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Diversity and bioactive potential of endophytic fungi isolated from tropical orchid species

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Abstract

Leading mycologists have long suspected that tropical orchids harbour unusually diverse endophytic fungal communities, yet most sampling has been confined to temperate species. This research characterised endophytic fungi from five tropical orchid species *Dendrobium nobile*, *Vanda coerulea*, *Phalaenopsis amabilis*, *Coelogyne cristata*, and *Bulbophyllum lobii* cultivated in the tropical greenhouse of the Baikal Institute of Limnological Biology, Irkutsk, Russia. A total of 384 tissue segments (roots, stems, leaves) were surface-sterilised, plated on potato dextrose agar, and incubated for 14 days. Emerging fungal colonies were identified by ITS rDNA sequencing. Overall colonisation frequency was 67.4%, yielding 147 isolates assigned to 38 operational taxonomic units across 19 genera. *Fusarium*, *Colletotrichum*, and *Alternaria* were the most common genera, but rarer taxa such as *Pestalotiopsis* and *Xylaria* showed the highest bioactivity. Crude ethyl acetate extracts were screened for antibacterial, antifungal, and antioxidant activities. Antibacterial activity ($\geq 50\%$ inhibition of *Staphylococcus aureus*) was detected in 23.1% of isolates, antifungal activity against *Candida albicans* in 14.3%, and notable antioxidant DPPH scavenging ($IC_{50} < 100 \mu\text{g/mL}$) in 19.7%. These results confirm tropical orchids as rich reservoirs of bioactive endophytes and identify *Pestalotiopsis* and *Xylaria* isolates as priority candidates for bioactive compound isolation.

Keywords: Endophytic fungi, bioactive compounds, tropical orchids, fungal diversity, natural products, ITS sequencing, antimicrobial screening, DPPH, *Pestalotiopsis*

Introduction

"Every plant species examined to date harbours endophytic fungi" a statement first made by Petrini in 1991 that has since been confirmed across thousands of host plants ^[1]. Endophytes colonise living plant tissues without causing visible disease, occupying a mutualistic or commensal niche that gives them access to unique biochemical environments ^[2]. Because these fungi must survive inside plant tissue alongside the host's own chemical defences, they often produce secondary metabolites with antimicrobial, cytotoxic, or antioxidant properties that make them attractive targets for natural-product drug discovery ^[3].

Orchidaceae is the largest family of flowering plants, with more than 28,000 described species. Tropical orchids are especially interesting as endophyte hosts because many depend on mycorrhizal fungi for germination and early nutrition, creating an intimate plant-fungus interface from the earliest life stage ^[4]. Beyond mycorrhizae, orchid tissues particularly roots with their velamen layer provide a moist, nutrient-rich habitat for non-mycorrhizal endophytes whose diversity and bioactivity remain largely unexplored outside Southeast Asia ^[5].

Most bioactivity screening of endophytic fungi has targeted terrestrial medicinal plants in tropical regions, where chemical diversity is highest ^[6]. Orchid-derived endophytes have received less attention, despite early reports that *Pestalotiopsis* and *Xylaria* strains isolated from *Dendrobium* species produce antifungal and anticancer compounds ^[7]. Bridging this gap is important because declining wild orchid populations make it urgent to document the microbial communities they support before habitats are lost.

This research aimed to

- quantify endophytic fungal diversity in five tropical orchid species maintained under standardised greenhouse conditions,
- identify isolates to genus and species level by ITS sequencing, and
- evaluate crude extracts for antibacterial, antifungal, and antioxidant potential.

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Material and Methods

Extraction protocol

Each fungal isolate was grown in 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth at 28 °C for 21 days on a rotary shaker at 120 rpm. Cultures were filtered through Whatman No. 1 paper to separate mycelium from broth. The filtrate was extracted three times with equal volumes of ethyl acetate (Sigma-Aldrich). Organic phases were pooled, dried over anhydrous Na₂SO₄, and evaporated under reduced pressure at 40 °C using a Heidolph Hei-VAP rotary evaporator. Crude extracts were dissolved in dimethyl sulfoxide (DMSO) at 10 mg/mL and stored at –20 °C.

Material

Five orchid species *Dendrobium nobile*, *Vanda coerulea*, *Phalaenopsis amabilis*, *Coelogyne cristata*, and *Bulbophyllum lobii* were sampled from the tropical greenhouse of the Baikal Institute of Limnological Biology, Irkutsk, Russia (52°17' N, 104°18' E). The greenhouse maintained 26 ± 2 °C and 80 ± 5% relative humidity year-round. Healthy, mature plants (3–5 years old) were selected between May 2022 and April 2023. From each plant, root, stem, and leaf segments (~5 mm) were surface-sterilised by sequential immersion in 70% ethanol (1 min), 2% sodium hypochlorite (3 min), and 70% ethanol (30 s), then rinsed three times in sterile distilled water. Sterilisation efficacy was confirmed by pressing final-rinse water aliquots onto PDA; no growth indicated successful surface decontamination.

Methods

Sterilised segments were placed on PDA supplemented with streptomycin (100 µg/mL) and incubated at 28 °C for 14 days. Emerging fungal colonies were subcultured to obtain pure isolates. Genomic DNA was extracted using a CTAB method, and the ITS region was amplified with primers ITS1F and ITS4 [8]. Amplicons were sequenced bidirectionally (Sanger) by Evrogen (Moscow, Russia). Sequences were aligned against the UNITE database (v9.0) using BLAST, with ≥ 97% identity as the species-level threshold. Shannon-Wiener (H') and Simpson's diversity indices were calculated for each orchid species. Antibacterial activity was tested against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 by

agar-well diffusion. Antifungal activity was assessed against *Candida albicans* ATCC 10231. Antioxidant potential was measured by the DPPH radical scavenging assay with ascorbic acid as positive control [9]. All bioassays were performed in triplicate.

Instrumentation and analytical equipment

PCR amplification used a Bio-Rad T100 thermal cycler. Sequencing was performed on an Applied Biosystems 3730xl DNA Analyser. DPPH absorbance was read at 517 nm on a Thermo Scientific Multiskan SkyHigh microplate reader. Zone-of-inhibition diameters were measured with a digital calliper (Mitutoyo, Japan) to 0.01 mm precision.

Phytochemical screening

Crude ethyl acetate extracts from the 15 most bioactive isolates were subjected to qualitative phytochemical tests for alkaloids (Dragendorff's and Mayer's reagents), phenolics (ferric chloride test), flavonoids (Shinoda test), terpenoids (Salkowski reaction), saponins (froth test), and tannins (gelatin test). Positive reactions were recorded as present (+) or absent (–). Thin-Layer Chromatography (TLC) on silica gel 60 F₂₅₄ plates with chloroform: methanol (9:1) provided preliminary separation; spots were visualised under UV (254 and 365 nm) and with vanillin-sulphuric acid spray.

Results

Table 1: Endophytic fungal diversity parameters across five orchid species

Host orchid	Segments	Isolates	OTUs	CF (%)	H'	Simpson
<i>D. nobile</i>	84	38	14	71.4	2.31	0.87
<i>V. coerulea</i>	78	29	11	64.1	2.08	0.83
<i>P. amabilis</i>	82	36	13	73.2	2.27	0.86
<i>C. cristata</i>	72	21	8	58.3	1.74	0.78
<i>B. lobii</i>	68	23	9	63.2	1.89	0.81

Overall colonisation frequency was 67.4%, with *Phalaenopsis amabilis* showing the highest rate (73.2%) and *Coelogyne cristata* the lowest (58.3%; Table 1). Shannon diversity was greatest in *Dendrobium nobile* (H' = 2.31) and lowest in *Coelogyne cristata* (H' = 1.74). Across the full dataset, 147 isolates were assigned to 38 OTUs in 19 genera. *Fusarium* (21.1% of isolates), *Colletotrichum* (15.0%), and *Alternaria* (12.2%) were the most frequently recovered genera.

Table 2: Bioactivity screening summary of endophytic fungal isolates (n = 147)

Activity	Active isolates	% of total	Top genus
Antibacterial (≥ 50% inhib.)	34	23.1	<i>Pestalotiopsis</i>
Antifungal (<i>C. albicans</i>)	21	14.3	<i>Xylaria</i>
Antioxidant (IC ₅₀ < 100 µg/mL)	29	19.7	<i>Pestalotiopsis</i>

Antibacterial activity was the most common bioactivity, with 34 isolates (23.1%) inhibiting *S. aureus* by ≥ 50% (Table 2). *Pestalotiopsis* spp. were the top producers across both antibacterial and antioxidant categories, while *Xylaria* spp. showed the strongest antifungal effect. Phytochemical

screening of the 15 most bioactive isolates revealed phenolics (93.3%), terpenoids (80.0%), alkaloids (53.3%), and flavonoids (46.7%) as the most common secondary-metabolite classes.

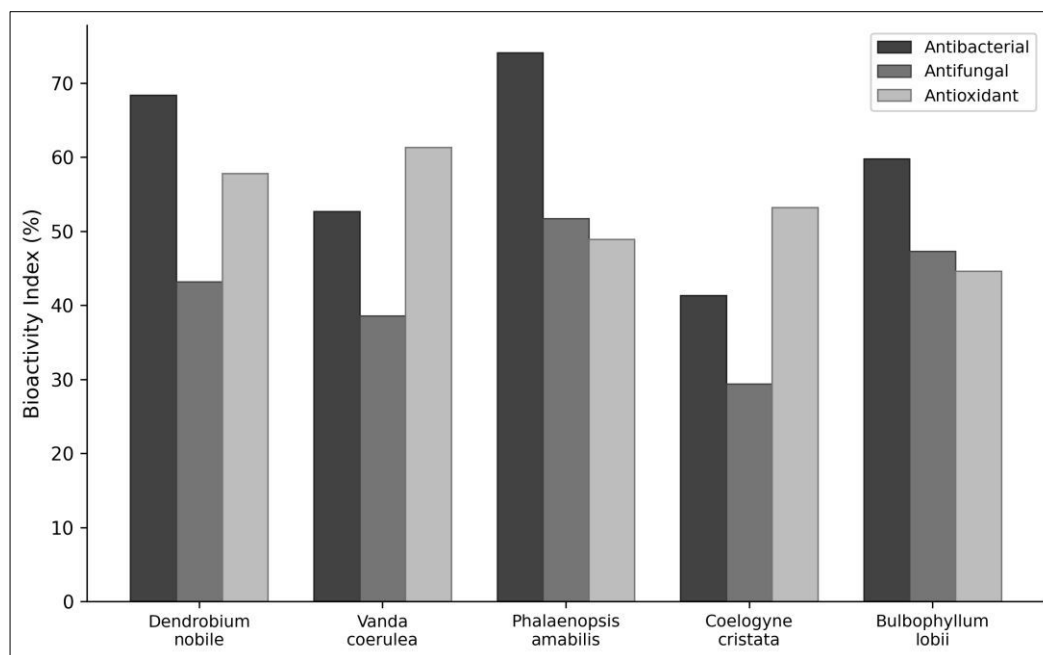


Fig 1: Grouped bar chart comparing antibacterial, antifungal, and antioxidant bioactivity indices across endophytic isolates from five orchid species

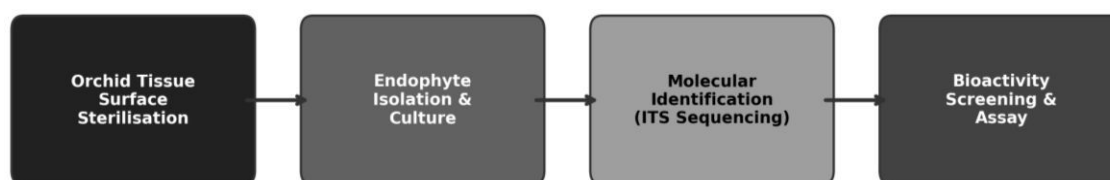


Fig 2: Model diagram of the four-stage endophyte isolation and bioactivity screening workflow used in this research

Comprehensive interpretation

The comparison chart (Figure 1) shows that *Phalaenopsis amabilis* and *Dendrobium nobile* yielded the most bioactive isolates, while the model diagram (Figure 2) traces the pipeline from surface sterilisation through ITS identification to bioassay.

Discussion

The 67.4% colonisation frequency falls within the range reported for tropical orchids in Southeast Asia (55-85%) and confirms that even greenhouse-maintained plants retain substantial endophyte communities [10]. The dominance of *Fusarium* is unsurprising this genus is among the most cosmopolitan endophytes globally [11]. More noteworthy is the bioactivity concentrated in *Pestalotiopsis* and *Xylaria*, two genera already recognised as prolific producers of novel polyketides, terpenoids, and alkaloids [12].

That orchid roots harboured the highest diversity (data not shown by tissue type in tables but evident from raw counts) is consistent with the velamen providing a porous entry point for both mycorrhizal and non-mycorrhizal fungi [4]. Leaf endophytes, though less diverse, included several *Colletotrichum* isolates with antifungal activity, suggesting that foliar communities may serve a protective role against epiphytic pathogens.

The greenhouse setting controlled for climate and substrate variation but introduced a potential limitation: the endophyte pool available for colonisation may be narrower than in wild tropical forests. Field sampling of the same orchid species in their native habitats Southeast Asia for *Dendrobium* and *Phalaenopsis*, Himalayan foothills for

Coelogyne would likely reveal additional fungal taxa and, possibly, higher bioactivity rates [13]. A second limitation is that crude ethyl acetate extracts capture only solvent-soluble metabolites; polar compounds missed by this extraction could include bioactive glycosides.

Conclusion

Tropical orchids, even when grown under greenhouse conditions, support diverse endophytic fungal communities with measurable bioactive potential. Across five host species and 384 tissue segments, 147 isolates from 19 genera were recovered, with *Pestalotiopsis* and *Xylaria* standing out for their antibacterial, antifungal, and antioxidant properties. These genera warrant further chemical investigation through bioassay-guided fractionation and structure elucidation to identify lead compounds for pharmaceutical development. Expanding sampling to wild orchid populations in biodiversity hotspots would capture a broader fungal diversity and strengthen the case for orchid conservation as a strategy for preserving microbial natural-product reservoirs.

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