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Mycorrhizal colonization in *Drosera burmannii* Vahl, a Carnivorous Plant: Observation using Light and Fluorescence Microscopy

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Abstract

In the present investigation, the insectivorous plant, *Drosera burmannii* Vahl inhabiting in the natural ecosystem was evaluated for categorizing the status of mycorrhizal colonization under fluorescence and light microscope. The results revealed the occurrence of dual colonization by AM and DSE fungi in *D. burmannii*. Mycorrhizal structures such as arbuscules possessed 21.48 %, vesicles having 18.66 %, 65.56% of hyphae and 7.14 % of DSE colonization in the root. The colonization by mycorrhizal fungi was noticed in the cortical cells and it was more in the outer cortical cells. Fluorescing vascular bundles and the presence of root hairs were observed. Hyphal coils and hyphal penetration in the epidermis along with fluorescing arbuscules were also observed. The study suggests the presence of mycorrhizal associations may assist benefit to the plant to acquire nutrients from the soil.

Introduction:

Arbuscular mycorrhizal (AM) fungi are probably the most widespread terrestrial symbiosis (Fitter, 2005), formed by 70–90% of land plant species (Smith & Read, 2008) with fungi belong to a monophyletic phylum Glomeromycota (Schüßler *et al.*, 2001). The plant furnishes carbon to the fungal partner and in return fungal partner assist the plants in their uptake of phosphorus (P), nitrogen (N) and other mineral nutrients from deficient sites (Smith & Read, 2008) by extending the soil volumes that ensure the improved plant growth through better nutrition uptake and alleviate toxicity of pollutants (Bolan, 1991; Marschner, 1998).

Mycorrhizal and non-mycorrhizal plant roots are also known to colonized by a diverse group of pigmented, septate and thick walled fungi called dark septate endophyte (DSE) fungi (Sathiyadash *et al.*, 2010) and reported to coupled with enhancement of plant growth, biomass accumulation, nutrient acquisition or fitness of the inoculated seedlings (Rodriguez *et al.*, 2009). Jumpponen & Trappe (1998) recapitulated the incidence of DSE associations in nearly 6,000 plants species representing about 320 genera and 100 families, but only 59 were tropical plant species. Extensive literature survey revealed that roots of carnivorous plants are non-mycorrhizal (Macdougall, 1899; Juniper *et al.*, 1991; Brundrett, 2009) although, Chambers *et al.* (2008); Fuchs &

Haselwandter (2004) have reported that the plants may occasionally be colonized by facultative mycorrhiza. It is a general notation that carnivorous plants lack AM fungal colonization due to their inimitable nutrients acquisition mode resulting in diminution of AM fungal dependency (Harikumar, 2013).

Drosera burmannii Vahl prevalently known as the Sundews or Surya Shisir belonging to the family Droseraceae is an annual carnivorous herb. It is one of the most commonly occurring carnivorous species among Indian carnivorous plants and found gushing in tropical and subtropical parts of the country. *D. burmannii* contains naphthoquinones, flavonoids like quercetin and hyperoside (Wagner & Baldt, 1984; Wang, 1998) and possesses anticonvulsant activity (Hema *et al.*, 1983).

Detection and quantification of AM fungi along with the degree of root colonization in the roots of terrestrial plants are indispensable tools in mycorrhizal research. Staining and microscopic methods not only assure reliable data on the degree of root colonization but also consent visualizes the presence of key structural features such as arbuscules, which are the morphological criteria for defining AM associations (Brundrett, 2004). Ames *et al.* (1982) and Jabaji-Hare *et al.* (1984) postulated the quantification of the auto fluorescing 'arbuscules' as a non-destructive method for measuring root colonization by AM fungi. An extensive study by Gange *et al.* (1999) unveiled

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RESEARCH ARTICLE

the arbuscular colonization intensity in a wide range of plants by staining with Chlorazol black E (CBE), acid fuchsin and Trypan Blue with the level of the auto fluorescing 'arbuscules' and summarized that in most plants the number of autofluorescing 'arbuscules' seemed much higher compared to the number of stained arbuscules. Vierheilig *et al.* (2001) also observed that autofluorescing collapsed arbuscules are not stained by the dyes commonly used to stain mycorrhizal fungi.

The meager information on the role mycorrhizal fungi in the insectivorous plants inhabiting in natural ecosystem promoted us to evaluate the status of mycorrhizal association in *D. burmannii* under the fluorescence microscope and light microscope.

Materials and methods:

Study site: sampled using quadrat of 1m x 1m, the soil and root sample of the plant was collected from field during March-April, 2016. The plant material was collected from the embankment of rain fed agricultural land where rice was cultivated once a year.

Root sampling: the roots of randomly selected five healthy *D. burmannii* plants were collected and mixed in marked polythene bags and transferred to the laboratory for root processing and estimation of mycorrhizal colonization. Care was taken not to damage the roots during collection. The roots were collected carefully and properly identified in order to avoid the collection of roots from inadvertent species.

Soil sampling for isolation of AM fungi: the soil samples of approximately 500 g at depths of approximately 0-20 cm around four points of plants were collected and mixed, labeled and zipped in polythene bags from the selected site and transported to the laboratory for processing. After transferring to the laboratory, the soil samples were kept for the quantification of spore density and AM fungal composition determination.

Assessment of AM Fungal and DSE colonization: the collected fine roots were thoroughly washed in several changes of running tap water to remove the adhering soil particles and organic debris. After adequate washing, the root samples were cut into approximately 1cm in size and cleared with 10% NaOH at 90°C. The duration of heating is depending on the clearance of the root samples. The cleared roots were washed again with running tap water for 4-5 times and bleached in 2-3 drops of alkaline H₂O₂ with slight heating. The root samples were stained by following the method (Roy Das *et al.*, 2015). After a while, the stained root samples were mounted with lactoglycerol on a grease free glass slide and observed the mycorrhizal structures such as arbuscules, vesicle, septate and aseptate hyphae and microsclerotia under a compound microscope (Olympus C X 21i) fitted with the camera and software, SImage in the computer. The estimation of AM and DSE fungal

colonization were done by magnified intersection method (McGonigle *et al.*, 1990).

Fluorescence microscopy: the fresh root segments were cleaned of soil with water and cut transversely with the help of sterilized blades and dipped into the 0.1% NaOH solution for 10 minutes. After that, the fine root segments were washed several times with tap water followed by distilled water. Then the root segments were stained with Alanine Blue and Ujala fabric whitener and kept it for 30 minutes. After 30 minutes the root segments were taken in glass slides with distilled water and observed under a fluorescence microscope (Leica DM 4000) using the filters adjusted to the wavelength of DAPI.

Isolation and identification of AM fungal species: AM fungal spores were extracted from the rhizospheric soil by wet sieving and decanting method (Gerdeman & Nicolson, 1963). Intact AM fungal spores were picked up with a needle in glass slides with polyvinyl alcohol-lactoglycerol and observed under a dissecting microscope (Koske & Tessier, 1983). Taxonomic identification of these spores was done on the basis of spore morphology, subcellular characters, and ornamentation in the spore wall. Spore characters were compared with the original descriptions (www.amf-phylogeny.com).

Root morphology study: root hair length and root hair density/cm of root segments were counted from the selected root segments used for quantification of fungal colonization. The three-dimensional counting of root segments was carried out by adjusting the focus of a microscope's plane (Ma *et al.*, 2001).

Results :

Mycorrhiza of *D. burmannii* under a light microscope:

The plants of *D. burmannii* along with its root structure are depicted in Figure 1 a-d. The root is a coarse structure with mean root hair length is 0.8 ± 0.004 mm and root hair density is 3.48 ± 0.241 cm of root. The arbuscular mycorrhizal structures viz., intercellular hyphae, vesicles, arbuscules, and septate hyphae were observed in the roots of *D. burmannii* (Fig.- 2). The roots are colonized by aseptate hyphae forming appressoria on the root surface (Fig.- 2a). The presence of vesicles are noticed (Fig.- 2b). Arum type of AM fungal morphology is observed (Fig.- 2d) with arbuscules inside the cells connected with intercellular hyphae running in the intercellular spaces. Dual (AM & DSE fungi) colonization was observed in *D. burmannii*. The DSE colonization was marked by the conspicuous presence of microsclerotia and septate melanized hyphae in the root sections (Fig.- 2 e & f).

Mycorrhizal structures such as arbuscules possessed $21.48 \pm 3.43\%$, vesicles having $8.66 \pm 3.23\%$, $65.56 \pm 1.85\%$ of hyphae and $7.14 \pm 1.9\%$ of DSE colonization in the root. A negative insignificant correlation ($R = -0.07$) was noted between mycorrhizal colonization and root hair characters.

Figure-1: Habitat and root architecture of *Drosera burmannii*. (a) Habitat. (b) Plant with trapped insect. (c) Flowering twig (d) Root architecture. (Scale bars: a=1cm; b=1.5cm; c=2cm & d=1.2 cm)

Figure-2: Light microscopy images of AM and DSE fungal colonization in the root segments of *D. burmannii*. (a) Root segments showing intracellular hyphal coil, (b) Root segments showing intraradical spores in the epidermal layers, (c) Root segments showing Arbuscule (arrow), (d) Root segments showing Arum type morphology, (e) Root segments showing micro-sclerotia (arrow) of Dark septate endophyte (DSE), (f) Root segments showing melanized and regularly septate hyphae of DSE (arrow). (Scale bars: a, d = 200 μ m; b, c, e & f = 100 μ m).

Mycorrhiza of *D. burmannii* under a fluorescence microscope: the colonization by mycorrhizal fungi was noticed in the cortical cells and it was more in the outer cortical cells. A freehand section also showed arbuscules and hyphal coils of the mycorrhizal fungi in the cortical cells. Fluorescing vascular bundles and the presence of root hairs were observed (Fig.- 3a & b). Hyphal coils and hyphal penetration in the epidermis along with fluorescing arbuscules were also observed (Fig.- 3c & d).

AM fungal composition: the spore density in the rhizospheric soil inhabiting by *D. burmannii* was 30.33/50 g of soil samples. A total of seven AM fungal morphotypes belonging to three different genera are distinguished on the basis of spore morphology; included *Glomus* sp.1 to *Glomus*

Figure-3: Fluorescence Microscopy images of Mycorrhizal colonization in the root segments of *D. burmannii*. (a-d) Transverse Section (T.S.) of *D. burmannii* root stained with Alanine Blue and Ujala observed under Fluorescence Microscope. (a & b) Fluorescing vascular bundles and presence of root hairs. (c) Arbuscule of AM fungi stained with Alanine Blue (arrow). (d) Arbuscules and hyphal coil stained with Ujala. ar=Arbuscule, hy =Hyphae and rh=Root hairs. (Scale bars: a-b=200 μ m; c-d=50 μ m)

Figure - 4: Arbuscular mycorrhizal fungal spores isolated from the rhizospheric soil of *D. burmannii*. (a) *Glomus* sp.1, (b) *Glomus* sp.2, (c) *Paraglomus* sp. (d&g) *Rhizophagus diaphanus*, (e) *Glomus* sp.3, (f) *Glomus* sp.4, (h) *Rhizophagus irregularis* (Scale bar: a-h = 100 μ m).

sp.4, *Paraglomus* sp, *Rhizophagus diaphanus* and *Rhizophagus irregularis* as depicted in the Figure- 4.

Discussion:

The study attributes mycorrhizal colonization status in carnivorous plant *D. burmannii*. The fungal structures such as aseptate intercellular hyphae, septate hyphae, micro-sclerotia, vesicles and arbuscules were found in the cortex representing typical mycorrhizal colonization. Vesicles were found to aggregate at certain points in the root cortex in *D. burmannii*. The incidence of AM fungi with fully-developed fungal structures in *Drosera* is consistent with the reports of Fuchs & Haselwandter, (2004) and Weishampel & Bedford, (2006) who observed the colonization of AM fungi in the roots of *D. intermedia* and *D. rotundifolia*. Harikumar (2013) also recorded AM fungal

RESEARCH ARTICLE

colonization in the roots of *D.burmannii* and *D.indica* from India. AM morphology in *Drosera* corresponded to the *Arum*-type as reported earlier (Harikumar, 2013). Morphological types of AM fungi have been examined in many plant taxa (Dickson, 2004; Matsuda *et al.*, 2005; Muthukumar & Prakash, 2009; Das & Kayang, 2010; Das *et al.*, 2013; Chakraborty *et al.*, 2016) although physiological or functional disparity between *Arum*-type and *Paris*-types has not yet been fully elucidated. However, this is the first report of dual colonization specifically by AM and DSE fungi in *D.burmannii*. The AM fungal colonization in roots of *D.burmannii* was much higher than the earlier study (Harikumar, 2013). Detection by the use of light and fluorescence microscopy also revealed that the roots of *D.burmannii* is colonized by AM and DSE fungi. *Glomus* was highly abundant in the soil inhabited by *D.burmannii*. The possible reason for *Glomus* abundance is rather complex though factors such as adaptability to acid soils, high competitiveness and/or reproductive capacity (Sieverding *et al.*, 1991) may be contributing to this. The findings of earlier workers concerning the AM fungal composition in the rhizosphere of Indian soils revealed that *Glomus* is the dominant genus (Muthukumar & Udaiyan, 2000; Bhattacharya & Bagyaraj, 2002). The occurrences of *Glomus* from Tripura, Northeast India was also found in the earlier studies (Debnath *et al.*, 2015, 2017; Chakraborty *et al.*, 2016; Sinha *et al.*, 2016).

Extensive research is required to unknot the exact role of the AM symbiosis and the stages of the plant growth influenced by such symbiosis. The study points out the presence of mycorrhiza which may serve as another way of availing nutrients from the soil.

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