glomeromycotean fungi resembling the *Paris*-type of arbuscular mycorrhizas in the parenchymatous cortical cells of rhizomes and gametophytes of *P. nudum*.

Read et al. (2000) suggested that there is a *prima facie* for considering the fungal association in *P. nudum* to be of mycorrhizal type as the fungal colonization was a prerequisite for a healthy gametophyte development. Further they suggested that an experimental approach is essential to test this hypothesis. Although several studies (Peterson et al., 1981; Gemma et al., 1992; Duckett & Lignore, 2005) presumed that the fungal association in *Psilotum* may be of arbuscular mycorrhizal (AM) type, there is no convincing evidence for this. So the aim of the present work is to describe the morphology of the fungal association in the sporophytes of *P. nudum* and to characterize the nature of the fungal endophyte.

## Material and methods

Rhizomes of six mature sporophytes of *P. nudum* were collected in January 2008 from the Bharathiar University Campus, Coimbatore, Tamil Nadu, India. The sporophytes in the present study were growing among the lawn grasses. We are not sure of the exact origin of this taxa in the University campus, but assume that it was probably introduced along with the lawn grasses.

A soil block containing the sporophytes was removed as the rhizome was highly fragile and breaks with slight pressure. The soil block was soaked in a tray of water. The soil attached to the plants was removed by agitating the plant and the rhizomes were separated. For observations longitudinal and transverse free-hand sections of the rhizomes were taken with a razor blade and stained with Chlorazol Black E in lactoglycerol.

### *Determination of soil characters*

Soil moisture was determined by drying over 100 ºC to a constant weight, and the weight loss was expressed as a percentage of dry weight. Available phosphorus (P) was determined colorimetrically with ammonium molybdate and stannous chloride reagents (Jackson, 1971). Total nitrogen (N) was extracted by micro-Kjeldahl digestion and measured on a Technicon Auto Analyzer (Gedko International Ltd., UK). Exchangeable potassium (K) was determined after extraction with ammonium acetate (pH 7) and measured on a digital flame photometer (Systronics, Mediflame-127).

#### *Measurement of rhizome characteristics*

Rhizome diameter was measured in twenty five 1-cm long rhizome segments. The rhizome segments were suspended in water on a microscopic slide and the rhizome diameter, rhizoids number, length and breadth were measured using an ocular micrometer under a binocular compound microscope (Itoh & Barber, 1991).

#### *Assessment of AM fungal colonization and spore numbers*

Rhizomes were washed free of soil, cleared in 2.5% KOH and stained in 0.05% Chlorazol Black E-lactoglycerol to determine mycorrhizal colonization (Muthukumar and Udaiyan, 2000). The AM fungal colonization was quantified according to magnified intersection method (McGonigle et al., 1990) under a compound light microscope at 200´ magnification**.** Two hundred intersections were observed.

Spores of AM fungi were recovered by a modified wet sieving and decanting technique (Muthukumar et al., 1996) and enumerated. Only entire and intact spores were counted. The isolated spores were examined using a dissecting microscope and spores of each morphotype were mounted in polyvinyl-lacto-glycerol (PVLG) and 1:1 PVLG/Melzer's reagent for identification (Koske & Tessier, 1983). Species identification was based on the examination of spore morphological and subcellular characteristics using stereo and light microscopes and compared to original descriptions and species descriptions on the International Culture Collection of Arbuscular and Vesicular-Arbuscular Mycorrhizal Fungi web site -http://invam.cag.wvu.edu.

#### *Rhizome trap culture*

The rhizomes were washed free of soil and cleared of adhering debris. The rhizomes were cut into 2 cm long bits, and 500 mg fresh wt. of rhizomes was layered 3 cm below the autoclaved sub soil in glass funnels. The soil was sterilized by autoclaving thrice for 90 min. with a 12 h interval between autoclaving. The funnels were seeded with either *Eleusine coracana* or *Sorghum bicolor* seeds for trapping AM fungi. Three funnels were maintained for each host. The control funnels received 500 mg of autoclaved rhizomes. The funnel contents were transferred to 15 cm diam. pots when roots started to emerge from the funnel. The plants were watered as necessary and irrigated with half-strength Hoagland's solution (without P) once a week. The trap plants were harvested after 80 to 90 days of growth. The roots and soil samples were processed to detect AM fungal colonization and the presence of AM fungal spores. The intact cytoplasm filled spores were isolated and identified as described earlier.

## Results

The soil in which *P. nudum* occurred was a sandy loam Alfisol with a pH of 8.3. The soil contained 10.3 mg N $kg^{-1}$, 1.68 mg P $kg^{-1}$ and 22 mg K $kg^{-1}$ of soil.

The aerial shoots of *P. nudum* originated from a semi erect rhizome and their diameter ranged from 1 - 4 mm (Fig. 1A). The rhizoids were present all over the rhizome and their number ranged from 9 - 21 $mm^{-1}$ of rhizome. The rhizoids were dark brown 208.3 - (565.39 ± 108.60) -1666.40 µm long and 9.09 - (12.31 ± 0.95) - 18.18 µm in diameter. All the rhizomes examined were colonized by fungi which was restricted to the cortex. The vascular tissue, and the meristematic tissue and the cells adjacent to meristematic tissue were free of fungal colonization (Fig. 1B, C). The fungal entry into the rhizome was mainly through the rhizoids (Fig. 1D,E) and occassionally directly through the epidermis. No appressorium formation was evident at the fungal entry point. The fungal colonization occurred about 312.45 – (2127.64 ± 1048.17) – 3124.50 µm behind the rhizome tip characterized by hyphal coils within cortical cells (Fig. 1F,G). A single hyphae arising from hyphal coils penetrated adjacent host cells with a hyphal constriction at the plant cell wall region (Fig. 1H). The hyphae were coarse, 1.5 – (3.11 ± 1.11) – 4.5 µm in diameter with uniformly thickened walls. Arbuscules were absent. Intracellular vesicles were present in cells containing hyphal coils and their number ranged from 1-12 per cells (Fig. 1I). The vesicles were globular, oval or irregular measuring 16- (28.11 ± 8.90) – 40 x 16 – (20.44 ± 4.37) – 24 µm. The degenerated and intact hyphal coils were observed in different cells (Fig. 1J). The extent of colonization ranged between 16-60% (25.33 ± 8.90). The percentage of rhizome length with vesicles was 24 ± 5.30.

The AM fungal spore numbers of the soil in which *P. nudum* occurred was 18 ± 3 spores 100 $g^{-1}$ soil. The isolated AM fungal spores belonged to *Acaulospora scrobiculata* Trappe, *Glomus aggregatum* Schenck & Smith emend. Koske, *Glomus geosporum* (Nicol. & Gerd.) Walker and *Glomus sinuosum* (Gerd. & Bakshi) Almeida & Schenck (Fig. 2).

The trap plants inoculated with *P. nudum* rhizomes developed typical AM fungal colonization. The extent of colonization was 56.21 ± 2.59 in *Eleusine coracana* and 49.37 ± 3.87 in *Sorghum bicolor*. The control trap plants inoculated with autoclaved *P. nudum* rhizome were free from AM colonization. The spores of *A. scrobiculata*, *G. aggregatum* and *G. geosporum* were isolated from trap cultures.

## Discussion

The roots containing mycorrhizae generally are used to initiate trap cultures (Brundrett, 1994) and also to establish the mycorrhizal nature of the fungal association in plant species (Muthukumar & Udaiyan, 2002). In the present study *E. coracana* and *S. bicolor* inoculated with the *P. nudum* rhizome developed typical mycorrhizal colonization of AM type. Thus, the present study confirms the glomeromycotean nature of the fungal endophyte in *P. nudum* sporophytes. Although, the presence of fungal symbionts in gametophytes and sporophytes of *P. nudum* has been documented (Holloway, 1939; Davis, 1975; Peterson et al., 1981; Gemma et al., 1992; Duckett & Lignore, 2005), nothing was so far known about the type or nature of the fungal associates. This study clearly demonstrates the Glomalean nature of the fungal associates as shown for Lycopodiaceae (Winther & Friedman, 2007) and Ophioglossales (Schimid & Oberwinkler, 1994; Kovács et al., 2003). The present study provides further evidence that non-seeded vascular plants form *Paris*-type AM morphology (Smith & Smith, 1997). Our observation of the endophytic colonization of *P. nudum* rhizome concords the previous observations of Peterson et al., (1981) and Duckett and Ligrone (2005) *viz*. fungal entry through rhizoids and the formation of intracellular coils and vesicles by aseptate hyphae. Gemma et al., (1992) also described AM association in *P. nudum* based on the abundance of non-septate hyphae colonizing the rhizomatous cells.

The distribution of the fungi within the sporophytic rhizome of *P. nudum* described in this study is similar to those previously reported for the gametophytes where the growing apices were reported to be devoid of the fungi. Hyphae were commonly present in the rhizoids a feature also observed by Janse (1897), Darnell-Smith (1917), Lawson (1917) and Peterson et al., (1981). However, this is not the only method of fungal entry into the rhizome, as direct entry of the fungal hyphae through the epidermal cells into the rhizome was also noted in the present study. In *Lycopodium cernuum*, a direct hyphal entry through the epidermal cells as observed in the present study has been reported. In the present study, contiguous cortical cells exhibited various stages of fungal development; containing intact fungal hyphae or degenerating hyphal plexus. However, the lack of reinvasion of rhizomatous cells containing degenerating hyphal plexus in the present study strongly contrasts these studies (Peterson et al., 1981; Duckett

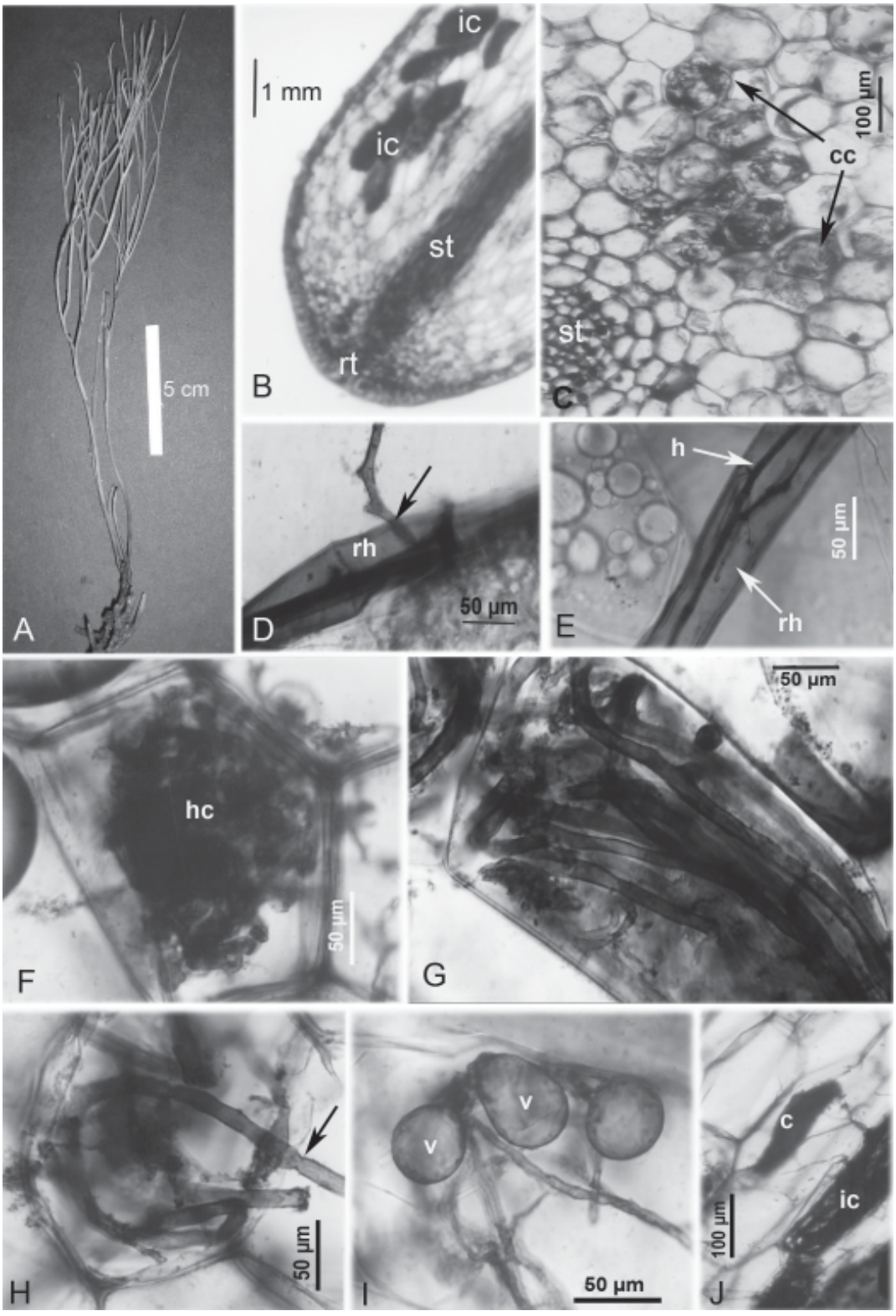


**Fig. 1: Habit and mycorrhizal association in *Psilotum nudum*.** A. **Habit showing rhizome.** B. **Longitudinal section of a rhizome tip showing fungal colonized cells (ic), stele (st) and colonization free rhizome tip (rt).** C. **Transverse section of the rhizome showing fungal colonized cortical cells (cc) surrounding a fungus free stelar region (st).** D. **Fungal entry (arrow) through a rhizoid (rh).** E. **Rhizoid (rh) with fungal hyphae (h).** F. **A rhizome cell packed with the fungal hyphal coil (hc).** G. **Magnified view of the intracellular hyphae.** H. **Hyphae transversing the walls of two cortical cells. Note the hyphal constriction (arrow) at the cell wall region.** I. **Intracellular vesicles (v).** J. **Intact (ic) and collapsed (c) hyphal coils in different cells.**

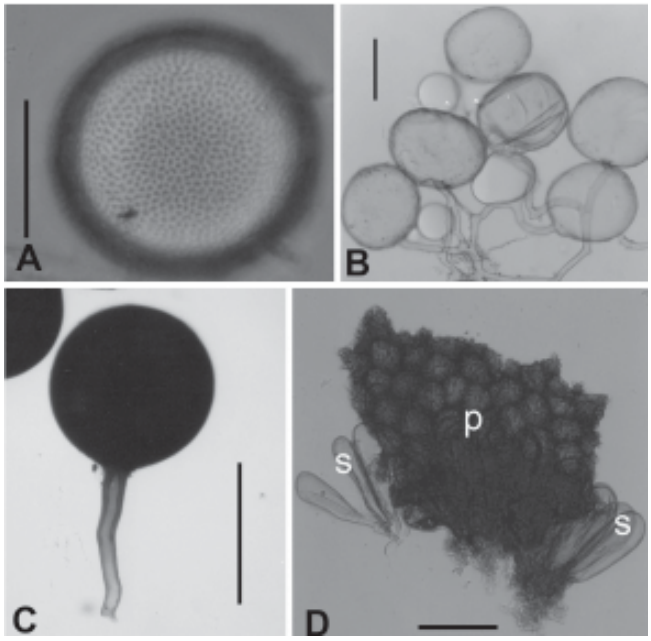


**Fig. 2: Arbuscular mycorrhizal spores isolated from the soils of *Psilotum nudum*. A. *Acaulospora scrobiculata*. B. *Glomus aggregatum*. C. *Glomus geosporum*. D. *Glomus sinuosum*. (s, spores; p, peridium) (scale bars = 100 μm)**

& Ligrone, 2005) where a reinvasion of cells containing degenerating hyphal plexus have been reported.

A comparison between the endophytic association in *Psilotum* and other pteridophytes like *Botrychium* (Schmid & Oberwinkler, 1994), Glechiniaceae (Schmid & Oberwinkler, 1995) and *Lycopodium clavatum* (Schmid & Oberwinkler, 1995) reveals several striking similarities with a few minor differences. In these pteridophytes the fungi invade the roots *via* root hairs or rhizoids. Furthermore, in all the above except in *Lycopodium* species (Goebel, 1891) the fungi are exclusively intracellular and confirm to the *Paris*-type of AM morphology (Shibata, 1902; Peterson et al., 1981). The *Paris*-type of AM morphology is characterized by intracellular hyphae, hyphal coils or arbusculate coils (Smith & Smith, 1997; Dickson et al., 2007). Further, vesicles that contain abundant lipids and numerous nuclei are intracellular in *Paris*-type. Peterson et al. (1981) observed that the fungal hyphae and vesicle-like structures in *Psilotum* gametophytes stored lipids, which appeared to be released into the host cytoplasm on hyphal degeneration. The sporophytes of *Psilotum* are massive autotrophic structures and are thought to be invariably, colonized by mycorrhizal fungi. However, there is no evidence that the colonization spreads from the gametophyte into the developing sporophyte (Read et al., 2000).

Bugreff (1938) reported the occurrence of arbuscules in *P. nudum*. However as observed in the present study and other studies (Peterson et al., 1981; Gemma et al., 1992; Duckett & Lignore, 2005) fungal association in *P. nudum* lacks arbuscules. In lower vascular plants, the arbuscules appear to be rare and are only well developed in Gleicheniaceae, whilst they are common in the hepatics and hornworts (Read et al., 2000).

In the present study only one type of hyphal morphology was noted. This contrasts the observation of Dangeard (1890) where at least three fungal genera were noted within rhizomes of *Tmesipteris*, the other genus in the family Psilotaceae. One of the three fungal genera was identified as *Cladochytridium* which was considered as probable parasite and not mycorrhizal fungi (Dangeard, 1890). This fungal genus was also identified in *Psilotum* rhizomes.

In a recent study Winther and Friedman (2007), using molecular techniques, confirmed the Glomalean nature of the fungal associates of *L. clavatum* which like *P. nudum* also possesses hyphal coils with relatively small vesicles and no arbuscules. These establish that the distinctive fungal structures found in many lower plants can be formed by AM fungi, there by providing further support for the view that lower tracheophytes like *Psilotum* are almost universally associated with Glomalean fungi (Read et al., 2000). Further studies are needed to understand the role of AM fungi in *Psilotum* nutrition.

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