

Study of morphology and orchid mycorrhizal associations in *Malaxis rheedei*

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ABSTRACT

The mycorrhizal association in orchids is a striking example of mutualism, where the orchid and the associated mycorrhizal fungi exchange essential nutrients and support each other's growth and reproduction. The present study focused on a selected terrestrial orchid which is noteworthy for its medicinal value. We aimed to investigate the specific mycorrhizal associations to understand how these interactions influence the morphology of their roots. The morphological characteristics of roots were studied using light microscopy. Transmission electron microscopy studies were conducted to analyze and measure fungal hyphae and peloton sizes as well as to know about fungal colonization distribution. Phylogenetic analysis was conducted to identify the mycorrhizal fungi. Colonization was seen in the 91% root cortex proving high mycorrhizal association. Hyphae appeared in the form of peloton patches at the cortex, occupying up to 62% of the cortical space. The root length with an intact peloton was noted 30% more than the degraded peloton. A single fungus, *Tulasnella calospora* was isolated from the roots. It was identified using phylogenetic analysis of *ITS* sequences. Identification of this orchid-mycorrhizal association has the potential to address ecological challenges, contribute to its conservation, and unlock the phytochemicals potential of plant-fungus interactions for drug development.

1. INTRODUCTION

Orchids, renowned for their captivating beauty and remarkable ecological adaptations, are a diverse and enigmatic group of flowering plants. Among the thousands of orchid species inhabiting various ecosystems worldwide, the terrestrial orchid *Malaxis rheedei*, syn *Malaxis versicolor* stands out as an intriguing subject of study [1]. It is an endangered species with medicinal value [2]. This species, known for its distinctive morphology and intriguing ecological interactions, has always fascinated botanists, ecologists, and conservationists. It is a terrestrial or lithophytic erect species. The stems are sheathed, swollen at the base, and purplish green. The flowers of *M. rheedei* are maroon, bracts lanceolate, dorsal sepals linear, and petals recurved or incurved [1]. Its pseudobulbs are used as a tonic and form one of the ingredients of important ayurvedic medicine known as "Astavarga" which has therapeutic value [3].

M. rheedei is a species of orchid native to various parts of Asia, including India. It is endemic to Silent Valley, Mysore, Hassan, Thalgoppa (Kodagu), Tarave forests, Charmadi ghats, Kotigehar, Kemmangundi, Dakshina Kannada in India [4]. It engages a complex symbiotic relationship with mycorrhizal fungi beneath the forest canopy or within its preferred habitat as it is a known quality of terrestrial orchids [5]. This relationship is critical for its survival and reproductive success, making it a compelling model for understanding orchid–fungus interactions and the broader ecology of terrestrial [6].

The mycorrhizal association in orchids, including *M. rheedei*, is a striking example of mutualism, where the orchid and the associated mycorrhizal fungi exchange essential nutrients and support each other's growth and reproduction [7]. Orchids depend on these fungal partners, especially for nutrient acquisition, as they often grow in nutrient-poor soils [8]. The most prevalent orchid mycorrhizal fungus in temperate and tropical areas are particularly members of the Tulasnellaceae family [9]. As a member of the fungal family Tulasnellaceae, *Tulasnella calospora* is known for its specific associations with orchids and its ability to facilitate their growth and development [10]. *Tulasnella calospora* is instrumental in assisting orchid seeds in germinating and developing into seedlings by providing the necessary nutrients and a suitable environment [11]. Several different consequences might

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result from the interaction of mycorrhiza with the orchid host. A post-embryonic plant structure called a mycorrhizal orchid protocorm, which has cells with intracellular fungi hyphae (pelotons) inhabited by them, is the result of a favorable association [12]. Usually, viable mycorrhizal protocorms grow into photosynthetic seedlings, but occasionally, they may become overtaken by the fungus' mycelium and decayed hyphae [13].

Investigating the specific mycorrhizal associations with *M. rheedei* and understanding how these interactions influence the morphology of the roots of plants are a central focus of this study.

2. MATERIALS AND METHODS

2.1. Study Location

Roots of *M. rheedei* (Lindl.) orchid species were collected from two different areas in the Western Ghats. Collection was done in September to identify plants at vegetative and reproductive stages. Non-flowering plants were considered vegetative and flowering plants were considered at the reproductive state of plants. The characteristics of the location of the study are presented in Table 1.

2.2. Sample Collection

Five individuals each attaining vegetative and reproductive growth stages were selected and their root samples were collected. When collecting, care was taken to avoid harming the roots. Roots were cleaned and kept until processing in a solution of FAA (formalin: 70% acetic acid: alcohol: 5:5:90).

2.3. Determination of Root Morphology

For the evaluation of root hair features, 10 randomly chosen root pieces (about 1 cm long) were put in water on microscope slides. $\times 20$ was used to count the number of root hairs per centimeter of root length using a dissecting microscope. Using a compound microscope equipped with an ocular scale, measurements were taken at $\times 100$ to determine the root hair's length and width.

2.4. Estimation of Orchid Fungal Colonization

To check for fungal colonization in the cortical cells, the plant material was dyed with trypan blue and examined under a compound microscope for the presence of the structure of fungi in the cortical cells. The magnified intersection method was used to calculate root length having mycorrhizal colonization [14]. In addition, the number of intersections having pelotons and fungal hyphae was counted. Root length having highly condensed hyphal pelotons (intact and digested) was selected through observation and their assessment was done through transmission electron microscopy [15]. The percentage of cells available for colonization was calculated by counting the number of colonized cells in the cortex divided by the total cells seen $\times 100$.

Each section's percentage of the cortical volume filled by pelotons was

Table 1: Characteristics of collection sites.

S. No.	Collection sites	Latitude	Longitude	Vegetation type
1.	Kogar, Shimoga	14.1483	74.9581	Evergreen and semi-evergreen climax forests
2.	Z-point, Chikmagalur	13.5474	75.7583	Grassland slopes

graded using a modified version of the eight-step scale (12.5–100%) developed by Rasmussen and Whigham [16]. A circular cover glass had crosshairs on it that divided the circumference into eight equal portions starting in the center. Eight portions of the circle were divided by 100%, making each portion a total of 12.5%. The divisions were divided into four pieces, each of 3.125%. Five hand sections taken from various root parts of a species were scored. The length and width of the 50 cells and pelotons in the chosen portions were measured.

2.5. Fungal Isolation and Purification

Mycorrhizal fungi were isolated in accordance with conventional methods known as plating method. The roots were surface sterilized on the suitable media. Roots were split into 5–10 mm pieces, in an aseptic manner and plated onto potato dextrose agar (PDA) medium, then allowed to set for 2 days at a temperature of 25°C. Fungi started developing from the chopped ends to produce the pure culture. These fungal isolates were subsequently grown on PDA at 25°C for 4 weeks in typical diurnal conditions before being tried for identification [17]. The isolated fungi were purified using the hyphal tip method.

2.6. DNA Isolation and Amplification

The CTAB technique was modified slightly to extract genomic DNA from the fungal mycelial. We used the universal primers (forward and reverse) for fungal *ITS* to carry out a polymerized chain reaction (PCR) to create many copies of a specific DNA. In the present study, Universal primers (forward and reverse) for fungal genome, *ITS* forward – (5' – TCC GTA GGT GAA CCT GCC G – 3'), and *ITS* reverse – (5' – TCC TCC GCT TAT TGA TAT GC – 3') sequences were used. The *ITS* sections were amplified using PCR reaction mixes in a volume of 50 μ L. Standard PCR settings were used to amplify the *ITS* sections of the fungal DNA [18].

2.7. Sequence Analysis

The chain termination approach was used to sequence DNA using a genetic analyzer from Applied Biosystems. For phylogenetic analysis, MEGA software version 6.0 was utilized, whereas ClustalW was employed for sequence alignments. A phylogenetic tree was created using the neighbor-joining (NJ) method. P-distance substitution model and bootstrapping value 1,000 were used for calculations [19].

2.8. Statistical Analysis

Mean and standard deviation were calculated to analyze mycorrhizal colonization and root hair morphology data. Unpaired t-tests were used to compare continuous variables that were reported. The analysis was conducted using SPSS version 22 statistical software. A 5% alpha level was regarded as significant.

3. RESULTS

3.1. Study of Root Characteristics

Roots of *M. rheedei* [Figure 1a] collected from Shimoga and Chikmagalur were analyzed thoroughly using a dissecting microscope, binocular compound microscope, and transverse electron microscope. Each centimeter of root length was observed under a dissecting microscope for the number of root hairs present in it. We noted 15.63 ± 1.12 and 16.14 ± 2.01 root hairs in Shimoga and Chikmagalur orchid samples, respectively. The root hair's length and width were measured using a binocular compound microscope and noted. Root hair's length was $92.45 \pm 9.32 \mu$ m and $91.12 \pm 9.98 \mu$ m at Shimoga and Chikmagalur locations, respectively. The breadth was noted at

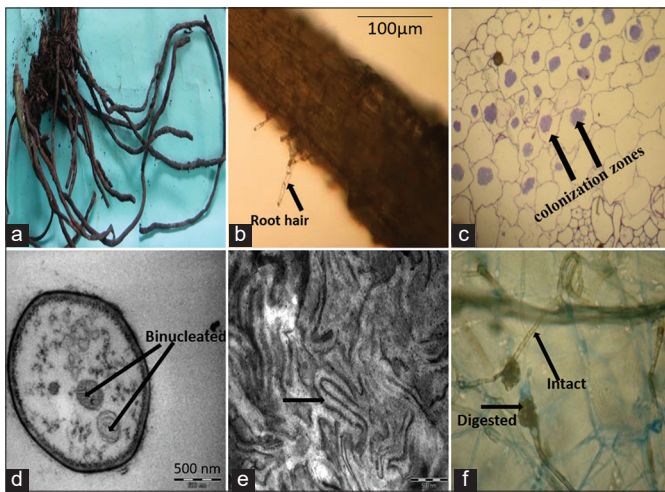


Figure 1: Microscopic study of *Entada rheedii* roots. (a) Roots of *M. rheedei*; (b) transverse section of root and root hair; (c) colonization zone of fungal pelotons in cortex; (d) binucleated hyphae; (e) hyphae in the form of peloton (f) intact and digested pelotons.

$10.24 \pm 1.45 \mu\text{m}$ and $9.58 \pm 2.15 \mu\text{m}$, respectively [Figure 1b]. These values were not statistically significant ($P < 0.05$), which concludes that variation in location does not have a significant impact on root characteristics.

3.2. Colonization of Roots

Fungal colonization was seen in the transverse section of the root cortical region. Root length with total colonization in the form of pelotons was approximately 91%. The diameter of the roots varied between 2 and 8 mm in the test species. The fungus' hyphae commonly invade the roots through the root hairs or the root surface itself. Colonization was seen in the cortex of roots of all ages, both vegetative and reproductive. Hyphae appeared in the form of patches in the cortex [Figure 1c], taking up to 62% of the space [Table 2]. In reproductive age roots, some deformation of root hairs cortex appeared to be related to endophytes within their roots in the areas of colonized roots. We noted a 30% difference in root length between the degenerating peloton and the intact peloton [Table 2]. We also noted width of the vascular bundles was narrower than the parenchymatous cortex [Table 2].

TEM studies noted that vegetative hypha was approximately $2.5\text{--}4 \mu\text{m}$ wide, hyaline, septate, binucleate [Figure 1d], constricted at branch points, sclerotia minute, mycelium composed of loosely arranged clusters of monoid cells which were hyaline, ellipsoidal to spherical, $7.5\text{--}14.2 \times 6.5\text{--}10.8 \mu\text{m}$, and in short branched or unbranched chains. In cells with degenerating peloton intact nuclei were seen.

TEM analysis also that *M. rheedei* root hairs did not remain straight and cylindrical and got distorted after the entry of fungal hyphae. Hence, we confirmed that the fungus entered into the roots only through the root hairs. Pelotons of loosely arranged clumps of hyphae were seen within the root's cortical cells. Although the colonization of the fungal hyphae is a continual process, we could not see that all of the cortex's cells were colonized at once. We also noted that colonization was mainly because of pelotonic hyphae [Figure 1e]. Pelotons were observed in the middle and inner cortex. The colonization density was up to 85% in the cortex. Both intact and digested pelotons were observed [Figure 1f]. The lysis of the peloton was from the periphery to the center of the cortical cell (centripetal lysis). Although 62% of the

Table 2: Root hair characteristics with and without pelotons.

S. No.	Characteristics	Kogar, Shimoga	Z-point, Chikmagalur
1.	Root hair number	15.63±1.12	16.14±2.01
2.	Root hair length	92.45±9.32	91.12±9.98
3.	Root hair breadth	10.24±1.45	9.58±2.15
4.	Root length with degenerating peloton	55.76±7.67	54.32±6.89
5.	Root length with the intact peloton	39.67±7.89	38.41±6.55
6.	Root length with total colonization	91.34±6.57	91.98±7.98
7.	Ratio of intact and lysed pelotons	1.65±0.21	1.70±0.19
8.	Percent cortical volume occupied	61.84±8.57	63.74±7.77

cortical volume was occupied with peloton, colonization was absent in the vascular region [Table 2].

3.3. Isolation of Fungi

The colony on PDA was pale white to cream, margin submerged, glabrous, with concentric zonation. The colony grew to 9 cm in diameter after 9 days of incubation. We yielded one fungal isolate from the roots of *M. rheedei* at both locations.

3.4. Molecular Characterization of Fungus

DNA was extracted and purified by the modified CTAB method from the fungal samples at both locations. The amplification success of loci, namely, *ITS1-5.8S-ITS2* was 95%. *ITS* sequence (581 bp) data of the fungal isolate from the roots of *M. rheedei* showed a close (95%) resemblance to many *Tulasnella* species on NCBI BLAST. The sequence has been submitted to NCBI GenBank with Accession No. KC920478. Higher bootstrap values were seen in the NJ-based *ITS* sequencing tree, which was also associated with other *Tulasnella* species. The phylogenetic tree of the fungal isolate is given in Figure 2. It showed 98% identity with *Tulasnella calospora*, hence identified as *T. calospora*.

4. DISCUSSION

According to the fact that mature orchids are mycorrhizal dependent and frequently contain mycorrhizal roots or tubers [20], we collected *M. rheedei* from various sites in the current research to find differences in their morphology and mycorrhizal relationship. We found the same fungal association at both locations and non-significant variations in root morphology, it proves that the specificity of the orchid-mycorrhizal association is not location dependent.

Our study found distortion of root hairs of *M. rheedei* roots, which made it evident that the root hairs served as the main point of entrance for the fungus. This supports Sathiyadash *et al.* observations of eight terrestrial orchids that the fungal entrance into roots was always restricted to the portion of the roots called root hairs [14].

The fungus commonly penetrates the root hairs near their tips, occasionally just slightly away from the tips and infrequently at their bases. Root hairs often lose their straight cylindrical shape and become crooked and bent to varying degrees after the introduction of the fungal hyphae, imitating the pattern of colonization including the nodulating bacteria [21]. Depending on the location of the hyphal entrance, the alteration of the root hair may just reach the tip or may continue to various lengths in the direction of the base [22]. In the present study,

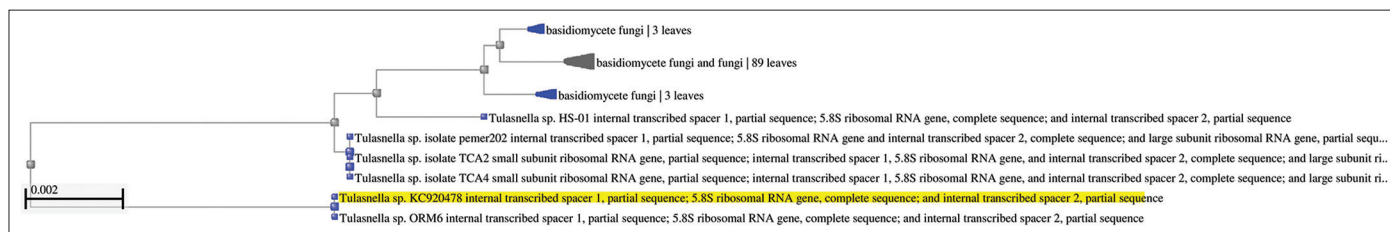


Figure 2: Phylogenetic tree of *Tulasnella calospora* isolated from the roots of *Entada rheedii*.

the deformation of the root hairs cortex can be considered a sign of the presence of fungi.

Members of Tulasnellaceae are noted as the key mycorrhizal symbionts of several orchids such as *Cypripedium*, *Chiloglottis Dendrobium nobile*, *Liparis japonica*, and *Ophrys* [23-28]. The mycorrhizal status of the *M. rheedei* species is being examined in the present study for the first time. Phylogenetic analysis of isolated fungi found that *T. calospora* is associated with the medicinal orchid *M. rheedei*. Similar to our study, this fungus has been isolated, sequenced, and phylogenetically analyzed from many orchid species using *ITS* primers, such as *Bipinnula fimbriata* by Steinfort *et al.* [29], *Cymbidium goeringii* and *C. faberi* by Yu *et al.* [30], and *Paphiopedilum barbigerum* by Tian *et al.* [31]. A case study by Hampejšová *et al.* (2022) noted that this fungal species is responsible for the accumulation of certain disease-resistant molecules in orchid tubers, which might increase the value of the tubers for pharmaceutical and therapeutic uses [32].

5. CONCLUSION

The study concludes that *M. rheedei* exhibits a robust mycorrhizal association, with 91% root cortex colonization by the fungal hyphae. Phylogenetic analysis of isolated fungi identified an association of the fungus *T. calospora* with *M. rheedei* roots. The morphological analysis reveals that intact and degraded peloton patches occupy up to 62% of the cortical space, influencing root length. This study on mycorrhizal association may help conservationists in the conservation and restoration of orchid species. This information is also important for researchers to find novel ways for the cultivation, propagation, and identification of bioactive compounds. Future research on mycorrhizal associations in *M. rheedei* may focus on elucidating the molecular mechanisms governing the specificity of interactions between the orchid and *T. calospora*.

6. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to the conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

All data generated during the study is available in the article.

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