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Diversity of Orchid Mycorrhizal Fungi in selected terrestrial orchids from Western Ghats, Karnataka, India

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Abstract

Orchids form mutualistic associations with a specialized group of fungi known as orchid mycorrhiza, particularly during early stages of development. This study investigates mycorrhizal associations in selected terrestrial orchid species. Transmission electron microscopy was used to examine fungal structures, measure hyphal and peloton dimensions, and assess the pattern of colonization within root tissues. Phylogenetic analysis was conducted to identify the associated fungal species. Fungal colonization was observed in 88% of the root cortex, indicating a strong symbiotic relationship. Entry of the fungus occurred mainly through root hairs, followed by the formation of tightly coiled hyphal structures, known as pelotons, within cortical cells. The spread of the fungus involved cell-to-cell penetration, and both intact and degraded pelotons were observed within the cortex. Fungal genera such as Tulasnella and Rhizoctonia were identified from root samples. Seed germination experiments using the isolated fungi demonstrated their role in promoting germination. These findings contribute to understanding the processes of colonization, isolation, and characterization of orchid mycorrhizal fungi, as well as the establishment of symbiotic relationships. Such knowledge may support conservation efforts and help address ecological challenges associated with orchid species.

Keywords: Orchid, mycorrhiza, pelotons, colonisation, seed germination.

Introduction

Orchids represent one of the most diverse and evolutionarily advanced families of flowering plants belonging to Orchidaceae. They are widely distributed across different ecosystems and are known for their complex ecological interactions, specialized reproductive strategies, and dependence on symbiotic associations[1]. Terrestrial orchids such as *Malaxis*, *Geodorum*, and *Nervilia* occupy distinct ecological niches and are commonly found on forest floors and shaded habitats in biodiversity-rich regions such as the Western Ghats of Karnataka[2].

A key biological feature of orchids is their obligate association with mycorrhizal fungi, particularly during early stages of development. Orchid seeds are minute and lack sufficient nutrient reserves, which prevents independent germination. Therefore, orchids establish symbiotic relationships with orchid mycorrhizal fungi that supply essential nutrients such as carbon, nitrogen, and minerals required for germination and seedling growth[3].

The orchid–fungus interaction is a specialized form of symbiosis characterized by the formation of intracellular hyphal coils known as pelotons within the cortical cells of roots. These structures function as sites of nutrient exchange between the plant and the fungal partner[4].

Pelotons undergo cycles of formation and degradation, which reflect a dynamic process of colonization and nutrient transfer essential for orchid survival[5]. Fungal colonization generally begins through root hairs or epidermal cells, followed by penetration into cortical tissues. The hyphae spread from cell to cell and form pelotons in successive cortical layers. Some pelotons remain intact for nutrient exchange, while others undergo lysis, releasing nutrients to the host plant[4]. The extent and pattern of colonization provide important information about the nature of the symbiotic relationship[4].

Several fungal genera, including *Tulasnella* and *Rhizoctonia*-like fungi, are commonly associated with orchid mycorrhiza and play a major role in supporting orchid growth and development[6]. The identification and characterization of these fungal partners are essential for understanding orchid ecology and for developing conservation strategies. Modern techniques such as transmission electron microscopy and molecular phylogenetic analysis have improved the study of orchid mycorrhizal associations. Transmission electron microscopy enables detailed observation of intracellular fungal structures, while molecular approaches allow accurate identification and classification of fungal taxa[7].

The Western Ghats are recognized as a global biodiversity hotspot and supports a high diversity of terrestrial orchids. However, many species are threatened due to habitat destruction, climate change, and anthropogenic activities.

Understanding their association with mycorrhizal fungi is therefore important for conservation and sustainable management[8].

The present study focuses on selected terrestrial orchids, namely *Malaxis rheedei*, *Geodorum densiflorum*, and *Nervilia crociformis*, collected from the Western Ghats region. The study aims to investigate the diversity of orchid mycorrhizal fungi associated with these species, to analyse fungal colonization patterns and peloton formation using transmission electron microscopy, to isolate and identify fungal partners using molecular methods, and to evaluate their role in seed germination. The study also aims to understand the ecological significance of orchid–fungus symbiosis in this region[9,10].

Materials and Methods

The present study was conducted using an integrated approach that included field sampling, laboratory analysis, ultra structural observation, and molecular techniques to investigate the diversity of orchid mycorrhizal fungi associated with selected terrestrial orchids.

Sample collection: Field sampling was carried out in selected locations of the Western Ghats of Karnataka, which is recognized as a biodiversity hotspot with high humidity and dense vegetation. Three terrestrial orchid genera, namely *Malaxis rheedei*, *Geodorum densiflorum*, and *Nervilia crociformis*, were selected for the study. Healthy and mature plants were identified in their natural habitats and carefully removed along with intact root systems. Special care was taken to avoid damage to roots during collection. Roots along with adhering rhizosphere soil were collected and placed in sterile polythene bags. Each sample was properly labeled with details such as location, date, and habitat conditions. The samples were transported to the laboratory under cool conditions for further analysis.

Preparation of root samples: In the laboratory, collected root samples were gently washed under running tap water to remove soil particles. Clean root segments were separated and used for different analyses, including microscopy, fungal isolation, and molecular studies. Fresh root tissues were processed immediately to maintain structural integrity.

Transmission electron microscopy (TEM) analysis: The ultra structure of fungal colonization and peloton development within root tissues was examined using TEM. For primary fixation, little root segments measuring one to two millimeters were fixed in glutaraldehyde. Post-fixation in osmium tetroxide was the next step. Following a graded ethanol series of dehydration, the samples were embedded in resin and sectioned using an ultra microtome. Lead citrate and uranyl acetate were used to stain the ultrathin slices. TEM was used to look at the produced slices.

The distribution of fungal colonization within cortical cells, hyphal shape and diameter, peloton size and layout, and the interaction between fungal hyphae and host tissues were all noted.

Assessment of fungal colonization: The extent of fungal colonization was evaluated using light microscopy. Root sections were stained with trypan blue or lactophenol-cotton blue to visualize fungal structures. Stained sections were observed under a compound microscope, and the presence of hyphae and pelotons within cortical cells was recorded. The percentage of colonization was calculated based on the proportion of infected root segments relative to the total number of segments examined.

Isolation of orchid mycorrhizal fungi: Standard isolation methods were used to extract fungal isolates from root tissues. Root segments were treated with 70% ethanol for 30 to 60 seconds and then 1% sodium hypochlorite for two to three minutes to surface sterilize them. To get rid of any remaining chemicals, the segments were then thoroughly cleaned with sterile distilled water. To avoid bacterial contamination, sterilized root fragments up to 1 cm were put on Potato Dextrose Agar (PDA) medium treated with antibiotics. The plates were incubated for 5–7 days at 25–28°C. In order to obtain pure cultures, emerging fungal colonies were monitored and subcultured onto new media.

Morphological identification of fungal isolates: Fungal isolates were initially identified using morphological traits. Features of the colony, including color, texture, and development pattern, were noted. The hyphal structure and spore production, if any, were examined under a microscope using lactophenol cotton blue staining. Fungal groups were tentatively identified using these traits.

Molecular identification and phylogenetic analysis: The identity of the fungal isolates was verified using molecular methods. Standard procedures were used to harvest genomic DNA from pure fungal cultures. The universal primers ITS1 and ITS4 were used to amplify the fungal DNA's internal transcribed spacer region. PCR amplification was confirmed by gel electrophoresis. After the amplified products were sequenced, the resulting sequences were compared to reference sequences found in databases like GenBank. To ascertain the isolates' evolutionary links, phylogenetic analysis was carried out. The fungal isolates were classified as belonging to genera like *Rhizoctonia* and *Tulasnella* based on sequence similarity.

Seed germination studies: To evaluate the functional role of fungal isolates, symbiotic seed germination experiments were conducted. Orchid seeds were surface sterilized before inoculation. Seeds were placed on agar medium containing fungal isolates, while control plates without fungal inoculation were maintained for comparison. All cultures were incubated under controlled environmental conditions.

Observations included seed swelling, protocorm formation, and subsequent growth stages. Germination percentage and growth rate were recorded to assess the effect of fungal association on seed development.

Data recording and documentation: All experimental observations were systematically recorded. This included percentage of root colonization, ultrastructural observations from electron microscopy, identification of fungal isolates, and seed germination data. Photographic documentation of microscopic observations, fungal cultures, and germination stages was carried out to support the analysis.

Results and Discussion

The present study provided detailed insights into mycorrhizal colonization, ultrastructural organization, fungal diversity, and their functional role in seed germination in selected terrestrial orchids, namely *Malaxis* spp., *Geodorum densiflorum*, and *Nervilia*spp (Figure-1). The findings confirm the ecological importance of orchid mycorrhizal fungi in sustaining plant growth and development.

Extent of mycorrhizal colonization: Microscopic analysis revealed a high level of fungal colonization in the roots of all studied orchids. Approximately 88% of cortical cells showed colonization, indicating a strong association between the host plants and fungal symbionts (Figure-2A, B). Colonization was largely restricted to the cortical region, while epidermal tissues showed minimal fungal presence. Fungal entry was primarily observed through root hairs, suggesting that these structures serve as initial points of infection. This observation is consistent with earlier reports on orchid mycorrhizal associations[11,12]. The high colonization percentage supports the concept that terrestrial orchids depend heavily on fungal partners for nutrient acquisition, especially during early stages of growth[1]. Microscopic observations showed both intercellular and intracellular hyphal growth, along with typical peloton formation. The presence of both intact and degraded pelotons within adjacent cortical cells indicates a continuous cycle of formation and digestion. This process enables the transfer of nutrients from the fungus to the host plant and is a key feature of orchid mycorrhiza[4,5]. The uneven distribution of colonization across cortical cells suggests that fungal proliferation is regulated by host cell condition and developmental stage. Similar spatial variation has been reported in other orchid species and reflects controlled symbiotic interaction[6].

Ultrastructural organization of fungal colonization: Transmission electron microscopy revealed detailed structural features of fungal colonization. Hyphae were septate and distributed within cortical cells, with diameters ranging from 2–5 µm. Pelotons were observed as tightly coiled hyphal masses occupying significant portions of cortical cells. Both intact and lysed pelotons were present (Figure-2C). Intact pelotons represent active nutrient exchange, while lysed pelotons indicate breakdown and release of nutrients into host cells[4]. Fungal penetration occurred through cell-to-cell movement without causing visible damage to host tissues. The absence of structural damage confirms the mutualistic nature of the association, where both partners benefit from the interaction[3]. The restriction of fungal structures to cortical tissues and their absence in vascular regions further supports the specificity of orchid mycorrhizal interactions[3,4]. The coexistence of intact and degraded pelotons suggests continuous turnover regulated by the host plant, allowing efficient nutrient uptake while maintaining cellular integrity[5].

Peloton formation and functional significance: Peloton formation was consistently observed in all studied orchid species and was mainly localized in the outer cortical region (Figure-3). These structures appeared as dense, coiled hyphal aggregates and varied in size depending on their stage of development. The simultaneous presence of intact and lysed pelotons indicates a dynamic process of nutrient exchange. Intact pelotons function in nutrient transfer, while lysed pelotons represent stages of nutrient release to the host plant[10]. This continuous cycle is essential for maintaining plant growth and metabolic activity. The ability of fungal isolates to form dense mycelial networks in culture suggests their adaptation to root environments. The absence of reproductive structures under laboratory conditions indicates that these fungi are primarily adapted for symbiotic growth rather than independent reproduction[13].

Isolation and identification of fungal partners: Fungal isolates obtained from root tissues showed consistent morphological characteristics. Colonies were initially white and cottony, later becoming cream to light brown. Microscopic observations revealed septate hyphae with branching patterns. Based on morphological features, the isolates were identified as *Tulasnella* spp. and *Rhizoctonia* spp. These genera are widely reported as dominant orchid mycorrhizal fungi and are known to establish stable symbiotic relationships with terrestrial orchids[5,6]. Their presence across all studied orchid species indicates ecological adaptability and a broad host range. The absence of host tissue damage further confirms the non-pathogenic nature of these fungal associations[13].

Molecular and phylogenetic confirmation: Molecular identification confirmed the identity of fungal isolates. Amplification of the ITS region was successful, and sequence analysis showed high similarity with *Tulasnella* (Accession no: KC920478) and *Rhizoctonia* species. Phylogenetic analysis grouped the isolates within established orchid mycorrhizal clades, confirming their classification. Molecular techniques improved the accuracy of identification and supported morphological observations[9,10]. The results also suggest that orchids may associate with a broader range of fungi than traditionally recognized. Recent studies indicate that orchid mycorrhizal communities are diverse and include both classical and non-classical symbionts[14,15].

Role of fungal isolates in seed germination: Symbiotic germination experiments demonstrated that fungal isolates significantly enhanced seed germination. Seeds inoculated with fungal cultures showed protocorm formation within a few weeks, whereas control seeds without fungal inoculation showed little or no germination (Figure-4). These findings confirm that fungal partners are essential for seed germination and early development in orchids[1]. Orchid seeds lack nutrient reserves and rely entirely on fungal symbionts for initial growth[12].

Ecological significance and interpretation: The high colonization levels observed in this study highlight the strong dependence of terrestrial orchids on fungal symbionts. Cortical tissues act as the primary site for nutrient exchange, while root hairs serve as entry points for fungal colonization. The identification of *Tulasnella* and *Rhizoctonia* species is consistent with previous studies that report these genera as dominant orchid mycorrhizal fungi[5,6]. Their widespread occurrence suggests ecological flexibility and adaptation to different environmental conditions. The dynamic formation and degradation of pelotons reflect a regulated process that ensures efficient nutrient transfer without damaging host tissues[4,5]. Such interactions are critical for plant survival in nutrient-limited environments.

Conservation implications and limitations: The findings emphasize the importance of mycorrhizal associations in orchid conservation. Protection of orchids alone is not sufficient without conserving their associated fungal communities and habitats[8]. The successful use of fungal isolates in seed germination experiments suggests that symbiotic propagation techniques can be applied in conservation and restoration programs. However, the study is limited to culturable fungi, and the actual diversity of fungal partners may be higher. Advanced techniques such as metagenomics and ecological studies are required to better understand orchid–fungus interactions[14,16,].

Figure-1: Plants studied: A. *Malaxis rheedei*, B. *Geodorum densiflorum*, C. *Nervilia crociformis.*

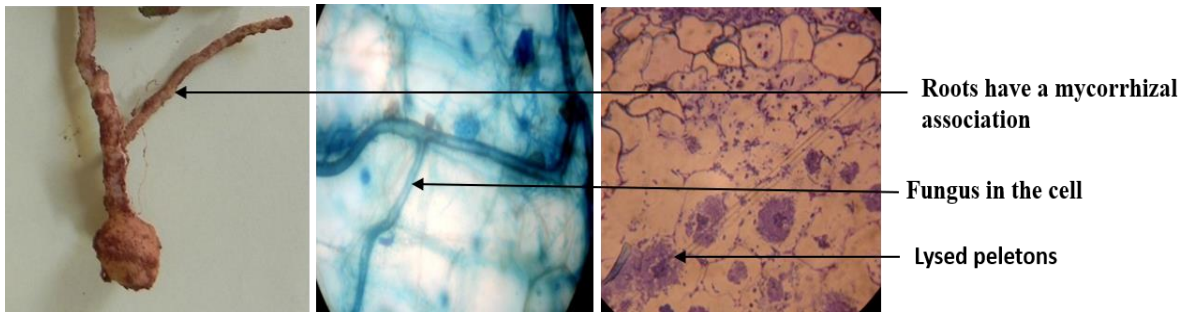

Figure-2: *Tulasnella* and *Rhizoctonia* fungal hyphae.

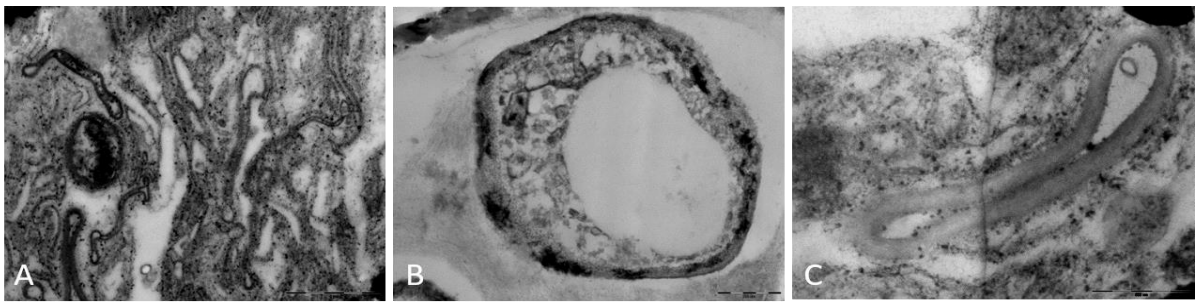

Figure-3: TEM analysis of fungal colonization in A. *Malaxis rheedei*, B. *Geodorum densiflorum*, C. *Nervilia crociformis.*

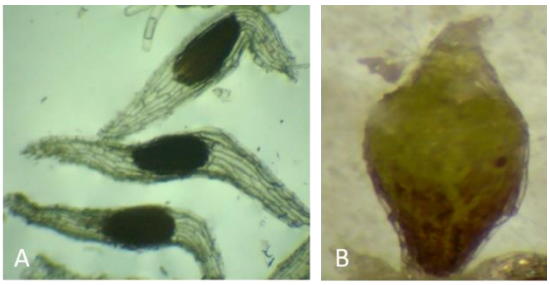

Figure-4: (A) Seed before germination, (B) Seed after germination (protocorm formation).

Table-1: Comparative observations of seed germination among orchid species.

Orchid Species	Colonization (%)	Fungal Isolates	Germination Response
Malaxisrheedei	80%	*Tulasnella*, *Rhizoctonia*	High
Geodorum densiflorum	82%	*Rhizoctonia*	Very High
Nervilia spp.	81%	*Tulasnella*	High

Conclusion

The present study demonstrates that orchid mycorrhizal fungi play a crucial role in colonization, nutrient exchange, and seed germination in terrestrial orchids. The integration of microscopic, ultrastructural, and molecular approaches provides a comprehensive understanding of orchid–fungus symbiosis. These findings contribute to plant ecology, mycology, and conservation biology, highlighting the importance of fungal associations in maintaining orchid diversity and ecosystem stability.

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