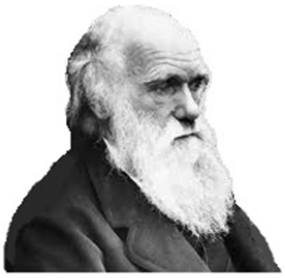
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Isolation and Evaluation of Endophytic Fungi from Two Endangered Orchids of the Western Ghats

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ABSTRACT

Four dominant non-mycorrhizal fungal endophytes isolated from surface-sterilized tissue segments of two indigenous orchids of the Western Ghats (*Acampe praemorsa* and *Luisia curtisii*) were selected to assess *in vitro* production of bioactive metabolites and plant growth promotion. *Beauveria bassiana* and *Botrytis cinerea* were dominant endophytes in *A. praemorsa*, while *Phialophora* sp. and *Sarocladium spinificis* dominated in *L. curtisii*. *Beauveria bassiana* produced the highest quantity of indole acetic acid (IAA), gibberellic acid (GA) and salicylic acid (SA). Culture suspension of all endophytes significantly induced higher germination compared to the control in seeds of green gram (*Vigna radiata*), while the radicle length was significantly higher in seeds treated with culture suspension of *S. spinificis*, followed by *Phialophora* sp. Being substantial producers of plant growth hormones (IAA and GA) and plant growth regulators (SA), *B. bassiana* and *B. cinerea* isolated from *A. praemorsa* deserve to be evaluated by fermentation techniques with different conditions to enhance their capability. This study also advocates inoculation of fungal endophytes of orchids studied into economically important legumes and cereals to enhance the productivity.

Keywords: Bioassay, Growth promotion, Hormones, Radicle growth, Seed germination, *Acampe praemorsa, Luisia curtisii*

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1. INTRODUCTION

The Orchidaceae constitute the second largest family among the flowering plants, with a worldwide distribution of about 30,000 species [1]. Orchids have adapted to various habitats as epiphytic, lithophytic and terrestrial plants; among them, 25% are terrestrial and up to 70% are epiphytic and/or lithophytic [2]. Orchids have ornamental, horticultural, medical, nutritional and industrial significance [3-5].

Orchid populations are dwindling as they are facing severe threats, like climate change, habitat loss, habitat fragmentation, overexploitation and human interference [6, 7].

Besides commercial values, orchids contain a wide range of bioactive compounds useful in treating various human diseases [8]. Orchids serve as reservoirs of many bioactive metabolites, such as alkaloids, hormones (indole acetic acid and gibberellic acid), phenolic acids, quinones, saponins, steroids, tannins, terpenoids, and they possess antimicrobial, insect repellent, anticancer and plant growth-promoting traits [9].

Orchids are mainly dependent on fungi (mycorrhizal and non-mycorrhizal) for adaptation to different habitats, completion of their life cycles and fetching the nutrients (mainly carbon, nitrogen and phosphorus) [10-12].

Studies on the association of orchids with fungi are largely confined to the mycorrhizal rather than non-mycorrhizal fungal mutualism [13-16]. Orchid mycorrhizal relationships have been studied widely, while exploration on the importance of non-mycorrhizal fungal associations is insufficient [12, 17].

Similar to mycorrhizal association, non-mycorrhizal fungal mutualism with orchids helps adaptation to varied environments, growth promotion, defending against pathogens, producing growth hormones and enhancing seed germination [18-20].

Xu and Guo [21] demonstrated the association of two fungal species with *Gastrodia elata* at different stages of its life cycle. Similarly, Leake [22] suggested that over 100 species of orchids are dependent on endophytic fungi in their lifetime. *Rhizoctonia zeae* isolated from *Vanda coerulea* possesses a significant role in promoting seed germination, growth of seedlings and increased survival rate during acclimatization [23].

Germination of orchid seeds is dependent on the influence of endophytic basidiomycete *piriformospora indica* [24]. Thus, fungal endophytes are crucial in orchid growth, development and adaptability, as they are coevolved [25].

The Indian subcontinent is known for the occurrence of up to 1,300 orchid species, while the Western Ghats possess 310 species, with 123 endemic species [26, 27]. The present study was envisaged to assess two endangered orchids, *Acampe praemorsa* and *Luisia curtisii*, growing in the Western Ghats of India for their association with dominant non-mycorrhizal endophytic fungi, production of bioactive metabolites and plant growth promotion potential.

2. METHODS

2. 1. Orchids

Two orchids, *Acampe praemorsa* (Roxb.) latt. & McCann and *Luisia curtisii* Seidenf. growing in the forest areas of Thirthamuthur (13.61° N, 75.24° E) and Moodbidri (13.07° N, 75.0° E), respectively, were collected to study endophytic fungi (Figure 1(A & B).



(A)



(B)

Fig. 1(A & B). Two endangered orchids assessed for isolation of endophytic fungi: **(A)** *Acampe praemorsa* (Roxb.) Blatt. & McCann, **(B)** *Luisia curtisii* Seidenf.

2. 2. Isolation of endophytic fungi

Endophytic fungi were isolated from the orchids based on the procedure outlined by Sudheep and Sridhar [15]. Six segments (4−5 × 30 mm) of matured aerial roots, stem and leaves were excised. Each set of segments was immersed in ethanol (95%, 1 min), followed by immersion in sodium hypochlorite (0.6%, 5 min) and ethanol (95%, 0.5 min). The sterilized segments were immediately rinsed in sterile distilled water repeatedly before plating onto Potato Dextrose Agar (PDA, 1.5%) medium supplemented with Chloramphenicol (250 mg/l).

Fungal colonies developed after one week of incubation at laboratory temperature (26 ±2 °C). Morphologically distinct and common fungi were subcultured on PDA without antibiotic until getting pure cultures. They were identified based on colony morphology, colour, nature of conidiogenous cells and conidia. They were deposited in Alva's Microbiology Culture Collections (AMCC).

2. 3. Bioactive metabolites

2. 3. 1. Indole Acetic Acid

Indole acetic acid (IAA) production by endophytic fungi was evaluated based on the Salkowski method [28]. Fungal strains were grown in potato dextrose broth (PDB) and incubated up to 3 days at 35 ±2 °C with agitation (120 rpm). After termination of incubation, cultures were centrifuged (Model R-4C Remi, Maharashtra, India) and the supernatant was mixed with Salkowski's reagent (150 ml of concentrated sulfuric acid, 250 ml of distilled water and 7.5 ml of 0.5 M $FeCl_3 \cdot 6H_2O$). The mixture was allowed to stand for 20 min with subsequent development of a pink colour. The amount of IAA produced was assessed by measuring the intensity of colour at 530 nm with a double-beam PC-based UV-Visible spectrophotometer (Model # 2202, Systronics India Ltd., Gujarat, India).

2. 3. 2. Gibberellic acid

Production of gibberellic acid (GA) produced by endophytic fungi was determined by following the procedure by Berríos et al. [29]. Similar to IAA estimation, the isolates were grown in PDB, incubated up to 7 days at 35 ±2 °C with agitation (120 rpm), centrifuged and the supernatant was extracted by ethyl acetate (2:1, v/v) after reducing the pH to 2.5 (with 1M HCl). The absorbance of the solution was measured at 254 nm using a double-beam PC-based UV-Visible spectrophotometer.

2. 3. 3. Salicylic acid

Salicylic acid produced by endophytic fungi was estimated based on the procedure by Warrier et al. [30]. Similar to GA estimation, fungal cultures grown on PDB were incubated up to 7 days at 35 ±2 °C with agitation (120 rpm) and centrifuged. Freshly prepared ferric chloride of volume 0.1 ml was mixed with 100 µl of culture supernatant. The volume of the reaction mixture was made up to 3.0 ml with distilled water and incubated up to 10 min. The absorbance of violet colour developed due to the complex of Fe^{3+} and salicylic acid was measured at 540 nm using a double-beam PC-based UV-Visible spectrophotometer.

2. 4. Plant growth promotion

The ability of endophytic fungi to promote plant growth was evaluated *in vivo* by

conducting a green gram [*Vigna radiata* (L.) R. Wilczek] seed germination assay. The isolated endophytes were grown on respective PDB in culture tubes and were incubated up to one week at 35 ±2 °C. Later, the cultures were centrifuged (10,000 rpm for 20 min). Seeds of green gram of uniform size were chosen, surface sterilized with 70% (v/v) aqueous ethanol and rinsed thrice in sterile distilled water. Sixty seeds were soaked in the culture supernatant for up to 12 hr. Later the 50 seeds each were rinsed in sterile distilled water, plated on sterile filter paper in standard Petri dishes and incubated at laboratory temperature (26 ±2 °C). Similarly, seeds treated with distilled water served as a control. Germination and radicle length were recorded 18 and 24 hours after incubation, respectively.

2. 5. Statistical analysis

A comparison was made in the quantity of IAA, GA and SA produced by *Beauveria bassiana* vs. other endophytes by *t*-test using Statistica version 8.0 [31]. The same test was applied to follow the significant difference in percent seed germination and radicle length between the control vs. treatment with fungal culture filtrates.

3. RESULTS AND DISCUSSION

3. 1. Endophytic fungi

Plating surface sterilized live segments of two orchids showed four dominant endophytic fungi (Table 1). Based on their morphological characteristics on PDA, they were identified as *Beauveria bassiana, Botrytis cinerea*, *Phialophora* sp. and *Sarocladium spinificis*. Anamorphic *B. bassiana* is related to the sexual state *Cordyceps bassiana*. It is an entomopathogenic fungus with worldwide distribution that serves as a parasite on different species of arthropods and is used as a bioinsecticide. Its association with aerial roots of *A. praemorsa* likely protects it from insect pests. *Botrytis cinerea* is known as a pathogen on many horticultural crops. But it exists as a dominant endophyte in *A. praemorsa*. *Phialophora* sp. dominated on the aerial roots of *L. curtisii*, which is parasitic as well as saprophytic. It has an association with leafcutter ants that serve as fungal gardens. *Sarocladium spinificis* is another saprobic fungus that is a dominant endophyte in leaves of *L. curtisii* and possesses antifungal potential.

Table 1. Morphological features of endophytic fungi isolated from orchids *Acampe praemorsa* and *Luisia curtisii* on PDA.

Endophyte	Orchid	Morphological features	AMCC code
Beauveria bassiana (Bals.-Criv.) Vuill.	Aerial roots (*A. praemorsa*)	White powdery colonies later turned to light yellow, possessed branched conidiophores and smooth hyaline, spherical to sub-globose conidia were borne on conidiophores and appeared as dry and powdery distinctive spore balls.	AMCC 5

Botrytis cinerea Pers.	Stem (*A. praemorsa*)	Initial white powdery colonies with aerial mycelia with concentric rings become greyish with age. Produce black sclerotia, conidiophores apically branched hyaline with hyaline single-celled ovoid conidia.	AMCC 6
Phialophora Medlar	Aerial roots (*L. curtisii*)	Phialides are short, flask-shaped and/or elliptical, distinct funnel-shaped, darkly pigmented with ellipsoidal and smooth-walled hyaline conidia.	AMCC 7
Sarocladium spinificis Y.H. Yeh & R. Kirschner	Leaves (*L. curtisii*)	Conidiophores, simple or occasionally branched with slender phialides, produce fusiform, small or elongated conidia.	AMCC 8

Although many of these dominant endophytes are saprophytic and pathogenic, they may also serve as endophytes in orchids to overcome stress and climate change.

3. 2. Bioactive metabolites

Endophytic fungi are well known for their capability to produce bioactive metabolites. The culture filtrates of four dominant endophytic fungi with orchids in our study produced hormones (IAA and GA) as well as a plant growth regulator (SA) (Table 2). Among them, *B. bassiana* produced significantly higher quantities of IAA, GA and SA ($p<0.01$), while *B. cinerea* is the second largest producer of GA.

The rest, *Phialophora* sp. and *Sarocladium spinificis*, showed low quantities of IAA, GA and SA. Associated fungal endophytes in these orchids have a potential role in the production of growth hormones as well as growth regulators.

Table 2. Production of indole-3-acetic acid, gibberellic acid and salicylic acid by the orchid endophytes (n=3, mean ±SD) (*t*-test: *, $p<0.01$; **, $p<0.001$).

Endophyte	**Indole-3-acetic acid (µg/ml)**	**Gibberellic acid (µg/ml)**	**Salicylic acid (µg/ml)**
Beauveria bassiana	55.74±1.12**	31.76±1.06*	21.53±0.94*
Botrytis cinerea	5.96±1.47	15.29±1.61	12.32±0.69
Phialophora sp.	1.30±0.39	0.06±0.02	0.31±0.03
Sarocladium spinificis	0.38±0.09	0.06±0.03	0.25±0.03

In addition, *B. bassiana* isolated from *A. praemorsa* may serve as a potential biocontrol agent against insect pests. *Sarocladium spinificis* isolated from *L. curtisii* may serve as a potential candidate for antifungal activity.

Several studies have demonstrated that endophytic *Beauveria, Botrytis, Phialophora* and *Sarocladium* are capable of increasing plant growth and resistance to abiotic and biotic stresses [32-36].

Macuphe et al. [37] opined that an endophytic *B. bassiana* enhanced plant growth by growth-promoting metabolites. Shaalan et al. [38] reported that inoculation of endophytic *B. bassiana* served a dual purpose, like enhanced growth and disease resistance.

3. 3. Plant growth promotion

The seed germination and radicle length enhancement in green gram by the orchid endophytes are presented in Table 3. All four endophytes induced significantly higher seed germination compared to the control ($p<0.05$), except for *Botrytis cinerea* ($p>0.05$), with the highest in *Phialophora* sp. (100%), followed by *Beauveria bassiana* (96%). Similar to seed germination, radicle length enhancement was higher in all endophytes ($p<0.05$), except for *Botrytis cinerea* ($p>0.05$), with the highest by *Sarocladium spinificis* (41%), followed by *Phialophora* sp. (32%).

Plant growth-promoting microbes (PGPM) are potential alternatives to the use of synthetic fertilizers. The PGPM directly aids in plant growth promotion by acquisition of nutrient resources (e.g., iron, nitrogen and phosphorous). They also modulate plant growth by supplying or regulating plant hormones (e.g., auxin, cytokinin and ethylene).

Saleemi et al. [39] reported that exogenous treatment of wheat seeds (*Triticum aestivum*) with IAA and GA enhanced the seed germination and increased root/shoot growth. Our study also showed that all endophytic fungi isolated from orchids produced IAA, GA and SA in their culture medium, thus deserving further investigation. These isolates could be evaluated under pot as well as field trials. Such approaches also help assess the impact of drought resilience in view of biological control of plant pathogens.

Table 3. Impact of endophytic fungal inoculum on seed germination and radicle length of green gram (n=50, mean ±SD) (*t*-test: *, $p<0.05$; **, $p<0.01$).

Control and Endophyte	Seed germination (%)	Length of radicle (mm)
Control (in distilled water)	85±1.63	4±0.381
Beauveria bassiana	96±2.94*	9±1.41*
Botrytis cinerea	92±2.94	7±1.41
Phialophora sp.	100±0.00**	32±6.48*
Sarocladium spinificis	90±1.63*	41±2.94**

4. CONCLUSIONS

Orchids are extensively dependent on associated mutualistic fungi to adapt and perpetuate in different habitats and climatic conditions. Among the four dominant endophytic fungi associated with two endangered orchids (*Acampe praemorsa* and *Luisia curtisii*) of the Western Ghats, *Beauveria bassiana* was the highest *in vitro* producer of indole acetic acid, gibberellic acid and salicylic acid. Significantly higher seed germination of green gram was shown by the culture suspension of all four endophytic fungi, with the highest by *Phialophora* sp. Enhanced radicle length was seen in culture suspension of all endophytes, with the highest by *Sarocladium spinificis*, followed by *Phialophora* sp. The strain *B. bassiana* isolated from *A. praemorsa* may serve to control insect pests efficiently. With limited antifungal agents, *Sarocladium spinificis* isolated from *L. curtisii* may serve as a future possible candidate for fungal infection control. Further investigations to enhance the hormones, growth regulators, plant growth promotion, biopesticide potential and antifungal metabolites by orchid endophytic fungi are warranted.

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