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PSATHYRELLA CANDOLLEANA (FR.) MARIE, A SAPROPHYTIC FUNGUS FORMING ORCHID MYCORRHIZA IN SATYRIUM NEPALENSE D. DON FROM INDIA

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ABSTRACT

Psathyrella candolleana (Fr.) Marie is a saprophytic fungus which inhabits litter in the forest ecosystem. *Satyrium nepalense var. nepalense*, a rare autotrophic terrestrial orchid was found associated with this fungus forming orchid mycorrhiza. Isolation from root and tuber yielded two different fungi on Potato dextrose agar medium. Molecular identification of the fungal cultures was carried out by sequencing ITS region of nuclear rDNA. The isolate from the root was identified as *Psathyrella candolleana* and the isolate from tuber as *Colletotrichum dematium* .Light microscopic studies of the roots have shown that 60% of the cortical cells contained both digested and intact pelotons. TEM studies of the root have revealed the fine structure of the pelotons with the hyphae observed in the form of fungal cells with thick chitin wall. Pelotons were surrounded by the host cell membrane as an electron dense interfacial matrix suggesting biotrophic relationship. The cortical cells showed shrinkage of protoplasm indicating digestion of the pelotons. An anatomical study of the tuber has not revealed any pelotons. This is the first report of *Psathyrella candolleana* as an orchid mycorrhizal fungus colonizing the roots of *Satyrium nepalense* from the Indian region.

Keywords: Orchid mycorrhiza, pelotons, *Psathyrella candolleana*, saprophytic fungus, *Satyrium nepalense*.

INTRODUCTION

Orchidaceae is the largest family of flowering plants comprising about 17,000-35,000 species which occur as epiphytes or as terrestrial. In nature, all orchids require a suitable fungal partner for their dust sized seeds to germinate and develop into a protocorm. The fungal symbiont invades the embryo and forms intracellular hyphal coils referred to as pelotons. All orchid species are considered to be mycoheterotrophic during this stage of their life cycle (Smith and Read, 2008). Orchid mycorrhizas are found intracellularly in the cortical cells and are generally confined to the roots and persist for a limited period before collapsing and degenerating (Hadley, 1982).

Satyrium nepalense is an endangered, ethnobotanically important terrestrial orchid. This species is represented by two varieties namely *S. nepalense* D. Don *var. nepalense* and *S. nepalense var. ciliatum* (Lindl.) Hook., distributed in Western and Eastern Himalayas. It occurs in Jammu and Kashmir, Uttarakand, Bhutan, Sikkim, Assam, Arunachal Pradesh and South India. The plant grows on grass hilly slopes at varying elevations of 600-4600m. It grows to a height of 30-45cm, with tubers which are oblong, leaves are oval to lanceolate, 7-10cm long, small, pink and fragrant flowers occur on an upright spike. The flower has an interesting shape with the lip which is a hood like having a terminal tip called flap. It has two spurs one on each side of the ovary. The indigenous people of upper Nilgiris call this plant as "Ezhtkwehhdr" which means "bullock horns", as the spur of these flowers look like bullock horns. It flowers from July-December. The plant has medicinal value-the tubers are powdered, dried and used as energizing drink, as an aphrodisiac, decoction of tubers, roots and stems are used to cure infectious diseases, as a nutritional supplement, in treating diarrhea, malaria and dysentery (Saklani *et al*., 2012). Review of literature revealed that not much work has been done on the mycorrhizal association of this endangered and ethno-botanically important orchid plant. Hence, the mycorrhizal association of *Satyrium nepalense* has been investigated.

MATERIALS AND METHODS

Collection of plant and rhizosphere soil samples

The underground part of the terrestrial orchid along with its rhizosphere soil was collected from Kemmangundi (13°33'N and 75°46'E; 1434m MSL) of Karnataka State, India. The plant was identified as *Satyrium nepalense* D. Don *var. nepalense* (Fig. 1a).

Isolation, purification and maintenance of mycorrhizal fungi

Pure cultures of the fungi were isolated from roots and

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tubers using Potato dextrose agar medium. The fresh root/tuber samples were cleaned with running tap water, surface sterilized using 0.1% $HgCl_2$, rinsed several times with sterile distilled water, cut into 0.5-1cm pieces, plated aseptically in petriplates containing PDA and inoculated at room temperature (28°C). The plates were observed periodically for the mycelial growth. The fungal mycelium emerging from the cut ends of the roots after suitable growth was selected for purification and further studies. Hyphal tip method was used for purification. Isolated fungi were sub-cultured and maintained on PDA.

Anatomical studies

Thin free hand sections of the plant material such as root, tuber were taken and stained with tryphan blue (0.1% in lactophenol) and observed under the light microscope for the presence of fungal hyphae in the cortical cells. Highly coiled hyphae called pelotons (intact and digested) were observed and assessed in each section. Ultrathin sections were taken for Transmission Electron Microscopy. Root bits of 0.5cm were excised and fixed overnight in 3% Glutaraldehyde in 0.1M Cacodylate buffer (pH 7.2).The roots were post fixed in 1% Osmium tetroxide, dehydration was done in a gradient series of ethanol and Propylene oxide, and later embedded in Epon-Araldite (Hoch, 1986). Semi-thin sections (1µm) were stained with 1% Toluidene blue. Ultrathin sections were stained with Uranyl acetate, Lead citrate (Reynolds, 1963) and observed with an FEI Tecnai 12 G^2 Electron microscope operating at 80 kV.

Identification of orchid mycorrhizal fungi using molecular techniques

Pure cultures of orchid mycorrhizal fungi were used to isolate genomic DNA. Extraction of DNA from fungal mat was performed using CTAB. Electrophoresis was performed in a horizontal submarine apparatus (Genei, Bangalore, India) as outlined by Sambrook and Russel (2001). To amplify the ITS region, PCR was performed using Thermocycler PTC-100 TM to produce multiple copies of a specified DNA. Universal primers (forward and reverse) were used. The DNA was purified using gel extraction kit according to manufacturer's specifications.

DNA sequencing and Data analysis

The purified PCR product was sequenced. Sequences were determined by the chain terminal method using ABI 3130 Genetic Analyzer. Sequencing was done in forward and reverse direction. The sequence was generated using data analysis software. Basic local alignment search tool (BLAST) was used to provide rapid searching of nucleotides and protein database. The rDNA gene sequence was used to carry out BLAST with the data base of the NCBI GenBank. Based on maximum identity scores first 10 sequences were selected and aligned using multiple sequence alignment software program Clustal -W.

RESULTS AND DISCUSSION

Patterns of fungal colonization

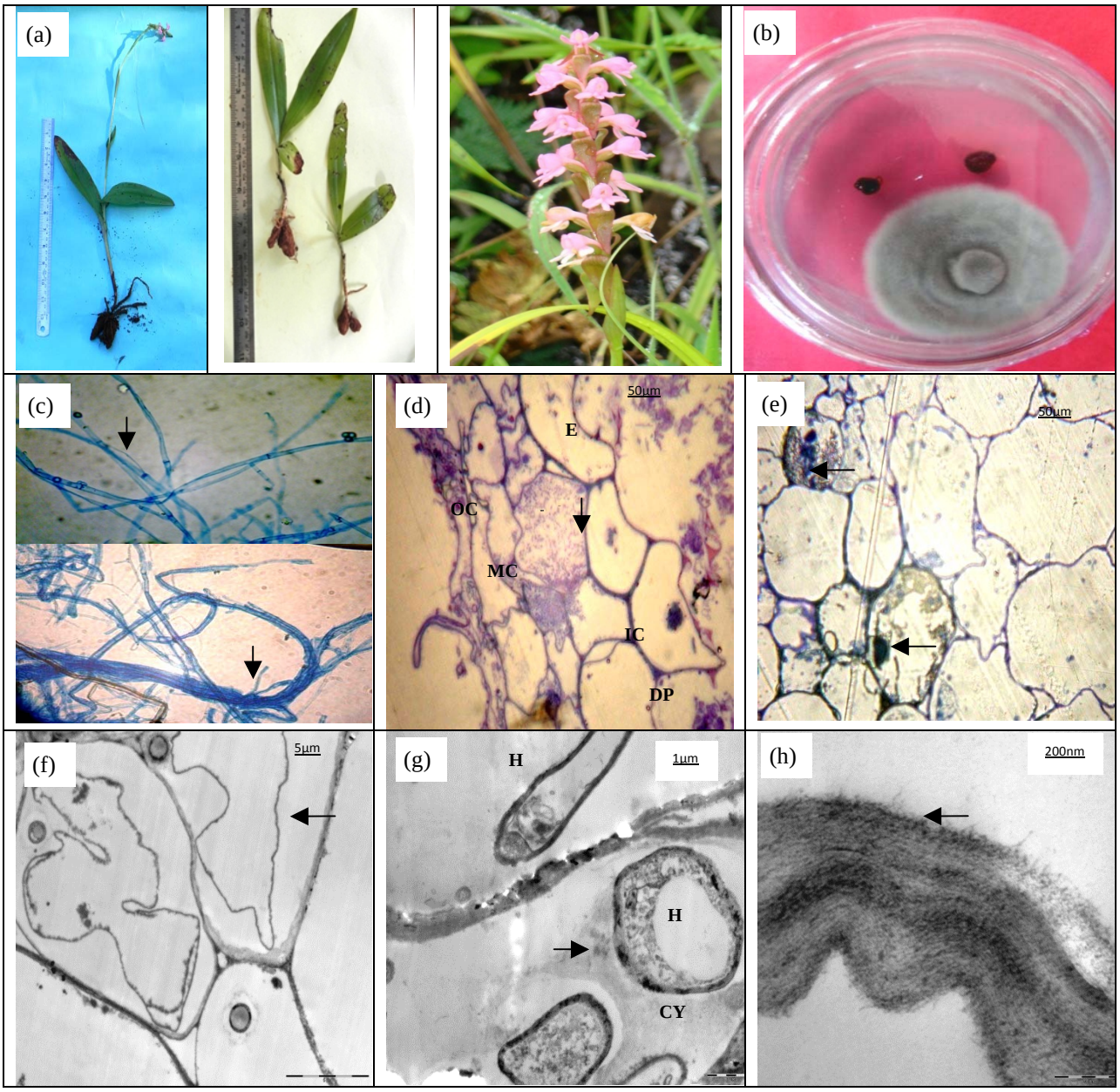
The transverse section of the root under the light microscope revealed the entry of the fungus through the root hairs. The root hairs lost their straight cylindrical nature and got distorted. After entry through the root epidermis the hypha entered the cortical cells and formed hyphal network called pelotons. Initially colonisation of the cortical cells was noticed in the epidermal cells and subsequently towards the outer and inner cortex. Two patterns of colonization by mycorrhizal fungi were observed. In the first pattern, the pelotons formed in the outer layer of cortical cells remained intact which may serve as a source of inoculum for the recolonization of cells deeper in the root cortex. The second pattern consisted of synchronously formed and degraded patches of pelotons in the inner cortex. Lysis of the pelotons, a characteristic of orchid mycorrhiza was observed in most of the cells and were referred as digestion cells (Fig. 1d,e).

Transmission Electron microscopy

Ultra-thin sections of the root revealed that the fungal hyphae were confined to the cortical cells. Epidermal cells were not colonized. The middle and inner cortex showed a multitude of fungal colonization as coils (pelotons) which were not clumped and free in the outer cortex, whereas in the inner cortex clumping of hyphae was observed. TEM revealed that the host cells showed increased cell cytoplasm in the cells colonized by fungal hyphae. The hyphal cells with large number of mitochondria indicates an increased activity of hyphae in the host cells. The hyphae were separated from the host cytoplasm by the host cell membrane as a layer of electron dense interfacial matrix suggesting a biotrophic relationship. No septal types were recognized. Digested and lysed pelotons were observed as typical collapsed hyphae (Figs. 1 f, g, h).

Colony characteristics

Two distinct fungal isolates were obtained, one each from the root and tuber of *Satyrium nepalense* on Potato dextrose agar (PDA). Macroscopic observation of the isolate obtained from the root showed white colony with wool like surface (Fig. 1b).The mycelial growth was observed in the range of 2-5cms for 7 days of incubation. The mycelium consisted of hyaline, thin walled, septate hyphae with clamp connections and formed mycelial strands. The other isolate from the tuber showed that the colony was initially white, turning dark gray, with felt like appearance and grew to a diameter of 3cm in 10 days. The mycelium consists of thick walled, closely branched, septate hyphae without clamp connections (Fig. 1c).



(a) Habit of *Satyrium nepalense* with inflorescence (b) Isolation of the fungus on PDA from the root (c) Mycelium showing septate hyphae and formation of rhizomorph (arrow heads) (d) Light microscopy - TS of root showing colonization of fungal hyphae in epidermal and cortical cells with pelotons (e) Digested pelotons in the cortical cells. (arrow heads) (f) TEM – showing digested peloton separated from the cortical cells (arrow head) (g) Transverse section of fungal hyphae in the cortical root cytoplasm with dense cell wall surrounded by host derived wall material (arrow head) (h) Fungal hyphae surrounded by electron dense layer of the host (arrow head); **CY** – Cytoplasm, **DP** – Digested pelotons, **E-** Epidermis, **H-** Hyphae, **IC**- Inner cortex, **MC** – Middle cortex, **OC**- Outer cortex

Molecular identification of mycorrhizal fungi

Two fungal isolates, one each from the root and tuber were identified by sequencing the fungal internal transcribed spacer (ITS) of ribosomal DNA. It revealed that the ground orchid in the root was colonized by *Psathyrella candolleana*, (GenBank Accession No. KC920474) a Basidiomycete. The isolate from the tuber was closely related to *Colletotrichum dematium*, (GenBank Accession No. KC920476) belonging to Deuteromycetes (Figs. 2a,b). Phylogenetic tree of both the fungal isolates showed 98% and 99% resemblance to the above species respectively. The novel aspect of the present work is that two fungi were found to be associated with the same orchid plant. Such dual infection of the

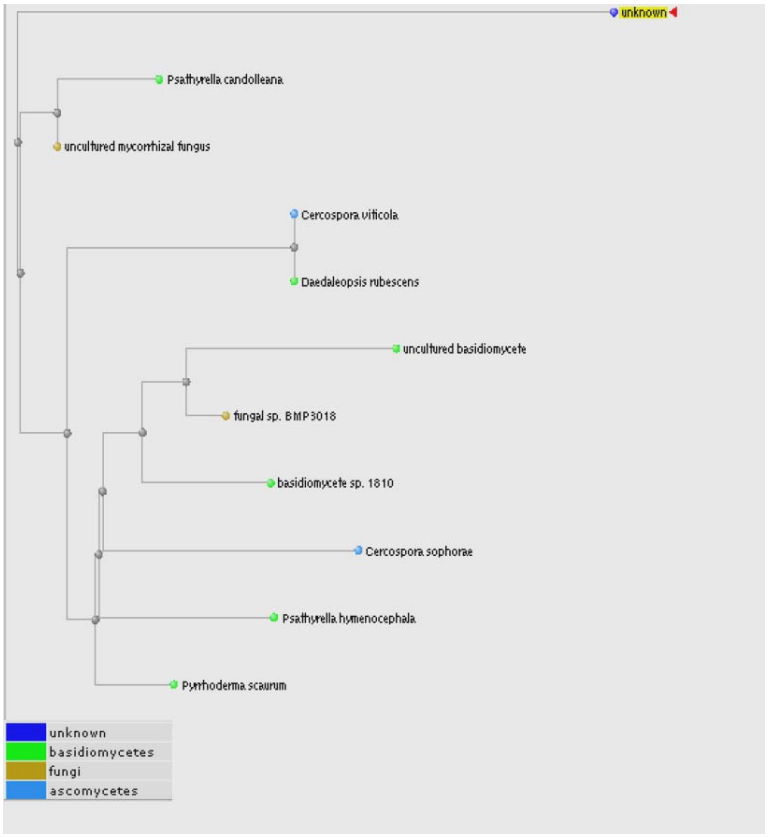


Fig. 2a. Phylogenetic Tree - *Psathyrella candolleana*.

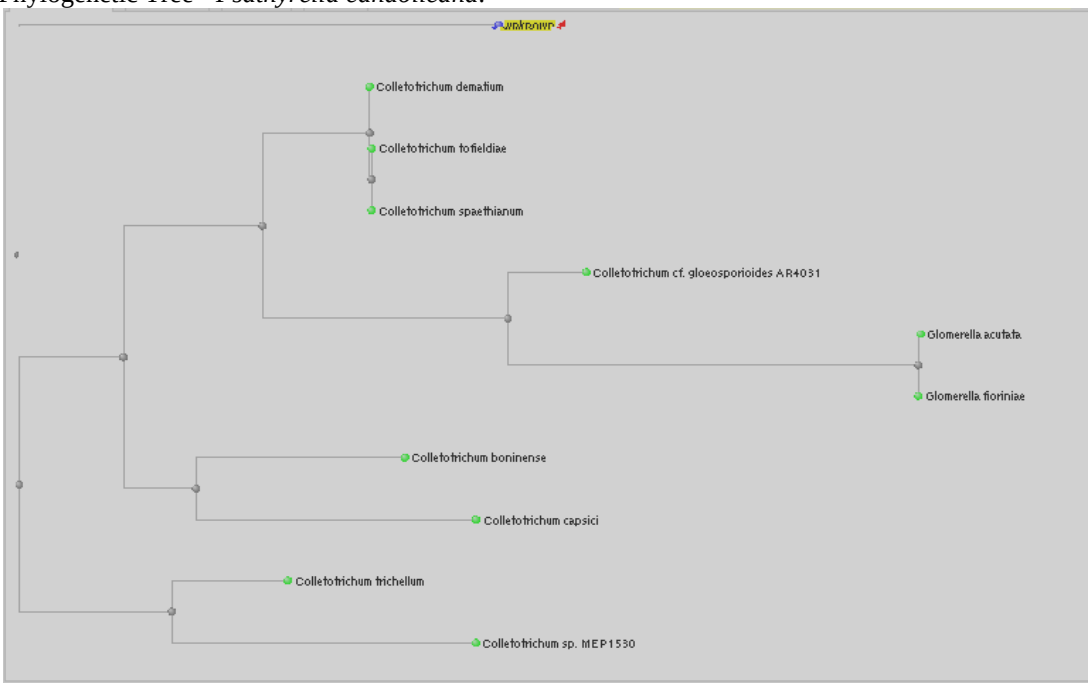


Fig. 2b. Phylogenetic Tree - *Colletotrichum dematium*.

same plant was considered a rare occurrence in orchid-fungal interaction (Kristiansen *et al*., 2001). These two phylogenetically distant taxa shared closely spaced tissues of the orchid. The presence of two different fungi in the same plant does not necessarily play a trophic role of mycorrhizal nature. It may be that the first fungi are the mycorrhizal partner and the second could be an endophyte whose possible role remains to be established.

Psathyrella candolleana is a saprophytic fungus growing on decomposing leaves and wood. It occurs in North America, Europe, Africa and many Asian countries like China, India, Japan and Sri Lanka. This is a new report on the mycorrhizal association of a saprophytic fungus with a terrestrial mycoautotrophic orchid. The saprophytic fungus with the flexibility to shift from saprophytism to mutualism is required for an orchid mycobiont (Rasmussen and Rasmussen, 2009). It is probable that due to the non-availability of regular symbionts, the terrestrial orchids may utilize the natively available saprophytic fungi like *Psathyrella* for their mycorrhizal requirements.

CONCLUSION

Isolation studies on mycobionts of *Satyrium nepalense* revealed the presence of *Psathyrella candolleana* in the root and *Colletotrichum dematium* in the tuber, which suggests that natively available fungal species can form mycorrhizal associations with terrestrial orchids. Interaction between the different fungal isolates and with that of plants would be worth investigating to understand the biology of orchid and mycorrhizal partners.

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