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RESEARCH ARTICLE

Isolation, Identification and Molecular Characterization of Endophytic Fungi from the leaves of *Coelogyne* species, and their role as an Antimicrobial agent

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ABSTRACT:

Endophytic fungi from Orchids believed to have an essential symbiotic relationship with the plant for both the germination of seeds and the development of young plantlets. Endophytes are microorganisms which live in the intercellular spaces of healthy host tissues without causing apparent symptoms. Endophytic fungi isolated from the medicinal plants are used for the development of drugs as they act as the source of bioactive compounds. This study has been designed to isolate the fungal endophytes from the leaves of three epiphytic orchid species (*Coelogyne nervosa*, A. Rich., *Coelogyne mossiae*, Rolf and *Coelogyne cristata*, Lindl) from the same genus *Coelogyne*, collected from Ooty flora, Coonoor. About five different endophytes were identified and their morphological characters were studied. *Coelogyne cristata* showed maximum colonization frequency. Among the five species, *Fusarium* species showed antibacterial activity against the gram-positive bacteria *Bacillus Subtilis*, a pathogen found in soil, water and food. Fungal genomic DNA isolated for molecular identification from the four fungal species.

KEYWORDS: Orchid endophyte, *Coelogyne*, Morphological studies, Antimicrobials, DNA isolation.

INTRODUCTION:

Orchidaceae family is highly evolved and consists of widely distributed monocots. It has about 750 genera and 30,000 species. Among these, over 210 genera have been investigated for their endophytic fungal diversity, which is less than 30% of the total orchid genera. Bioprospecting studies of endophytic fungi for pharmaceutical and biotechnological purposes are essential for the discovery of new substances for human therapeutics, includes antibiotics, anti-malarial, and anticancer.

Microorganisms are significant sources of bioactive natural products with enormous potential for the detection of new molecules for drug discovery, industrial use and agricultural applications. Endophytic fungi from Orchid plants believed to have an essential symbiotic relationship with the plant for both the germination of seeds and the development of young plantlets. Endophytes are microorganisms which live in the intercellular spaces of healthy host tissues without causing apparent symptoms. Fungal endophytes have been investigated in a large number of orchid species from around the world.¹ Nowadays endophytic fungi take attention from many scientists as they produce secondary metabolites that have anticancer, antidiabetic, insecticidal, and immuno-suppressive compounds.²⁻⁴

Endophytes belong to a wide range of organisms (e.g. bacteria, fungi and algae) inhabiting the healthy plant tissues without causing visible disease symptoms at any specific moment.⁵⁻⁶ *Coelogyne cristata* has been used as a remedy for acute and chronic bronchitis, toothache, duodenal ulcer and fracture healing. It is proved to be

the storehouse of phytochemicals including alkaloids, flavonoids, glycoside, benzyl derivatives, phenanthrene, terpenoids, phenolic compound, pyrone derivatives, etc. with promising biological activities for antitumor, anti-inflammatory, anticancer, anticonvulsive, antidiabetic, osteoprotective, etc.⁷⁻⁹

Relatively half of the identified fungal natural products sourced from endophytic fungi, and hence endophytic fungi became the new sources of novel active compounds with biological activity including antimicrobial action. Endophytic fungi from the genera *Penicillium*, *Ampelomyces*, and *Fusarium*, isolated from *R. mucronata* reported by Hamzah et al.,¹⁰ effective on controlling *E. coli*. Despite endophytic fungi considered as novel sources of unique active compounds, their real potentiality stays largely left underexplored.¹¹ Leaves hosts and harbours different fungal endophytes concerning other plant plants.¹²⁻¹³ Endophytic fungi have been established for their varied biological activities; antibiotic, anticancer, antimicrobial and insecticidal attributes.⁹ Endophytic fungi isolated from leaves of *Indigofera suffruticosa* Miller, showed promising results especially as an antimicrobial agent with *Nigrospora sphaerica* and *Pestalotiopsis maculans* being the most prominent, exhibiting antimicrobial activity against both Gram-negative and Gram-positive bacteria.¹³ Endophytes and Endophytic fungi have also been explored for their other environmental applications like bioremediation.¹⁴⁻¹⁸

The current study aimed to use the endophytic fungi isolated from the leaves of three species of the same genus (*Coelogyne*) for its antimicrobial activity to control pathogens found in soil, water and food.¹⁹

MATERIALS AND METHODS:

Selection of plant:

Coelogyne species of orchids chosen to isolate the endophytic fungi. These orchids were terrestrial type of orchids which have endophytic fungi in their leaves.¹⁶

Collection of plant:

Coelogyne species (Orchidaceae) collected from Ooty flora, Katteri, Coonoor, The Nilgiris Dt.

Medium preparation:

Fresh potatoes were taken and washed; their skin was peeled and sliced into small pieces. The sliced potato was cooked in 500mL of water for 15min and autoclaved. The potato extract was filtered using muslin cloths. About 20g of Dextrose added to the potato extract, and made up to 1L with distilled water. The pH of the medium was adjusted to 6.5 using pH meter. The pH maintained by using 1N NaOH or 1N HCl. About 20 grams of Agar was added and autoclaved for 1h at

121°C. Streptomycin was added to the medium at the time of inoculation to avoid bacterial growth.¹⁷

Sterilization:

Laminar air-flow chamber wiped with 75% ethanol, and UV light switched on for 20min. The medium, handling materials and glassware were placed inside the chamber, and UV light again switched on for 5-10min. Initially, the leaves washed in running tap water before washing with distilled water to remove all adhere soil particles and debris, followed by dipping into 90% ethanol for 1 min, 5% sodium hypochlorite for 10s, 96% ethanol for 1 min. At last, the plant parts were rinsed three times in sterile distilled water for 2 min.²⁰⁻²²

INOCULATION:

The leaves were inoculated in a fresh PDA medium using forceps. About 2 leaf segments were inoculated in each Petri dish. All Petri dishes were sealed with sterile Parafilm to protect them from contamination during repeated handling while examining endophytes and from desiccation. The plates incubated at 26±2°C for 7days.¹⁰

Anatomical studies:

The leaves of the orchid species were hand sectioned, and Safranin stain used for better understanding of some anatomical structures of the sections. Sections were examined with a light microscope and photographed by Optika digital camera. Various measurements performed on microscopic images.⁸

Statistical analysis and Relative percentage occurrence:

The colonization frequency (CF) percentage of Endophytic fungi and Relative percentage occurrence, of different groups of fungi (RPO), calculated using the method employed by Kannan et al.¹⁴

Colonization frequency (%):

The colonizing rate calculated using eq (1)

$$CF = \frac{\text{No. of species isolated}}{\text{No. of segments screened}} \times 100 \quad \dots\dots\dots(1)$$

Relative percentage occurrence (RPO) of different groups of fungi (%):

Relative percentage occurrence (RPO) arrived at using the formula (eq 2)

$$RPO = \frac{\text{Density of colonization of one group}}{\text{Total density of colonization}} \times 100 \quad \dots\dots\dots(2)$$

Subculture:

The endophytic fungi collected from the inoculated plates, and they were sub-cultured in fresh PDA medium plates. The plates were tightly packed with parafilm for several weeks until the mycelium grows more prominent, then it was stored at lower temperature 6°C, which would slow down the rate of metabolism and the growth of the fungus.¹⁹

Morphological studies:

Morphological characteristics such as colony topography, colour, and growth pattern were studied. Slides were prepared from cultures by hyphal tipping, stained with trypan blue and viewed under microscope.¹⁹

Screening for antibacterial activity:

Antagonistic assay was done by agar diffusion method. Isolated endophytic fungal extracts were tested for the antibacterial activity. The gram-positive bacteria *Bacillus subtilis* was used in this screening process. Bacterial pathogens were spread on Nutrient agar plates. Then wells were made, and 50µl crude extract of each strain inoculated into a separate well. Antagonistic activity detected after an incubation of 24 to 48h at 35°C. The presence of a clear zone on agar plates used as an indicator for the antibacterial activity.²³⁻²⁴

Molecular Identification: Fungal Genomic DNA Extraction:

Fungal mat extracted from 15days culture and it is completely dried using tissue, and the fungal mat weighed in a pre-weighed Petri dish. 2g of the fungal mat was taken in a sterilized pestle and mortar, and ground with acid wash sand to a fine powder. 3ml of CTAB buffer and 0.5ml of 0.2% mercaptoethanol added. Then it is transferred into sterilized centrifuge tube. It is shaken vigorously and kept in a water bath at 60°C for 1h. The sample cooled to room temperature. Then it is centrifuged at 10000rpm for 20 minutes. The supernatant was collected and transferred to a fresh centrifuge tube. Then equal volume of chloroform: Isoamyl alcohol (24:1) mixture was added. It is shaken vigorously and centrifuged at 15,000rpm for 15min. The supernatant was collected and transferred to a fresh tube. Then 0.7 volume of ice-cold isopropanol was added to the supernatant. Then the sample was kept in cold condition for 1 hour and centrifuged at 10,000rpm for 5min. The soup discarded, and the pellet was washed twice with 70% ethanol. The pellet was dried and dissolved in 100µl of Millipore water or TAE buffer. DNA samples were allowed to run in the agarose gel electrophoresis tank. Then it is viewed under the gel doc for further studies.¹⁰

RESULTS:

Morphological studies and Colonization frequencies of endophytic fungi:

From the leaves of the three orchid species, five different endophytic fungi isolated. Of these, three isolated from *Coelogyne nervosa* and one from *Coelogyne mossiae* and one from *Coelogyne cristata*. They have different colours, and the frequency is maximum in *Coelogyne cristata* i.e. 100%. The relative percentage occurrence is maximum in *Fusarium* sps.1, *Cladosporium* sps and *Nodulisporium gregarium*, i.e. 100% and minimum in *Fusarium* sps.2, i.e. 25%. Spores seen in each fungal species.

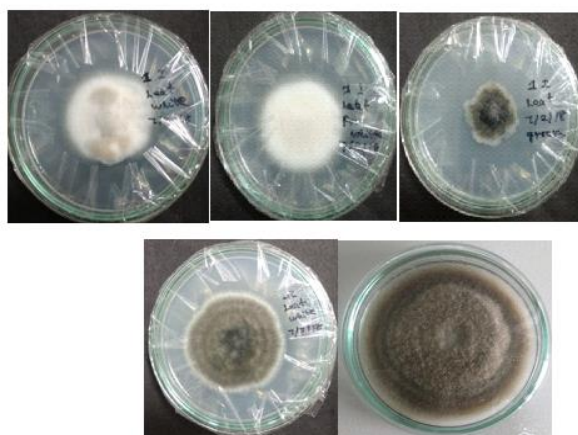


Figure 1: Isolated five different endophytic fungi

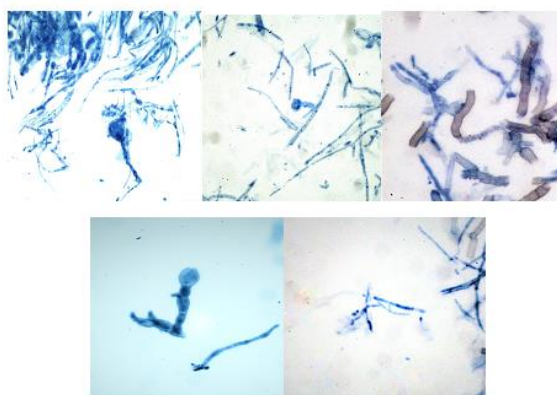


Figure 2: Anamorph Morphology of the Isolated Endophytic Fungal species



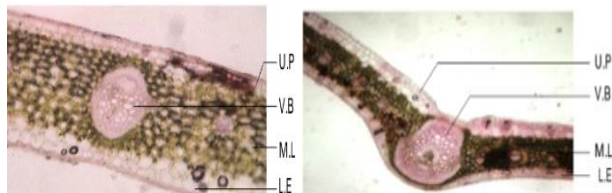
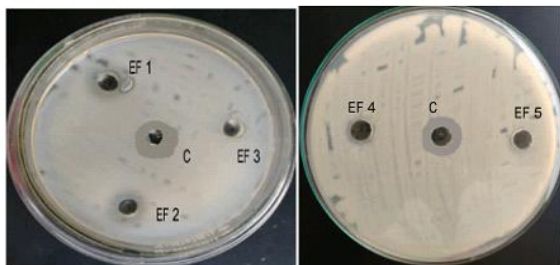
Figure 3: Cross-section of the three *Coelogyne* Species

Table 1: percentage occurrence of isolated Endophytic fungi.

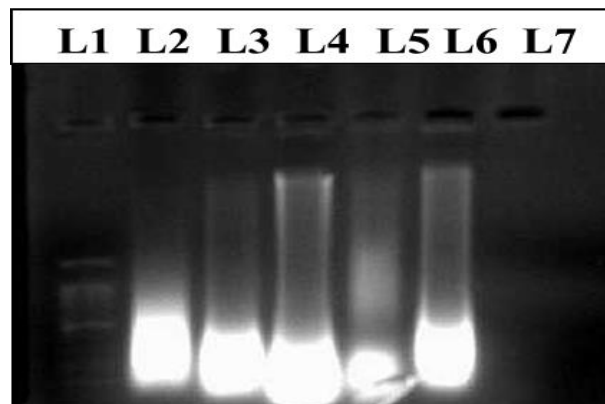
Plant name	Name of the identified fungal species	Relative percentage occurrence (%)	Colony frequency (%)
<i>Coelogyne nervosa</i>	<i>Fusarium sps. 1</i>	100	50
	<i>Fusarium sps. 2</i>	25	
	<i>Lasioidiplodia theobromae</i>	50	
<i>Coelogyne mossiae</i>	<i>Cladosporium sps</i>	100	50
<i>Coelogyne cristata</i>	<i>Nodulisporium gregarium</i>	100	100

Antibacterial activity:

Antibacterial activity against gram-positive bacteria *Bacillus subtilis* shows a zone of inhibition in two species, where 50µl of crude extract of *Fusarium sps.1* and *Fusarium sps.2* shows 0.3cm and 0.2cm of clear zone respectively.¹⁰



EF1 – *Fusarium sps.1* EF4 – *Cladosporium sps.* EF2 – *Fusarium sps.2* EF5 – *Nodulisporium gregarium* EF3 – *Lasioidiplodia theobromae* C – Control

Figure 4: Zone of inhibition shown by fungal extracts against *Bacillus subtilis*

L1 – Marker L2 – *Fusarium sp. 1* L3 – *Fusarium sp. 2* L4 – *Lasioidiplodia theobromae* L5 – *Cladosporium sp* L6 – *Nodulisporium gregarium*

Figure 5: DNA Bands viewed under Gel Doc

Table2: Zone of inhibition against *Bacillus subtilis*

Plant species	Zone of inhibition (cm)	
	Streptomycin (50µl)	Fungal crude extract (50µl)
<i>Fusarium sp 1</i>	0.4	0.3
<i>Fusarium sp 2</i>	0.4	0.2
<i>Lasioidiplodia theobromae</i>	0.4	-
<i>Cladosporium sp</i>	0.4	-
<i>Nodulisporium gregarium</i>	0.4	-

Three species of *Coelogyne* genus collected from Ooty flora, Katteri, Coonoor, Nilgiris Dt., the leaves inoculated in PDA medium, and five different fungal species were isolated and identified. Two *Fusarium* species and one *Nodulisporium gregarium* and two unknown species isolated. Anatomical studies of leaves of *Coelogyne nervosa*, *Coelogyne mossiae*, *Coelogyne cristata* carried out. The internal structures of epidermis, vascular bundles are studied. Similar studies were reported by.²³⁻²⁵ The morphology of the fungal species was studied and their colony colour, colony margin, colony diameter and anamorph morphology recorded. Similarly, the morphology of the endophytic fungi according to their colony, mycelium and spore characteristics were identified by Kannan et al.¹⁴ The colonization frequency of the endophytic fungi was calculated, wherein *Coelogyne cristata* showed 100% frequency and both *Coelogyne mossiae* and *Coelogyne nervosa* showed 50% frequency. Similar studies were done by Deshmukh et al.² calculated the percentage colonization frequency was more in stem 14.28% followed by roots 12.5%, inner leaf sheath 5.33% and outer leaf sheath 2.5%. The relative percentage occurrence for the five endophytic fungi also calculated, where *Fusarium sps.1*, and *Nodulisporium gregarium* showed 100% relative occurrence. Similarly, Kannan et al.¹⁴ estimated the relative percentage occurrence of endophytic fungi in Hyphomycetes group is more in stem 47.5%, outer leaf sheath 61.76% and 50% in roots, while Coelomycetes in inner leaf sheath is 50%. The antibacterial activity against gram-positive bacteria *Bacillus subtilis* was analyzed where *Fusarium sps.1* and *Fusarium sps.2* shows 0.3 and 0.2cm of clear zone. Similar studies were also reported by (Mousa et al. 2013) where *Aspergillus terreus* showed the maximum zone of inhibition was 11.6±0.57mm against *Salmonella typhi*. The isolation of DNA carried out for the five fungal species from which the fungal genomic DNA isolated for four species.²⁶⁻³⁰

CONCLUSION:

The present study concludes that the traditional method of fungal species identification using morphological characters replaced by molecular characterization. Molecular studies invariably rely on ITS sequencing to differentiate fungal morphotypes; however, due to the

inherent shortcomings associated with this approach, a 90% ITS sequence similarity is considered a good match for designating species names for these fungi. Even while using the molecular approach, the sterile morphotypes isolated in the present study gave a maximum sequence similarity of 94 % only suggesting that more effort needs to be focused on exploring the true diversity of unculturable and sterile endophytes, especially from tropical hosts. Both *Fusarium* spp. exhibited antimicrobial activity and suitable for control of pathogens in the environment. There are vast biological and chemical diversity hidden in tropical fungi and the fact that these fungi can act as an alternative source of plant metabolites and considered for other environmental applications like bioremediation.

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