

RESEARCH ARTICLE



Ultrastructural studies and molecular characterization of root-associated fungi of *Crepidium acuminatum* (D. Don) Szlach.: a threatened and medicinally important taxon

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Abstract. *Crepidium acuminatum* (Orchidaceae) is a threatened medicinal orchid that grows under shady and moist forest floor where light remains for a very short period of time. Mycorrhizal association is known to be essential for seed germination and seedling establishment in a majority of orchids. Identification of fungi that form mycorrhizae with orchids is of crucial importance for orchid conservation. We used both morphological as well as molecular approaches to study this plant–fungal interaction. Scanning electron microscopy showed that fungi grow and proliferate in the middle layers of the cortex. Also, spiral-root hairs were found along with root hairs, which is an unusual observation. Spiral-root hairs provide more surface area for fluid absorption and entrance of colonizers. Further, total root genomic DNA was isolated and fungal internal-transcribed spacer (ITS) regions were polymerase chain reaction (PCR)-amplified using specific primer combinations ITS1F/ITS4 and ITS1/ITS4ul. ITS sequences were obtained and analysed to know the closest sequence matches in the GenBank using BLASTn hosted by NLM-NCBI. Subject sequences were identified to be belonging to three main genera, namely, *Tulasnella*, *Aspergillus* and *Penicillium*. Results indicate that mycorrhizal association is necessary for the growth and development of the plant. In addition, this symbiosis influences the distribution and rarity of this medicinally valuable taxon. Specific fungal partners may lead to an enhanced seed germination rate and increased efficiency of nutrient exchange between both the partners. Hence, knowledge of mycorrhizal fungi is essential for future *in vitro* germination and seedling establishment programmes, because they rely on fungi for germination. Identification of mycorrhizal fungi can be used for orchid propagation and conservation programmes.

Keywords. *Aspergillus*; internal-transcribed spacer; mycorrhizae; orchids; *Penicillium*; *Tulasnella*; *Crepidium acuminatum*.

Introduction

Crepidium acuminatum (D. Don) Szlach. (Syn. *Malaxis acuminata* D. Don) is a terrestrial orchid that occurs in patches in shady sites under oak or conifer forests on moist moss-laden soil. The species is distributed in Bhutan, China, India, Java, Malaysia, Myanmar, Nepal, Philippines, Thailand and Vietnam. In India, it is mostly found in temperate to subtropical Himalayas in Himachal Pradesh, Uttarakhand to Arunachal Pradesh, Assam, Nagaland, Manipur, Mizoram, Sikkim, Tripura and Khasi hills (Meghalaya) at an altitude of 1500–2400 m (Chauhan 1999; Balkrishna *et al.* 2012). Pseudobulb of *C. acuminatum* is an important ingredient of polyherbal Ayurvedic formulation ‘Astavarga’: a group of eight medicinal plants. Since Vedic period, it is used in Ayurveda for the

preparation of Chyawanprash and Astavarga Churna. Chyawanprash protects against cough, cold and other infections by revitalizing the body’s natural immunity. Pseudobulb contains several bioactive compounds that aid in smooth functioning of the circulatory, nervous and respiratory systems. It is used as adaptogenic, immunomodulator, health promoter and rejuvenator and helps in improving digestion and bowel movements. It is also used in formulation of traditional medicine which is used for curing tuberculosis (Sharma *et al.* 2011). Thus, this plant is overexploited from its natural populations and has become a threatened plant and notified in CITES Appendix-II for ensuring its protection and conservation (Lange and Schippmann 1999).

C. acuminatum harbours mycorrhizal fungi in roots, pseudobulbs and rhizomes (Uma *et al.* 2015). *In vitro*

regeneration protocols using internodes, embryos and pseudobulbs as explants have been standardized (Cheruvathur et al. 2010; Kaur and Bhutani 2010; Arenmongla and Deb 2012; Deb and Arenmongla 2013, 2014). The results demonstrated that orchids take longer duration to grow and multiply (Tamta et al. 2015).

Orchid mycorrhizae are restricted to the angiosperm family, Orchidaceae, which is one of the largest families of the flowering plants, with 736 genera distributed throughout the world except in polar regions and deserts (Hajra and De 2011; Chase et al. 2015). Mycorrhizal association is known to be beneficial for orchids (Rasmussen 2002; Dearnaley and Le Brocque 2006). In nature, orchids rely on infection by mycorrhizal fungi to provide the carbon sources for seed germination and seedling establishment (Yam and Arditti 2009). In the majority of orchids, fungal association occurs with the embryo cells of germinating seeds (Peterson et al. 1998) as well as with the roots of seedlings and mature plants.

Root hairs are extensions of root epidermal cells, mostly unicellular, but may be uniseriate (Stern et al. 1993), branched or unbranched, with spiral or reticulate thickening (Leitgeb 1865) and with a form of polarized growth known as apical growth (Heckman et al. 1997; Sieberer et al. 2005). Some plant possess have a life cycle of a few days, but in others it can last for a lifetime. The key function of root hairs is to increase the absorption area for water and nutrients from the soil (Fahn 1990; Evert 2006). Characterization of ultrastructure of cells, cell wall and septal pore of root samples using microscopy (Currah and Sherburne 1992; Wells and Bandoni 2001; Suárez et al. 2006) is also one of the methods that help in identification of orchid mycorrhizae.

Previously, knowledge about orchid mycorrhizae has been procured from *in vitro* isolation of fungi (Warcup 1971; Clements 1988). In recent years, fungal taxonomy is studied, especially by isolating fungal DNA from host roots and sequencing the nuclear ribosomal DNA (Seifert 2009). The fungal partners of orchid mycorrhizae can be more accurately identified directly from orchid protocorms, roots, tubers and rhizomes (Bougoure et al. 2005; Martos et al. 2009; Swarts et al. 2010). Polymerase chain reaction (PCR) amplification of the colonized orchid tissues using fungus-specific primers is commonly used (Dearnaley and Le Brocque 2006; Dearnaley and Bougoure 2010). Sequencing of the internal-transcribed spacer (ITS) of the nuclear ribosomal DNA after PCR amplification using a variety of primer combinations (White et al. 1990; Gardes and Bruns 1993) is now a rapid (Bernal et al. 2015) and reliable method for identifying orchid mycobionts.

Thus, the aim of this paper was to investigate symbiosis between the mycorrhizal fungi and roots of *C. acuminatum* using ultrastructural studies and identification of the associated fungi in the roots to determine the nature of

association and long-term conservation of this medicinally valuable threatened taxon.

Materials and methods

Collection sites

Soil and root samples were collected from Jageshwar, Uttarakhand (29°39.452'N 079°50.457'E, elevation: 1800–2000 m), India. Soil samples were taken which were near the roots, pooled into a composite sample per plot and analysed for pH, moisture, total carbon and hydrogen and nitrogen contents.

Sample collection and soil analysis

A portion of fresh soil from each sample was used to measure pH with a methodology modifying Schlichting et al. (1995). Root samples were washed thoroughly with double-distilled water and fixed in respective fixatives for microscopic analysis and some were stored at –80°C for DNA extraction.

Root clearing

Cleaned root segments of a length of ca. 0.5–1.5 cm were heated at 90°C in 10% KOH for 1–2 h, depending on the colour and thickness of the roots. Cleared root segments were rinsed with tap water for a few minutes followed by heating in 20% HCl for 1 h. Roots were then stained with trypan blue and the rest of the samples were stored and destained in 50% glycerol (Phillips and Hayman 1970; Brundrett 2014).

Scanning electron microscopy (SEM)

Root samples were collected and washed thoroughly with sterile-distilled water. further they were fixed in 2% glutaraldehyde at 4°C for 6–8 h (Ruzin 1999) and stored in 0.1 M phosphate buffer (pH=7.2) at 4°C until further analysis. Later, the material was dehydrated in a series of ethanol and subjected to critical point drying with CO₂ as the transition liquid, mounted on aluminium stubs using double-adhesive tape and sputter coated with gold (Bozola and Russell 1991). These root samples were observed under a scanning electron microscope (JSM-6610LV-JEOL, Japan) installed at the Department of Botany, University of Delhi. Images were captured at various magnifications at 3.0 kV.

Transmission electron microscopy (TEM)

For transmission electron microscopy study, roots were fixed overnight in 2.5% glutaraldehyde and 2% paraformaldehyde, prepared in 0.1 M sodium phosphate buffer (pH 7.2), then postfixed in 1% osmium tetroxide in the same phosphate buffer for 4 h, later dehydrated in graded acetone series and embedded in resin. Ultrathin sections (1 μ m) were cut and stained with 1% toluidine blue and contrasted with uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963) and observed at 120 kV using a TEM JEOL JEM-2100F, USA, installed at Advanced Instrumentation Research Facility (AIRF), Jawaharlal Nehru University (JNU). Images of cortical cells were obtained and processed using SI Viewer software.

Molecular characterization of root-associated fungi

Total genomic DNA was extracted from the roots of *C. acuminatum* using a DNeasy Plant Mini kit (Qiagen, Germany) using manufacturer's instructions. The total DNA was recovered in 50 μ L elution buffer. The fungal ITS sequence was amplified using two sets of primers: ITS1F/ITS4 and ITS1/ITS4tul (Gardes and Bruns 1993). The quality and quantity of DNA samples were estimated on 1% agarose gel and spectrophotometer, respectively. PCR was carried out in 50 μ L, with final concentrations of 66 μ M for each dNTP, 0.6 μ M for each of the primers (Laboratoires Eurobio, Les Ulis, France), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mg/mL gelatin, 0.1% (v/v) Triton x100, 5% (v/v) dimethyl sulphoxide and 1.5 units of *Taq* DNA polymerase. We used an AB Verity Thermal Cycler (USA) for amplification: initial denaturation at 94°C for 4 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. A 2 μ L sample of the amplified ITS was then migrated on 1.5% agarose gel in 1 \times Tris acetate EDTA (TAE) buffer stained with ethidium bromide (0.5 μ g/mL) to ensure the success of the amplification.

PCR products were purified using a MinElute PCR purification kit (Qiagen, Germany) using the manufacturer's instructions. PCR products were recovered in 40 μ L double-distilled water and sequenced using ITS1F/ITS4 and ITS1/ITS4tul. All the 11 sequences generated have been deposited in the GenBank hosted by the National Center for Biotechnology Information (NCBI) (GenBank: MG734749–MG734760). Searches for similar sequences were conducted using blast on the NCBI page (Altschul *et al.* 1997).

Phylogenetic analyses

In the present study, 12 new sequences were generated and 45 sequences were retrieved from the GenBank.

Close relatives of our sequenced taxa were identified through Discontinuous MegaBLAST searches of the GenBank and masked FASTA searches of the website hosted by the Berkeley University of California, Plant and Microbial Biology Department (<http://biotech.inbre.alaska.edu/fungal-metagenomics/>). The chromatograms obtained were edited using DNA Baser ver. 3.5 (2011). The consensus sequences for all the accessions were imported to one fasta file and that fasta file was imported to Clustal X (Thompson *et al.* 1997), in which all the sequences including the downloaded GenBank sequences were aligned followed by manual final adjustment to fine tune the alignments before analyses in Mesquite ver. 3.1.3 (Maddison and Maddison 2014) and also the authors tried an online multiple sequence alignment tool webPRANK with default settings (Löytynoja and Goldman 2010) (<http://www.ebi.ac.uk/goldman-srv/webprank/>).

The phylogenetics analyses were conducted using neighbour joining (NJ) and maximum-likelihood methods (ML). An unrooted NJ tree was constructed using multiple sequence alignments obtained after some manual adjustment in Mesquite (ver. 2.0). T-Rex (Tree and Reticulogram REConstruction) is used for NJ and ML analyses, both resulted in an identical topology. The statistical support obtained is estimated at each node by bootstrap parameters (≥ 75).

Results and discussion

Physico-chemical properties of soil

Physico-chemical properties of the soil were analysed at University Science Instrumentation Centre, Department of Chemistry, University of Delhi, India. The soil was brown sandy and moist type, pH 5.88, with high per cent of carbon content, i.e. 4.09%, followed by low hydrogen (0.90%) and nitrogen (0.33%) content. The dry weight of 10 g of fresh weight of soil was 6.64 g. Mycorrhizal fungi grow well in acidic soil. Mycorrhizae lower the rhizospheric pH due to selective uptake of ammonium ions (NH₄⁺) and release of H⁺ ions. A decreased soil pH increases the solubility of phosphorus precipitates. The hyphal uptake of NH₄⁺ also increases the flow of nitrogen to the plant as NH₄⁺ is adsorbed in the soil's inner surfaces and must be taken up by diffusion (Kheyroodin 2014).

Fungal colonization

The root clearing experiment showed colonization of roots by fungal hyphae (figure 1b). A total of 50 roots were viewed under the microscope and all of them showed fungal colonization. The mycorrhizal fungal hyphae are much finer than plant roots, and hence they could easily penetrate into small spaces between the soil particles.

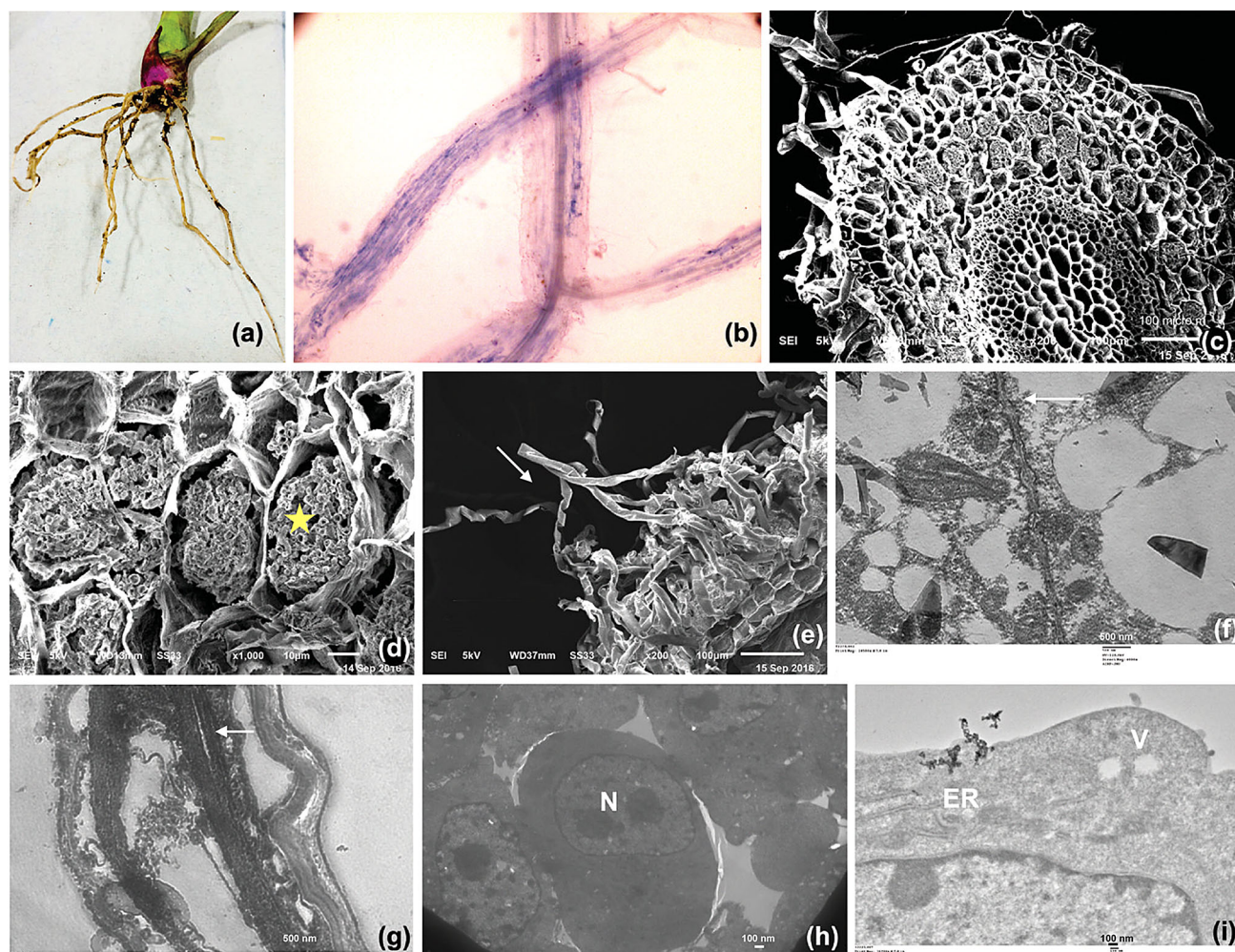


Figure 1. *C. acuminatum* (a). Roots (b). Root-clearing image showing fungal colonization (c). SEM image of transverse section of root (d). SEM image of cortical cells showing fungal pelotons (represented by star) (e). SEM image of spiral-root hairs (arrow) (f). TEM image of longitudinal section of root showing fungal septum (arrow) (g). TEM image of transverse section of root showing fungal cell wall (arrow) (h). Fungal nucleus (N) (i). Endoplasmic reticulum (ER) and vesicles (V).

The thread-like hyphae are structured such that there are linear cytoplasmic units that can extend for a metre or more, fan out into the soil to scavenge even highly immobile nutrients, efficiently absorb the maximum amount of available nutrients and deliver these nutrients back to the plant inside the root cell wall.

Ultrastructure of root and fungal hyphae

Scanning electron microscopy of the transverse sections of *C. acuminatum* roots showed that fungal hyphae penetrate into the epidermal cells of the host root through root hairs and spread out from one cell to another. The entry of fungal hyphae through the root hairs has been reported in many other orchid species, e.g. *Malaxis latifolia* (Muthukumar et al. 2003), *Zeuxine gracilis* (Muthukumar et al. 2011), *Eulophia epidendrea* (Sathiyadash et al. 2012), *Malaxis versicolor* (Sathiyadash et al. 2012) and

Geodorum densiflorum (Agustini et al. 2009; Jyothsna and Purushothama 2013). Then, the hyphae penetrate into the cortex and proliferate in the middle and inner layers of the cortex, forming pelotons in the cortical region of host's roots (figure 1, c&d). Both young and old pelotons were found in the root's cortex cells. Connections between the pelotons in the adjacent cortical cells through the cell wall were observed, which is a typical orchid mycorrhizal feature. It has been observed that hyphae did not penetrate or pass the endodermis and stele. In general, pelotons initially composed of loosely coiled hyphae filling the cell (the 'host' phase), followed by the hyphae collapsing into the centre of the cell around the nucleus (the 'digestive' phase) (Wahrlich 1886; Magnus-Levy 1990), from which the orchid derives the nutrition (Frank 1891; Burgeff 1909, 1936, 1943, 1959, reviewed by Arditti 1992). The transmission electron microscopy also showed the presence of hyphae, septum,

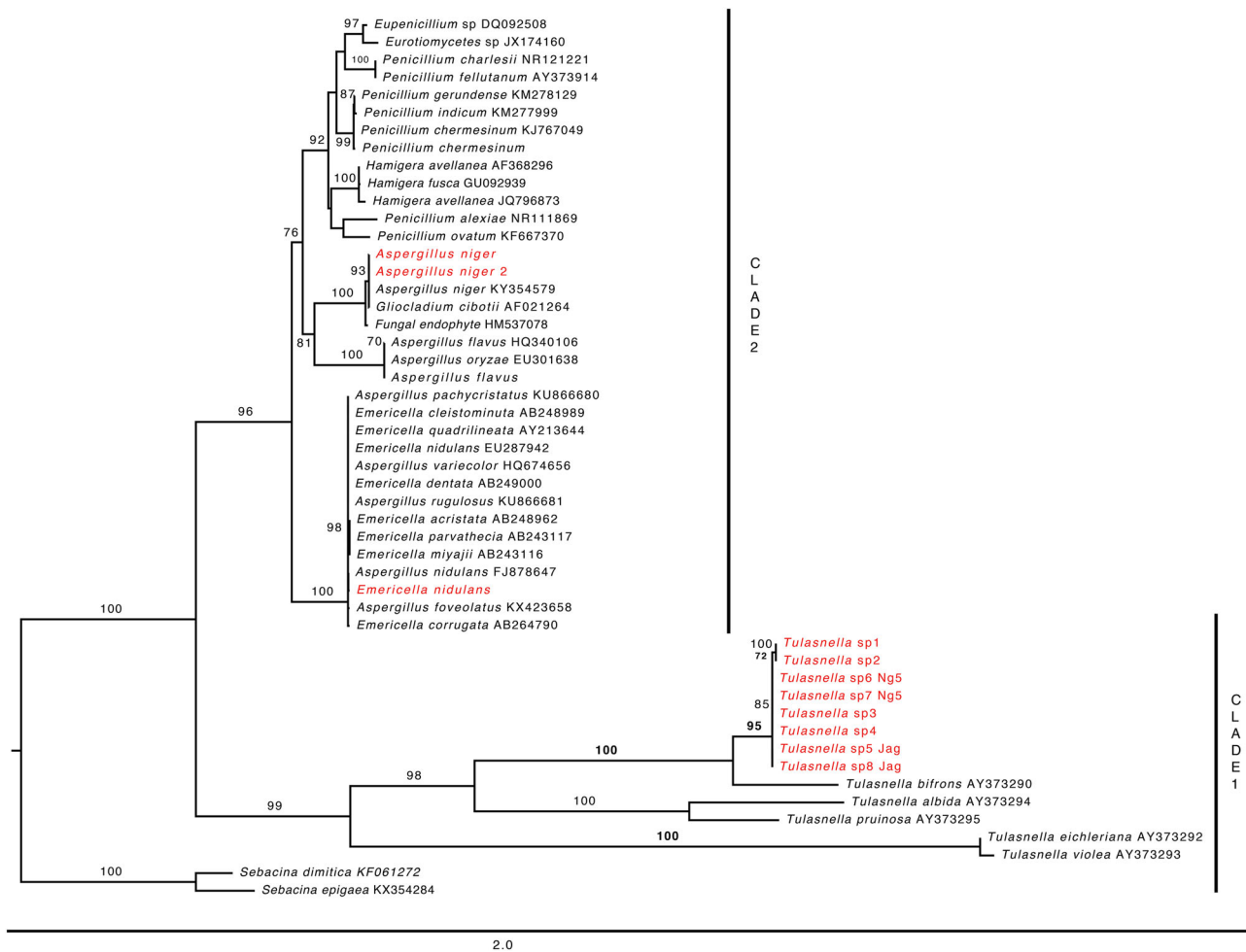


Figure 2. Best ML and NJ unrooted trees obtained from analyses of ITS for 58 sequences (45 from GB+13 sequences) analysed under the Tamura and GTR-CAT model. Taxa sequenced for the present study and included in the analyses have been marked in red font.

nuclei and cytoplasmic organelles in the root cytoplasm (figure 1, f–i).

Scanning electron microscopy also showed the presence of spiral-root hairs along with normal root hairs (figure 1e). It has been believed that the spiral-root hairs improve fluid absorption and entrance of colonizers (Bernal *et al.* 2015). Further, spiral-root hairs may facilitate the entry of water by capillarity (due to the wall opening), and offer greater adherence to the substrate (Bernal *et al.* 2015). The distributions of different types of root hairs in different groups of Orchidaceae as well as its value for systematic studies are not very well known (Bernal *et al.* 2015). Considering the genetic, hormonal and environmental variables in the development of root hairs in plants (Shi and Zhu 2002), it has been hypothesized that the spiral-root hairs are the final phase of differentiation of some root hairs. According to Lersten and Curtis (1977), the spiral shape of the root is caused by cell stretching, which causes the microfibrils of the outer wall to break down. Lersten and Curtis (1977) also speculated that these wall thickenings represent the original primary wall. The

presence of spiral-root hairs appear to be genetically established and are not influenced by environmental conditions (Bernal *et al.* 2015). Thus, the microscopic analysis demonstrated that, the roots of *C. acuminatum* are moderately colonized by mycorrhizal fungi that help in seed germination and seedling development.

Molecular characterization of root-associated fungi

The peloton isolation from the root cortex is the most preferable method for isolation of orchid symbionts (Warcup and Talbot 1967; Taylor and Bruns 1997; Rasmussen 2002). However, isolation of pelotons from many orchids is difficult because the roots of several orchid species do not possess massive mycorrhizal infections (Bayman *et al.* 2002; Otero *et al.* 2002, 2007). Further, under a dissecting microscope it was not possible to distinguish between the live and dead pelotons. Hence, total root genomic DNA was extracted to carry out this study.

Two primer combinations, i.e. ITS1F/ITS4 and ITS1/ITS4_{ul} were used for amplification. The results yielded a similar topology for both (NJ and ML) the analyses. The unrooted tree depicts that the identified sequences clearly fall into two clades (1 and 2) with a bootstrap support at nodes greater than 70% (figure 2). The majority of species identified belong to the genus *Tulasnella*, which belongs to the family Tulasnellaceae and division Basidiomycota. Also, the ITS sequences were not able to provide clear diagnosis for *Aspergillus* and *Penicillium* as the former is embedded within the latter (figure 2, clade 2). Experimental studies have shown that these fungi can sustain underground development of some orchids by supplying carbon from soil organic matter to developing seedlings (Smith and Read 2010). Some species belong to several species of *Aspergillus* and *Penicillium* which belong to division Ascomycota. Bidartondo et al. (2004) also found ascomycetes associated with orchid roots. Several species of *Tulasnella* form a monophyletic group (bootstrap 100) consisting of accession from different localities in Uttarakhand. Almost all mycorrhizae associated with terrestrial orchid are *Rhizoctonia* including anamorphic of *Tulasnella*, *Ceratobasidium* and *Thanatephorus* (Otero et al. 2002; Bonnardeaux et al. 2007). *Penicillium* and *Emericella* are grouped together with other accession of *Penicillium* retrieved from the GenBank (bootstrap 98). *Aspergillus* is grouped with other accessions of *Emericella* and *Aspergillus* retrieved from the GenBank. Sudheep and Sridhar (2012) reported the presence of *Aspergillus niger*, *A. flavus* and a few species of *Penicillium* in the roots of two orchids, namely *Bulbophyllum neilgherrense* and *Vanda testacea*.

In conclusion, Mycorrhizal association is necessary for the growth and development of orchid species. Specificity in orchid mycorrhizae has always been controversial; previous studies showed that some orchids are specific in their association with mycorrhizae and some orchids are generalist. However, data from an extensive study of Indian orchids are required to suggest that the degree of specificity is variable among orchid species. Terrestrial orchids have creeping, fibrous to fleshy rhizomes and roots with a wide range of functions. The function of roots in terrestrial orchids includes nutrient absorption and substrate fixation, storage as in tuber-like roots and roots specialized in storage, absorption and fixation. *C. acuminatum* is thus likely to derive its nutrition from orchid mycorrhizae present inside the root cortical region. In addition, these symbioses influence the distribution and rarity of this medicinally valuable taxon. Specific fungal partners may lead to an enhanced seed germination rate and increased efficiency of nutrient exchange between both the partners. Hence, knowledge about association of mycorrhizal fungi is essential for future *in vitro* germination, seedling establishment, orchid conservation and reintroduction in natural habitats.

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