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The diversity of fungal associates of *Dendrobium ovatum* (L.) Kraenzl., an endemic orchid of the Western Ghats of India

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ABSTRACT *Dendrobium ovatum* is a tropical epiphytic orchid endemic to the Western Ghats of India and has been listed as a threatened species in recent research due to its declining populations and changes in flowering and fruit set patterns. This study aims to investigate the mycoflora associated with the roots, stems and leaves of *D. ovatum*. Both surface-associated and endophytic fungal associates were isolated and identified using morphological and molecular methods. The study resulted in the isolation of 139 cultures, which were divided into 24 morphotypes, 99% of which belonged to Ascomycota. The most dominant members, *Trichoderma harzianum* and *Colletotrichum gloeosporioides*, were consistently observed across all the study sites. Tissue-specific fungal diversity analysis revealed that each organ was dominated by a distinct fungal group, forming characteristic communities specific to each tissue. The roots of *D. ovatum* exhibited the highest species richness and diversity, compared to the stem and leaves. This research also represents the first documentation of fungal associates of the threatened orchid *D. ovatum*.

KEYWORDS *Colletotrichum*; *Dendrobium ovatum*; Endemic; Endophytes; Fungal associates; Threatened; *Trichoderma*

1. Introduction

Orchids are a vast and diversified group of plants, highly valued for their long lasting and captivating blooms. India boasts a rich diversity of orchids, with nearly 1,300 recorded species distributed throughout the country (Adit et al. 2022; Pal et al. 2022). They are widely distributed in the tropical and temperate regions of the world with high humidity. Though cosmopolitan in distribution, they are particularly vulnerable to habitat loss and deforestation (Purwantoro et al. 2023; Vitt et al. 2023), and conservation efforts can help decrease their extinction rates (Ilham et al. 2022). A comprehensive investigation of the threatened plants in India by Barik et al. (2018) underscored the family Orchidaceae as the most susceptible group, encompassing approximately 23% of all the threatened plant species in the country.

D. ovatum, commonly known as the Green-Lipped *Dendrobium*, is an epiphytic orchid endemic to the Western Ghats and its spillover regions. The genus *Dendrobium*, with approximately 1,400 species, holds significant importance in horticulture, floristry, and traditional medicine due to its wide range of benefits (Pujari et al. 2021). Also, it is the source of 'Moscatilin', a bibenzyl active principle with anti-cancerous, anti-inflammatory, antioxidant, anti-mutagenic activity, and forms a component of many ayurvedic formulations (Pujari et al. 2021). Due to the massive trading and marketing of the genus, *Dendrobium* has been included in Annexure II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) (Pujari et al. 2021; Vitt et al. 2023).

D. ovatum blooms during November to December, following the post-monsoon season in the tropics, when the temperatures are conducive for the flowering and fruit setting. The seeds require a minimum of two years to reach the reproductive stage, with a germination rate of less than 20% in the wild, even under favourable conditions encompassing all factors (Pujari et al. 2021; Pujari and Sankar Babu 2022). Moreover, the absence of beneficial fungal biota, the presence of parasitic fungi in the soil, and seasonal changes can lead to delayed seed germination and a prolonged vegetative phase, ultimately leading to their decreased population in the wild (Pujari et al. 2021; Pyati 2022).

Orchids can be considered as flagship species that reflect the health of an ecosystem, as their well-being relies on active interactions with numerous other life forms

throughout their life cycle (Liu et al. 2023). Notable among these interactions is their dependence on fungi for nutrient acquisition, particularly during seed germination, growth, and the development of protocorm, as they lack endosperm (Fay 2018). Some of these fungal associations may persist into the adult stage, including in photosynthetic orchids. Thus, the diversity and distribution of the latter can be correlated with the composition of their associated fungi (Pecoraro et al. 2018).

Endophytes are microorganisms that colonise the internal tissues of plants asymptomatically, showing no symptoms of infection. The distribution of orchids relies on the interaction between orchid mycorrhizal fungi (OMF) and diverse orchid non-mycorrhizal fungi (ONF) (Li et al. 2021). In many cases, ONF (distributed over 110 genera) outnumber OMF (Ma et al. 2015) and exhibit tissue or organ specificity (Fitzpatrick et al. 2020), that may in turn modify the host-fungal interactions. Understanding such interactions between the above ground and below ground fungi is imperative in shaping the host phenotype (Dastogeer et al. 2020). Many of such associated fungi have been isolated and successfully integrated in the conservation efforts of wild orchids (Yang et al. 2020). For instance, ecological-specific fungi of *Dendrobium officinale* have been isolated and used to prepare orchid seed-fungi bags for restoring them in natural habitats (Wang et al. 2021). Despite the ecological significance and threatened status of *D. ovatum* (Barik et al. 2018), little is known regarding their above ground and below ground fungal associates. This knowledge gap is of paramount significance as these associations play pivotal role in promoting growth and general fitness of the host, improving stress tolerance, and mediating mycorrhizal associations (Chand et al. 2020; Shan et al. 2021). Moreover, the comparative study of the epiphytic and endophytic mycoflora of orchids helps in comprehending fungal ecology and generating approaches suitable for plant conservation, by offering deeper insights into plant-microbial interactions (Salazar-Cerezo et al. 2018). Hence, our hypothesis revolves around the potential presence of discrete fungal species within the distinct tissues of *D. ovatum* collected from diverse habitats, which may throw light onto the complex web of relationships that fortify its ecological success through orchid-fungal co-evolution.

2. Materials and Methods

2.1. Study area and plant collection

Twigs of *D. ovatum* were collected from various locations of Pathanamthitta district, Kerala state, India and taken to the laboratory in polythene bags for inoculation within 24 hours of collection. Roots, stems, and leaves were used to isolate fungal associates. The collection sites were limited to natural wild habitats, excluding cultivated areas, and the specimens were collected during May to October (2020-2022), corresponding to the period of vegetative growth in the plant. The plants were devoid of leaves during the flowering and fruiting time (November to April) (Figure 1).

2.2. Isolation of fungal associates of D. ovatum

The root, stem, and leaf segments of *D. ovatum* were surface-sterilized and used for the isolation of endophytic fungi, whereas non-surface-sterilized tissue segments were used for the isolation of surface associated fungal isolates. Surface sterilization was performed following the methods of Ma et al. (2015) and Sarsaiya et al. (2019). To summarize, the tissue segments were treated using 70% ethyl alcohol for 30 seconds followed by 0.5% $HgCl_2$ for 3 -5 minutes, and then rinsed four times with sterile distilled water. These sterilized tissues were dried on a sterile filter paper, cut into small pieces, teased with sterile blade and inoculated into Potato Dextrose Agar (PDA) plates and Czapek Dox Agar plates supplemented with 50 µg/mL ampicillin, to prevent the growth of endophytic bacteria. To test the effectiveness of surface sterilization, the sterilized tissue segments were imprinted onto PDA plates and checked for the growth of fungal hyphae. All the plates were incubated at 28–30 °C and regularly monitored for the emergence of fungal hyphae up to 2 weeks. The isolates were transferred to fresh PDA plates, checked for its purity by observing the growing hyphal tips under compound microscope, later transferred to PDA slants and stored at 4 °C.

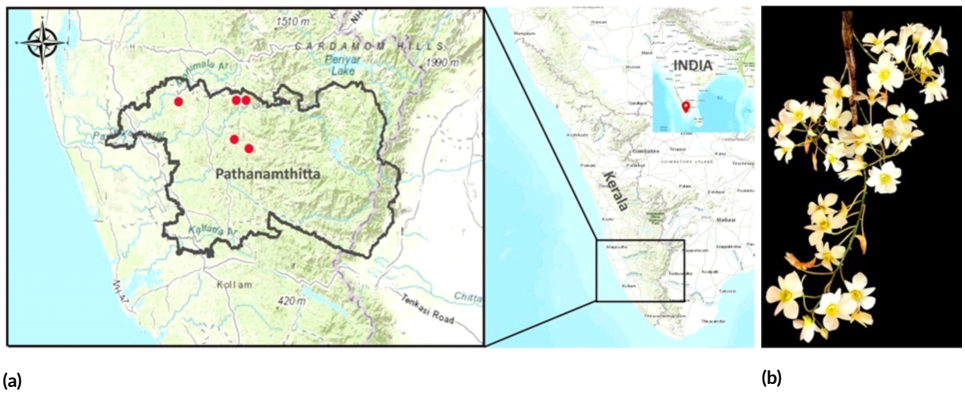

FIGURE 1 (a) Sampling sites of *D. ovatum* in Pathanamthitta district, Kerala, India (b) Flowering twig of *D. ovatum*

2.3. Identification of fungal associates

The species-level identification of the fungal isolates was conducted using a combination of cultural, micromorphological, and molecular characteristics. The observed cultural characters included color of the colony (front and reverse), growth rate, and color of spores produced (if any). Additionally, the microscopic details of conidiophores and conidia were noted in each case. The morphological identity of the isolates was confirmed using the analytical services of Agharkar Research Institute, Pune, India.

The molecular sequencing of the isolates was done utilizing the microsatellite DNA fingerprinting and DNA barcoding services of Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram, for confirming the species level identification. The DNA was isolated in pure form, and its quality was checked using agarose gel electrophoresis for all the isolates. For the species-level identification of fungal isolates, the Internal Transcribed Spacer (ITS) region of rRNA was amplified using the primers ITS_1F (TCCGTAGGTGAACCTGCGG) and ITS_4R(TCCTCCGCTTATTGATATGC) for all the isolates (White et al. 1990). In cases where ITS did not work well, large subunit (LSU) of the rRNA was amplified, as they also form valuable in species identification of fungi (Raja et al. 2017). Forward primer LR0R (ACCCGCTGAACTTAAGC) and reverse primer LR7 (TACTACCACCAAGATCT) were amplified for LSU. The DNA sequencing was performed using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) adhering strictly to the manufacturer's guidelines. All the sequences were submitted to NCBI, accession numbers were obtained.

2.4. Analysis of fungal diversity

The total number of isolates (n) was used as the basis for assessing the fungal diversity. The isolation rate, measuring the fungal richness was calculated by dividing the total number of isolates from specific tissues or sites by the total number of tissue segments analyzed (Huang et al. 2008). The colonization rate was determined by dividing the total number of isolates obtained from specific tissue by the total number of taxa from all tissues and was expressed in percentage. The relative frequency, which indicates fungal density, was expressed as a percentage. It was calculated by dividing the number of isolates of a particular species by the total number of isolates and multiplying by 100 (Huang et al. 2008).

Camargo's index (1/S) was calculated to determine the fungal dominance, where S determined the species richness. If Pi > 1/S, a species was considered dominant, where Pi is the number of species in an area. The Simpson's index (D), Simpson's Diversity index (1-D) and Simpson's Reciprocal Index (1/D) and Shannon Diversity Index (H) were calculated for each tissue and each collection site. The Plieou's Evenness Index/Species Evenness (E) was determined according to the formula E=H/ln(S). Jaccard's index was calculated using the following formula and was used to compare the similarities between fungal taxa isolated from different tissues and sites (Magurran 1988).

$$J = a/(b + c - a) \quad (1)$$

where, a= number of taxa shared by both communities b and c= number of taxa in each of the two communities (tissues/sites).

The spatial distribution of the isolated fungal strains in different tissues were analyzed using the VENNTURE tool (Martin et al. 2012).

3. Results and Discussion

A total of 270 tissue segments of *D. ovatum* (nine surface sterilised and nine non- surface sterilised root, stem and leaf segments from five different locations) were analysed for the isolation and identification of fungal associates. 139 fungal strains were isolated from the 270 tissue segments examined and grouped into 24 morphotypes based on their consistency in the morphological characteristics on PDA and Malt Extract Agar (MEA). Of the 24 morphotypes, 11 were found as surface associates, and 13 were isolated as endophytes from different tissue segments. These morphotypes were distributed across eight genera, namely *Trichoderma*, *Colletotrichum*, *Fusarium*, *Alternaria*, *Malassezia*, *Diaporthe*, *Lasiodiplodia*, and *Starmerella*. The morphology of all the sporulating cultures were authenticated and the cultures were deposited at the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune (ARI), and accession numbers were obtained (Supplementary Table 1). The molecular sequencing of all the isolates were performed, and the data was compared against the morphological characters to finalise the identification of the isolates. The 24 morphotypes isolated along their systematic position, NFCCI, and NCBI accession numbers are listed in Table 1.

Out of the total isolates, 99% (138 cultures) belonged to the Ascomycota, represented by six orders, namely Hypocreales (54.17%), Glomerellales (25%), Pleosporales (8.34%), Diaporthales (4%), Saccharomycetales (4%) and Botryosphaeriales (4%), while the remaining one isolate belonged to Basidiomycota, represented by a single member *Malassezia japonica* (Figure 2). Relative frequency measuring the fungal density was found to be high for *Trichoderma harzianum* (23.02%) followed by *Colletotrichum gloeosporioides* (20.86%)

To isolate the endophytic members, surface sterilization formed a crucial step as the imprinting method confirmed the absence of any surface isolates. Twelve fungal taxa were isolated from the roots, six from the leaf, and six from the stem segments. *Trichoderma* sp. was found to be the most prominent surface associate and was isolated from the root, stem, and leaf segments examined. These also showed endophytic representation in the

TABLE 1 The fungal associates of *D. ovatum* along with their NFCCI and NCBI accession numbers.

Nature	Identification	Division, Order, Family	NFCCI Accession Number	NCBI Accession number	Primer used
Root Surface associate	*Trichoderma harzianum* Rifai	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5172	OQ552832	ITS
	Trichoderma lentiforme (Rehm) P. Chaverri, Samuels & F.B. Rocha	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5173	OQ552839	ITS
	Trichoderma reesei E.G. Simmons	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5174	OQ552833	LSU
	Trichoderma asperellum Samuels, Lieckf. & Nirenberg	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5175	OQ552842	ITS
	Trichoderma lixii (Pat.) P. Chaverri	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5176	OQ552840	ITS
Root Endophyte	*Colletotrichum coccodes* (Wallr.) S. Hughes	Ascomycota, Glomerellales, Glomerellaceae	NFCCI 5177	OQ552841	ITS
	Fusarium oxysporum Schltdl.	Ascomycota, Hypocreales, Nectriaceae	NFCCI 5178	OQ552845	ITS
	Trichoderma viride Schumach. 1803	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5179	OQ552844	LSU
	Trichoderma asperellum Samuels, Lieckf. & Nirenberg	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5180	OQ552846	LSU
	Trichoderma harzianum Rifai	Ascomycota, Hypocreales, Hypocreaceae	-	OR116249	ITS
	Alternaria alternata (Fr.) Keissl.	Ascomycota, Pleosporales, Pleosporaceae	-	OR119829	LSU
	Malassezia japonica Sugita, M. Takash., M. Kodama, Tsuboi & A. Nishikawa	Basidiomycota, Malasseziales, Malassesziaceae	-	OR116193	LSU
Leaf surface associate	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. complex	Ascomycota, Glomerellales,Glomerellaceae	NFCCI 5279	OQ552855	LSU
	Trichoderma harzianum Rifai	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5280	-	Morphology
Leaf endophyte	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. complex	Ascomycota, Glomerellales, Glomerellaceae	NFCCI 5273	OQ552848	ITS
	Alternaria alternata (Fr.) Keissl.	Ascomycota, Pleosporales, Pleosporaceae	-	OR116195	ITS
	Colletotrichum gigasporum Rakotonir. & Munaut	Ascomycota, Glomerellales, Glomerellaceae	NFCCI 5274	OQ552849	LSU
	Diaporthe tulliensis R.G. Shivas, Vawdrey & Y.P. Tan	Ascomycota, Diaporthales, Diaporthaceae	-	OQ552850	ITS
Stem surface associate	*Trichoderma* sp.	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5276	-	Morphology
	Colletotrichum gloeosporioides (Penz.) Penz. & Sacc. complex	Ascomycota,Glomerellales, Glomerellaceae	NFCCI 5277	OQ552852	LSU
	Trichoderma harzianum Rifai	Ascomycota, Hypocreales, Hypocreaceae	NFCCI 5278	OQ552851	LSU
	Starmerella etchellsii (Lodder & Kreger-van Rij) C.A. Rosa & Lachance	Ascomycota, Saccharomycetales,Incertae sedis	-	OQ555614	LSU
Stem Endophyte	*Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. complex	Ascomycota,Glomerellales, Glomerellaceae	NFCCI 5275	OQ552853	LSU
	Lasiodiplodia theobromae (Pat.) Griffon & Maubl.	Ascomycota, Botryosphaeriales, Botryosphaeriaceae	-	OQ552856	LSU

root segments. The major endophytic representation was *Colletotrichum* sp. These strains were also isolated as surface associates from the leaf and stem segments analyzed. While considering the other six strains, apart from *Starmerella*, all the others were exclusively isolated as endophytes (Figure 3).

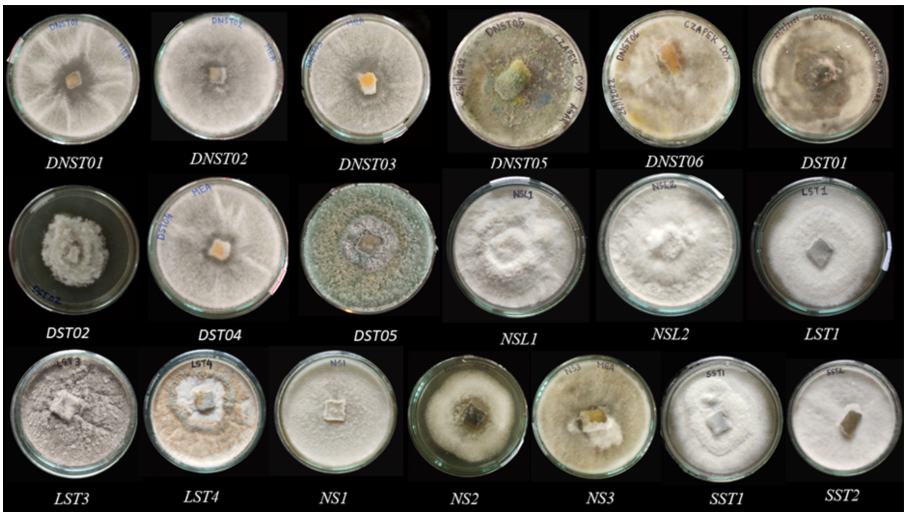

FIGURE 2 The culturable fungal associates from root, stem and leaves of *D. ovatum*

The relative frequency of isolation from different tissues and different sites of collection is represented in Figure 4. *Trichoderma* and *Colletotrichum* were consistently isolated from every collection site, indicating their strong association with the host plant. Maximum number of isolates was obtained from the roots (45.32%), followed by stem (36.69%) and leaves (17.98%). Collection site 2 with 25.89% isolates was the most diverse, followed by site 1 with 21.58% isolates.

The roots of *D. ovatum* exhibited higher diversity than the leaves and stems, as indicated by Simpson's Diversity index (1-D) and Simpson's Reciprocal index (1/D). Additionally, the Shannon diversity index also highlighted the roots as the most diverse tissue (H=2.08) (Table 2). The Jaccard's indices showed the that similarity between stem and leaf was highest (J= 0.25), followed by root and leaf (H= 0.154), and the lowest similarity was between root and stem (J= 0.071) (Figure 5). Collection site 1 and site 2 showed maximum similarity (J= 0.8125). Jaccard's similarity index between different collection sites is represented in Table 3.

3.1. Discussion

The Western Ghats are one of the richest centres of biodiversity, housing almost 123 endemic orchid species (Jalal and Jayanthi 2012). *D. ovatum* is an endemic species more common to many regions in Central and Southern Western Ghats. The flowering and fruit set of the endemic orchid,

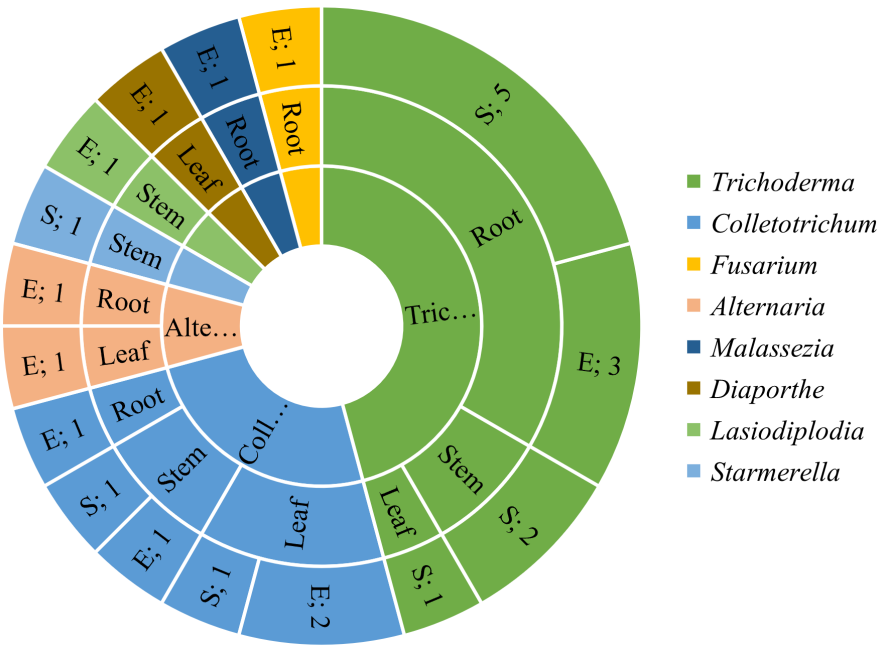

FIGURE 3 Diversity of surface associates and endophytes in various tissue segments of *D. ovatum*; E- Endophyte; S- Surface Associate. The numbers following the letters denote the number of species.

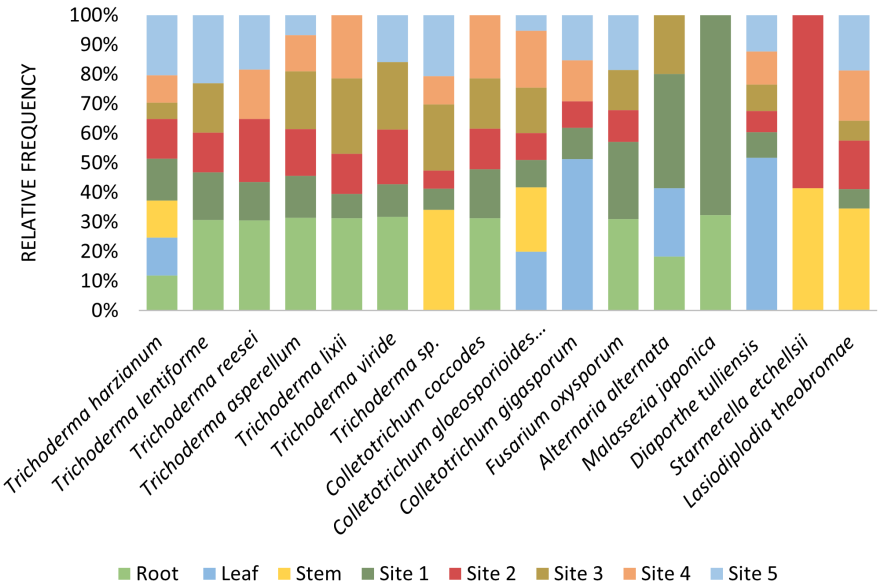

FIGURE 4 Relative frequency of isolation from root, stem and leaves of *D. ovatum* and various locations.

D. ovatum has been significantly impacted by changing climatic conditions, further exacerbated by human activities such as urbanization, and deforestation (Pujari et al. 2021). The availability of suitable OMF and ONF fungi is crucial for orchid seed germination as well as their continued existence, and the lack thereof can negatively affect its propagation in the natural environment. However, the microbiological associations of *D. ovatum* have not been explored yet. Hence, this study focussed on investigating the diversity of fungal associates of *D. ovatum* is of paramount significance for the development of scientific knowledge as well as the ecological and conservational applications they have.

The present work showed the dominance of Ascomycetes both in surface-associated and endophytic fungal isolates of *D. ovatum*. *Trichoderma*, *Colletotrichum*, *Fusarium*, *Alternaria*, *Lasiodiplodia*, and *Diaporthe* were the strains frequently observed in the present study, of which, the first three were listed as the most prevalent non-mycorrhizal endophytes in orchids (Ma et al. 2015; De Mers 2022). The results are also consistent with the findings of Yuan et al. (2009) in their study on *Dendrobium nobile*. Previous reports, also show that the majority of these non-mycorrhizal fungal associates of orchids are represented by Ascomycota and less frequently by Basidiomycota (Li et al. 2021; Adit et al. 2022). The absence of mycorrhizal fungi in the present study might be attributed to the limited occurrence of extensive mycorrhizal infections in tropical adult epiphytic orchids (Wang et al. 2022), which instead exhibit greater non-mycorrhizal diversity (Ma et al. 2015).

In the present study, the genus *Colletotrichum* and *Trichoderma* were identified as the predominant endophytic and surface associated fungal taxa of *D. ovatum*, respectively. These were isolated from all the plant parts analysed, with a higher representation of *Colletotrichum* sp.

TABLE 2 Diversity analysis between various plant tissues and collection sites.

	Plant Tissues			Collection sites				
	Root	**Leaf**	**Stem**	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**
Total no. of isolates (n)	63	25	51	30	36	29	23	21
Isolation Rate	0.70	0.57	0.28	0.56	0.67	0.54	0.43	0.39
Colonisation Rate (%)	45.32	36.69	17.99	18.87	22.64	18.24	14.47	13.21
Species richness (S)	10	5	5	15	14	12	10	11
Camargo's index (1/S)	0.1	0.2	0.2	0.067	0.071	0.083	0.100	0.091
Simpson's index (D)	0.13	0.22	0.25	0.1	0.1	0.11	0.15	0.15
Simpson's Diversity Index (1-D)	0.87	0.78	0.75	0.9	0.9	0.89	0.85	0.85
Simpsons Reciprocal Index (1/D)	7.57	4.55	3.97	10.12	9.84	9.23	6.84	6.77
Shannon Diversity Index (H)	2.08	1.45	1.43	2.38	2.34	2.21	1.99	2.05
Plieou's Eveness Index/Species Eveness (E)	0.904	0.904	0.889	0.877	0.885	0.891	0.864	0.857

TABLE 3 Jaccards Similarity index between the five different collection sites.

Sites	1	2	3	4	5
1		0.8125	0.6875	0.666	0.733
2			0.733	0.714	0.786
3				0.571	0.643
4					0.615

Numbers 1 to 5 represent the five different locations shown in the map of study area.

in the stem and leaves, while *Trichoderma* sp. was more prevalent in the roots (Figure 5). Notably, our findings revealed that the genus *Colletotrichum* and *Trichoderma* were consistently seen across all the study locations, highlighting their ecological plasticity and indispensable role in the growth and reproduction of orchid species. This observation was supported by the Jaccard's Similarity Index. Several pathogenic and non-pathogenic *Colletotrichum*, like *C. boninense*, *C. camelliae-japonicae*, *C. fructicola*, *C. jiangxiense*, *C. gloeosporioides* species exhibit an asymptomatic endophytic nature in living plant tissues, including orchids (Ma et al. 2018; Parthibhan et al. 2017), and most of them are known to promote host plant growth and provide resistance against stress and disease (Ma et al. 2018; da Silva et al. 2020). Additionally, they are latent pathogens that remain in a quiescent stage, until any alterations in the physiological state of the host plant leading to stress conditions emerge. This statement is reinforced by the fact that many of the endophytic fungi are closer relatives of pathogens on the same or related host, and might have evolved directly from the pathogenic strains (Carroll 1988). Our findings align with previous studies where *Trichoderma* species, particularly, *T. reesei*, *T. harzianum*, *T. koningiopsis*, *T. atroviride* with plant growth promoting capabilities, were isolated and identified from healthy orchid tissues (Hajong and Kapoor 2020; Alomía et al. 2022; Morgan et al. 2022; Woo et al. 2023). This may be due to their rapid colonization capability and ability to form symbiotic associations with plants, particularly in the rhizosphere region (Sood et al. 2020).

Additionally, *Colletotrichum gloeosporioides* and *Starmerella etchellsii* were identified as surface associates of stem and leaf of *D. ovatum*. Literature analysis indicates that *Starmerella* sp. is a less frequent plant endophyte but is commonly found as an episphere inhabitant in various plant species (Félix et al. 2022). Our findings are consistent with this report since, *Starmerella* was isolated as a surface associate from the stem of *D. ovatum* and was found at only one location in our study, with an RF of 1.44%. Our study, also identified *Malassezia japonica* as a root endophyte from a single location, with a relative frequency (RF) of 0.72%. While *Malassezia species* is a common skin pathogen, they have been reported to associate with the plant root surface (Amend 2014). *M. japonica* was recently reported as endophyte of *Silybum marianum* (Abdusattorova et al. 2024). The genus *Malassezia* serves as endophytes in orchids (Wang et al. 2022), but their association with *Dendrobium* species has not been extensively documented.

Microbial diversity, also tends to vary among different plant tissues due to differences in plant-derived resources, variations in tissue structures, as well as exposure to different environmental conditions (Fitzpatrick et al. 2020). Tissue-specific diversity of associates, species richness, colonisation rate, and isolation rate were found to be higher in roots, suggesting that roots may be providing a better ecological niche for the colonisation of associated

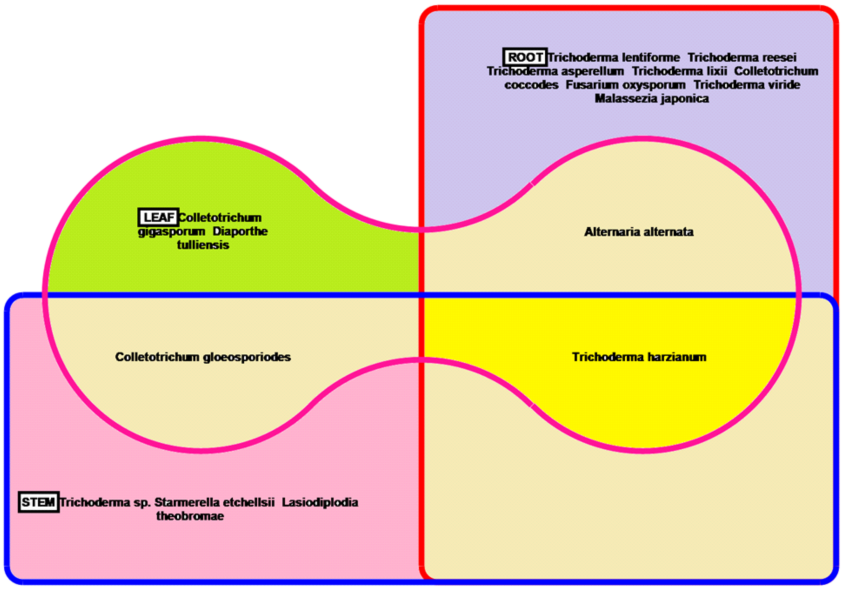

FIGURE 5 Venn diagram showing distribution of fungal isolates in various tissues of *D. ovatum*.

mycobiota. Moreover, the humus-rich barks of the tropical host trees may be an ideal habitat for a various fungal species colonising the roots of orchids. This is in accordance with the findings of Jin et al. (2017), who reported higher diversity of endophytes in the roots of *D. officinale*. Several studies have reported limited overlap between the leaf and root microbial communities (Rodriguez and Redman 2008), which aligns with our observation. Tissue specific fungal diversity analysis revealed that each organ is dominated by a distinct fungal group, forming characteristic communities specific to each tissue, as visualised in Figure 5.

The concept of holobiont has gained much attention over the past few years, and an increased number of research works have been popping out that emphasize the role of associated microbiota in conserving the hosts. This aspect is particularly significant for threatened orchid species on the brink of extinction. Many of the associated microbiota isolated in our study have been reported to possess various plant growth-promoting activities such as the production of plant growth hormones, solubilization of inorganic phosphates, nitrate assimilation, and siderophore production (Jin et al. 2017; Chand et al. 2020; Deepthi and Ray 2021). Additionally, these microorganisms exhibit indirect effects such as HCN production, catalase activity, and antibacterial and antifungal properties, thereby inhibiting the growth of phytopathogens and improving the overall plant health (Rajan et al. 2022). The fungal associates of orchids have also found application in symbiotic seed germination of rare and threatened orchids, and in their reintroduction programs (Yang et al. 2020). Several endophytic fungal strains from orchids have also proved to promote the vegetative growth of ornamental orchid species (Deepthi and Ray 2021). Overall, the consistent occurrence of the isolates from all the locations studied, highlight the potential of these fungal associates in shaping the phenotype and ecology of the hosts, thereby modifying its overall physiology and interactions with the environment. Therefore, the successful conservation of any organism should consider the associated microbiota, because a healthy microbiome serves as an indicator of a healthy organism.

4. Conclusions

The present study resulted in the isolation and identification of fungal associates from the roots, stem, and leaves of the threatened orchid *D. ovatum*. Amidst the challenges posed by climate change, identifying, preserving, and nurturing the diverse microbiota associated with any host can contribute to their successful conservation and long-term survival. Hence, this study act as an initial step towards a more comprehensive understanding of the ecological and conservation aspects of orchid-fungal relationship.

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Authors' contributions

RR conducted the experiment, did data analysis and wrote the manuscript, PR, SM and RAR participated in the experiment and gave valuable suggestions to the experiment, JJ designed the project, reviewed, revised the manuscript and helped with data analysis, EC supervised the study, participated in the experiment design, reviewed and revised the manuscript. All the authors have gone through the manuscript and was well informed about the work.

Competing interests

All authors declare that there are not any conflicts of interest.

References

Abdusattorova NU, Narmuhamedova MK, Xusanov TS. 2024. Isolation and identification of endophytic microorganisms from *Silibium marianum* plant. Sci. Innov. 3(3):24–28. doi:10.5281/zenodo.10783105.

Adit A, Koul M, Kapoor R, Tandon R. 2022. Topological analysis of orchid-fungal endophyte interaction shows lack of phylogenetic preference. South African J. Bot. 149:339–346. doi:10.1016/j.sajb.2022.06.025.

Alomía YA, Otero JT, Jersáková J, Stevenson PR. 2022. Cultivable fungal community associated with the tropical orchid *Dichaea andina*. Fungal Ecol. 57-58:101158. doi:10.1016/j.funeco.2022.101158.

Amend A. 2014. From dandruff to deep-sea vents: Malassezia-like fungi are ecologically hyper-diverse. PLoS Pathog. 10(8):e1004277. doi:10.1371/journal.ppat.1004277.

Barik SK, Tiwari ON, Adhikari D, Singh PP, Tiwary R, Barua S. 2018. Geographic distribution pattern of threatened plants of India and steps taken for their conservation. Curr. Sci. 114(3):470–503. doi:10.18520/cs/v114/i03/470-503.

Carroll G. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. Ecology 69(1):2–9. doi:10.2307/1943154.

Chand K, Shah S, Sharma J, Paudel MR, Pant B. 2020. Isolation, characterization, and plant growth-promoting activities of endophytic fungi from a wild orchid *Vanda cristata*. Plant Signal. Behav. 15(5):1744294. doi:10.1080/15592324.2020.1744294.

da Silva LL, Moreno HLA, Correia HLN, Santana MF, de Queiroz MV. 2020. *Colletotrichum*: species complexes, lifestyle, and peculiarities of some sources

of genetic variability. Appl. Microbiol. Biotechnol. 104(5):1891–1904. doi:10.1007/s00253-020-10363-y.

Dastogeer KM, Tumpa FH, Sultana A, Akter MA, Chakraborty A. 2020. Plant microbiome–an account of the factors that shape community composition and diversity. Curr. Plant Biol. 23:100161. doi:10.1016/j.cpb.2020.100161.

De Mers M. 2022. Alternaria alternata as endophyte and pathogen. Microbiol. (United Kingdom) 168(3):001153. doi:10.1099/mic.0.001153.

Deepthi AS, Ray JG. 2021. Ecological relevance of the endophytic fungal diversity in velamen roots of tropical epiphytic orchids. Czech Mycol. 73(1):91–108. doi:10.33585/CMY.73107.

Fay MF. 2018. Orchid conservation: how can we meet the challenges in the twenty-first century? Bot. Stud. 59:16. doi:10.1186/s40529-018-0232-z.

Félix CR, da Silva Nascimento BE, Valente P, Landell MF. 2022. Different plant compartments, different yeasts: The example of the bromeliad phyllosphere. Yeast 39(6-7):363–400. doi:10.1002/yea.3804.

Fitzpatrick CR, Salas-González I, Conway JM, Finkel OM, Gilbert S, Russ D, Teixeira PJPL, Dangl JL. 2020. The plant microbiome: From ecology to reductionism and beyond. Annu. Rev. Microbiol. 74:81–100. doi:10.1146/annurev-micro-022620-014327.

Hajong S, Kapoor R. 2020. An amalgam of pathogenic and beneficial endophytic fungi colonizing four *Dendrobium* species from Meghalaya, India. J. Basic Microbiol. 60(5):415–423. doi:10.1002/jobm.201900631.

Huang WY, Cai YZ, Hyde KD, Corke H, Sun M. 2008. Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers. 33:61–75.

Ilham M, Puspitasari F, Semiarti E. 2022. The effectivity of thidiazuron and 1 naphthaleneacetic acid on somatic embryo induction in transgenic *Dendrobium phalaenopsis* Fitzg. carrying 35S:: GR:: AtRKD4. Indones. J. Biotechnol. 27(3):133–141. doi:10.22146/ijbiotech.70833.

Jalal JS, Jayanthi J. 2012. Endemic orchids of peninsular India: a review. J. Threat. Taxa 4(15):3415–3425. doi:10.11609/jott.o3091.3415-25.

Jin Z, Li D, Liu T, Yu F, Zhang Z, Su C, Wang Y, Guo Q, Liu Z. 2017. Cultural endophytic fungi associated with *Dendrobium officinale*: Identification, diversity estimation and their antimicrobial potential. Curr. Sci. 112(8):1690–1697. doi:10.18520/cs/v112/i08/1690-1697.

Li T, Wu S, Yang W, Selosse MA, Gao J. 2021. How mycorrhizal associations influence orchid distribution and population dynamics. Front. Plant Sci. 12:647114. doi:10.3389/fpls.2021.647114.

Liu Q, Wu X, Xing H, Chi K, Wang W, Song L, Xing X. 2023. Orchid diversity and distribution pattern in karst forests in eastern Yunnan Province, China. For. Ecosyst. 10:100117. doi:10.1016/j.fecs.2023.100117.

Ma X, Kang J, Nontachaiyapoom S, Wen T, Hyde KD. 2015. Non-mycorrhizal endophytic fungi from orchids. Curr. Sci. 109:72–87.

Ma X, Nontachaiyapoom S, Jayawardena RS, Hyde KD, Gentekaki E, Zhou S, Qian Y, Wen T, Kang J. 2018. Endophytic *Colletotrichum* species from *Dendrobium* spp. In China and northern Thailand. MycoKeys 43:23–57. doi:10.3897/mycokeys.43.25081.

Magurran AE. 1988. Diversity indices and species abundance models. Netherlands: Springer. doi:10.1007/978-94-015-7358-0_2.

Martin B, Chadwick W, Yi T, Park SS, Lu D, Ni B, Gadkaree S, Farhang K, Becker KG, Maudsley S. 2012. VENNTURE-A novel Venn diagram investigational tool for multiple pharmacological dataset analysis. PLoS One 7(5):e36911. doi:10.1371/journal.pone.0036911.

Morgan T, Custódio FA, Tavares MP, Pereira OL, Guimarães VM, de Oliveira Mendes TA. 2022. Genome sequencing and evolutionary analysis of a new endophytic *Trichoderma* species isolated from orchid roots with reduced repertoire of protein-coding genes. Mycol. Prog. 21(7):60. doi:10.1007/s11557-022-01811-2.

Pal R, Meena NK, Dayamma M, Singh DR. 2022. Ethnobotany and recent advances in Indian medicinal orchids. Springer. doi:10.1007/978-3-030-38392-3_26.

Parthibhan S, Rao MV, Senthil Kumar T. 2017. Culturable fungal endophytes in shoots of *Dendrobium aqueum* Lindley – An imperiled orchid. Ecol. Genet. Genomics 3-5:18–24. doi:10.1016/j.egg.2017.06.004.

Pecoraro L, Caruso T, Cai L, Gupta VK, Liu ZJ. 2018. Fungal networks and orchid distribution: New insights from above- and below-ground analyses of fungal communities. IMA Fungus 9:1–11. doi:10.5598/IMAFUNGUS.2018.09.01.01.

Pujari I, Sankar Babu V. 2022. Precocious *in vitro* flowering in threatened ornamental orchid, *Dendrobium ovatum* – Decoding the causal factors. Curr. Plant Biol. 31:100257. doi:10.1016/j.cpb.2022.100257.

Pujari I, Thomas A, Rai PS, Satyamoorthy K, Babu VS. 2021. *In vitro* bioproduction and enhancement of moscatilin from a threatened tropical epiphytic orchid, *Dendrobium ovatum* (Willd.) Kraenzl. 3 Biotech 11(12):507. doi:10.1007/s13205-021-03059-1.

Purwantoro A, Setiawan AB, Nugraha RS, Mujtaba SB, Setyadi AH. 2023. Genetic variation and genomic constitution in orchid *Dendrobium* hybrid section Spatulata derived from interspecific hybridization based on sequence related amplified polymorphism marker. Indones. J. Biotechnol. 28(2):119–126. doi:10.22146/ijbiotech.83595.

Pyati AN. 2022. *In vitro* propagation of orchid (*Dendrobium ovatum* (L.) Kraenzl.) through somatic embryogenesis. Plant Tissue Cult. Biotechnol. 32(1):53–66. doi:10.3329/ptcb.v32i1.60472.

Raja HA, Miller AN, Pearce CJ, Oberlies NH. 2017. Fungal identification using molecular

tools: A primer for the natural products research community. J. Nat. Prod. 80(3):756–770. doi:10.1021/acs.jnatprod.6b01085.

Rajan P, Mathew S, Raj R, Cherian E, Remakanthan A. 2022. Improved designing and development of endophytic bioformulations for plant diseases. Academic Press. doi:10.1016/B978-0-323-88478-5.00003-1.

Rodriguez R, Redman R. 2008. More than 400 million years of evolution and some plants still can't make it on their own: Plant stress tolerance via fungal symbiosis. J. Exp. Bot. 59(5):1109–1114. doi:10.1093/jxb/erm342.

Salazar-Cerezo S, Martinez-Montiel N, Cruz-Lopez MdC, Martinez-Contreras RD. 2018. Fungal diversity and community composition of culturable fungi in *Stanhopea trigrina* cast gibberellin producers. Front. Microbiol. 9:612. doi:10.3389/fmicb.2018.00612.

Sarsaiya S, Shi J, Chen J. 2019. A comprehensive review on fungal endophytes and its dynamics on Orchidaceae plants: current research, challenges, and future possibilities. Bioengineered 10(1):316–334. doi:10.1080/21655979.2019.1644854.

Shan T, Zhou L, Li B, Chen X, Guo S, Wang A, Tian L, Liu J. 2021. The plant growth-promoting fungus MF23 (*Mycena* sp.) increases production of *Dendrobium officinale* (Orchidaceae) by affecting nitrogen uptake and NH^{4+} assimilation. Front. Plant Sci. 12:693561. doi:10.3389/fpls.2021.693561.

Sood M, Kapoor D, Kumar V, Sheteiwy MS, Ramakrishnan M, Landi M, Araniti F, Sharma A. 2020. *Trichoderma*: The "secrets" of a multitalented biocontrol agent. Plants 9(6):762. doi:10.3390/plants9060762.

Vitt P, Taylor A, Rakosy D, Kreft H, Meyer A, Weigelt P, Knight TM. 2023. Global conservation prioritization for the Orchidaceae. Sci. Rep. 13(1):6718. doi:10.1038/s41598-023-30177-y.

Wang T, Wang X, Gang Y, Cui X, Lan H, Liu Z. 2022. Spatial pattern of endophytic fungi and the symbiotic germination of *Tulasnella* fungi from wild *Cymbidium goeringii* (Orchidaceae) in China. Curr. Microbiol. 79:139. doi:10.1007/s00284-022-02826-4.

Wang XJ, Wu YH, Ming XJ, Wang G, Gao JY. 2021. Isolating ecological-specific fungi and creating fungus-seed bags for epiphytic orchid conservation. Glob. Ecol. Conserv. 28:e01714. doi:10.1016/j.gecco.2021.e01714.

White T, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Academic Press. doi:10.1016/b978-0-12-372180-8.50042-1.

Woo SL, Hermosa R, Lorito M, Monte E. 2023. *Trichoderma*: a multipurpose, plant-beneficial microorganism for eco-sustainable agriculture. Nat. Rev. Microbiol. 21(5):312–326. doi:10.1038/s41579-022-00819-5.

Yang WK, Li TQ, Wu SM, Finnegan PM, Gao JY. 2020. *Ex situ* seed baiting to isolate germination-enhancing fungi for assisted colonization in *Paphiopedilum spicerianum*, a critically endangered orchid in China. Glob. Ecol. Conserv. 23:e01147. doi:10.1016/j.gecco.2020.e01147.

Yuan ZL, Chen YC, Yang Y. 2009. Diverse non-mycorrhizal fungal endophytes inhabiting an epiphytic, medicinal orchid *(Dendrobium nobile)*: Estimation and characterization. World J. Microbiol. Biotechnol. 25:295–303. doi:10.1007/s11274-008-9893-1.