



RESEARCH ARTICLE

Seasonal diversity and spatiotemporal distribution of fungal endophytes associated with the medicinal plant *Coleus forskohlii* Briq.

Grace Leena Crasta^{1,2*} & Koteswar Anandrao Raveesha^{1,3}

¹Department of Studies in Botany, Center for Innovative Studies in Herbal Drug Technology, University of Mysore, Mysuru-570 006, India

²Department of Botany, St. Joseph's University, Bengaluru-560027, India

³School of Life sciences, JSS Academy of Higher Education and Research, Mysuru-570 015, India

*Email: graceleena@sju.edu.in



ARTICLE HISTORY

Received: 23 June 2023
Accepted: 26 September 2023
Available online
Version 1.0 : 28 October 2023
Version 2.0 : 02 January 2024



Additional information

Peer review: Publisher thanks Sectional Editor and the other anonymous reviewers for their contribution to the peer review of this work.

Reprints & permissions information is available at https://horizonepublishing.com/journals/index.php/PST/open_access_policy

Publisher's Note: Horizon e-Publishing Group remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Indexing: Plant Science Today, published by Horizon e-Publishing Group, is covered by Scopus, Web of Science, BIOSIS Previews, Clarivate Analytics, NAAS, UGC Care etc. See https://horizonepublishing.com/journals/index.php/PST/indexing_abstracting

Copyright: © The Author(s). This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited (<https://creativecommons.org/licenses/by/4.0/>)

CITE THIS ARTICLE

Crasta G L, Raveesha K A. Seasonal diversity and spatiotemporal distribution of fungal endophytes associated with the medicinal plant *Coleus forskohlii* Briq. Plant Science Today. 2024; 11(1): 223–233. <https://doi.org/10.14719/pst.2729>

Abstract

Fungi that colonize the healthy tissues of the plants without showing any disease symptoms in the host plants are termed as fungal endophytes. The presence of fungal endophytes provides a positive effect on the host's growth and development and also triggers the production of some essential bioactive compounds in the host. This study was undertaken to isolate, identify and understand the spatiotemporal distribution and seasonal diversity of fungal endophytes associated with the leaf, stem and root of *Coleus forskohlii*, an important and endangered medicinal plant. Sampling was done for a period of 12 months between May 2020–April 2021. A total of 950 fungal endophytes were isolated from a total of 1680 tissues of the leaf, stem and root of *C. forskohlii*. The fungi were identified based on their morphological features and some of them were identified by molecular identification by 18S rRNA sequencing. The endophytic isolates belonged to 10 different orders belonging to 3 different classes-Sordariomycetes (Hypocreales, Xylariales, Microascales, Trichosphaeriales, Glomerellales and Sordariales), Dothiomycetes (Pleosporales, Capnodiales, Botryosphaeriales) and Eurotiomycetes (Eurotiales). About 81.26% of the isolates belonged to Ascomycota and 2.63% of the isolates belonged to Mucoromycota. *Chaetomium globosum*, *Collariella bostrychodes*, *C. robusta*, *Colletotrichum gloeosporioides*, *Fusarium chlamydosporum*, *Sterile hyaline mycelia*, *Aspergillus niger*, *Xylaria curta*, *X. grammica*, *Mucor circinelloides* and *Trichoderma harizianum* were the frequently isolated species of fungi. *C. globosum*, *C. bostrychodes*, *C. gloeosporioides*, sterile hyaline mycelia and *X. curta* were found distributed in all the tissues of the plant. *C. forskohlii* has thus revealed a rich diversity of fungal endophytes that could be isolated and cultured to yield some pharmacologically important bioactive compounds.

Keywords

Coleus forskohlii; fungal endophytes; seasonal diversity; spatiotemporal distribution; Ascomycota

Introduction

The Royal Botanic Gardens, Kew, estimates that nearly 390,900 plant species exist on earth, and studies have shown that almost all plants are associated with endophytes (1–4). Endophytic fungi are important group of micro-organisms that colonize the healthy tissues of the plants without instigating any disease symptoms into them. The term “Endophytae” was given by Heinrich Friedrich Link in 1809, who considered endophytes as fungi that are somewhat parasitic. It was Anton de Bary in 1866 who first gave the term “Endophytes” for those fungi residing inside the plant tissues. Fossil evidences of many plants indicate that plants have been associated with fungal endophytes for >400 million years (5–7). Plants have posed new niches

to endophytic fungi by establishing a beneficial symbiotic relation with them. Endophytic fungi colonize almost all parts of the plants including the leaves, stems, roots, flowers and seeds (8–10). The symbiotic association, organ preference and ecological functioning of fungal endophytes with their hosts could be vastly capricious. As much as endophytic fungi depend on the host for colonization and survival, the host plants also depend on the fungal endophytes for their growth and development, to solubilize minerals, to synthesis secondary metabolites, to synthesis phytohormones, to gain resistance against many pathogens and to withstand the environmental conditions (11, 12). Some endophytes are able to produce similar bioactive compounds as the host plant, while others are able to produce bioactive compounds that are not naturally synthesized by the host. These bioactive compounds could exhibit a wide range of applications in the field of agriculture and medicine (13).

Although fungal endophytes are reported from various plants distributed in different ecological habitats, their choice of host varies with the variation in climatic conditions and geographical regions. Seasonal changes have also known to affect the potential of the endophytic community harbouring the different tissues of the host plant. Variations in the climatic conditions such as temperature and atmospheric humidity is known to influence the endophytic community within the host tissues, because these factors affect the germination and growth of the fungal spore on the host. Only conducive environments can favour the above process. Alongside the external conditions, endophytic fungi also show tissue preference. They are sometimes explicitly found and isolated from specific tissues such as leaves, bark, root, etc., implying to their spatiotemporal distribution within the host (14).

Owing to the unexplored roles and bioactive potential of the endophytic fungal community associated with the tissues of *Coleus forskohlii*, the present study was conducted to decipher their diversity and tissue preference over the different seasons. This could pave way for isolating some useful bioactive compounds by fermenting these endophytic fungi.

C. forskohlii, a member of Lamiaceae is a well-known ancient perennial aromatic medicinal plant originating in the Indian sub-continent which occupies an important position in traditional medicine in countries like India, Kenya, Congo, Brazil, Gabon, Somalia, Rwanda and China (15). The wild variety of *C. forskohlii* is extensively cultivated for its fasciculate root tubers as they yield essential bioactive compounds of pharmacological repute (16). Nearly 68 pharmacologically important compounds are isolated from different parts of *C. forskohlii*, some of the most important ones are forskolin, rosmarinic acid, barbatusin, cyclobutatusin and essential oils (17). Harvesting the plant to obtain bioactive compounds is a plant devastating process and hence an alternative eco-friendly method needs to be employed to isolate the essential compounds. One such method is to isolate endophytes from the plant to obtain the necessary compounds.

Isolating bioactive compounds from the endophytic fungi associated with *C. forskohlii* is essential and hence as a first step. The present study was conducted mainly to seasonally isolate the fungal endophytes, understand their spatiotemporal distribution in different tissues of the plant and identify them.

Materials and Methods

Sampling site for collecting plant material

In order to isolate endophytic fungi, the selection of the host plant could be based on the following strategies: plants which grow in unusual environmental conditions, plants growing endemically in the areas hosting rich biodiversity or plants having a history of being used in folk medicine. *Coleus forskohlii* was chosen because of its medicinal value and ethnobotanical use. Based on the unreported studies that were conducted on the diversity of fungi from the soil of St. Joseph's University campus, this location was chosen hypothesizing that we would obtain a rich diversity of fungal endophytes from the host plant.

Leaves, stem and root of *C. forskohlii* were collected from the medicinal plant garden of St. Joseph's University, Bengaluru located in the altitude of 3020 ft/900 m above sea level, longitude: 77°35'49.56" E and latitude: 12° 57'45.72" N, Karnataka, India. The average maximum and minimum temperature and average rainfall during different seasons between May 2020 to April 2021 was 33.07°C and 20.32°C and 57.7 mm rainfall during summer (February to May), 28.4°C and 20.52°C and 173.07 mm rainfall during monsoon (June to October) and 27.6°C and 17.93°C and 33.27 mm rainfall during winter (November to January). Voucher specimen of *C. forskohlii* was submitted to Foundation for Revitalisation of Local Health Traditions for authentication and identification bearing the voucher number FRLH Coll. No. 124372. Healthy and mature plants of *C. forskohlii* were harvested every month, for a period of twelve months, between May 2020–April 2021 to obtain the leaves, stem and roots. The collected plant material was transferred to an aseptic plastic bag, taken to the laboratory, and immediately subjected to surface sterilization.

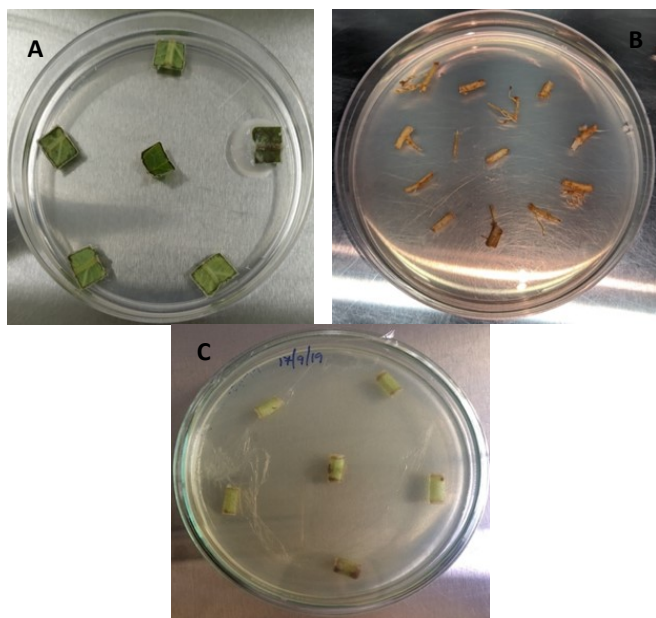
Isolation and identification of fungal endophytes

The plant parts were segregated into leaves, stem and roots and were washed thoroughly under running tap water for 10–15 minutes to get rid of the debris and rinsed with sterile distilled water to wash off the epiphytic microbes. Surface sterilization was done following the methodology of Suryanarayanan *et al.* and Srivastava and Anandrao (18, 19). The standardized surface sterilization protocol is mentioned in Table 1.

The surface sterilized plant materials were dry blotted using a sterile blotting paper. The leaf segments were cut to a size of approximately 5 × 5 mm, the stem was cut to small cylinders of size 6 × 6 mm (Fig. 1) followed by a longitudinal cut to obtain two halves and the roots were cut to small cylinders of size 4 × 4 mm using a flame sterilized scalpel.

Table 1. Standardized protocol for surface sterilization of *C. forskohlii*.

Plant part	Root	Stem	Leaf
70% ethanol	2 min.	30s	30s
Sterile distilled water	Rinse in sterile distilled water		
4% sodium hypochlorite	1 min.	10s	30s
Sterile distilled water	Rinse in sterile distilled water		
70% ethanol	30s	10s	10s
Sterile distilled water	Rinse in sterile distilled water (twice)		

**Fig. 1.** Surface sterilized leaf (A), root (B) & stem bits (C) of *C. forskohlii*.

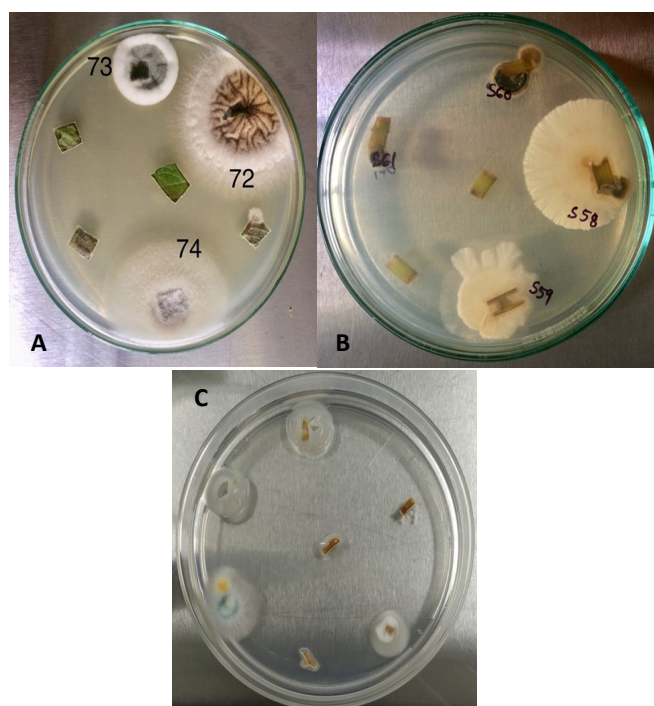
About 35 young leaf segments, 35 old leaf segments, 20 stem cylinders (40 halves) and 30 root cylinders were taken from the plant every month for isolation of endophytic fungi. In total, around 420 young leaf segments, 420 old leaf segments, 240 stem cylinders (480 halves) and around 360 root cylinders were placed on PDA (Potato Dextrose Agar) medium supplemented with chloramphenicol (200 mg/L) in order to prevent the growth of bacteria. The plates were incubated at natural conditions of light and darkness, and were regularly monitored for endophytic fungal growth, the emerging mycelia were sub-cultured on the PDA media for further studies.

The fungal endophytes were then identified based on their morphological features like colony characters, mycelia, structure and arrangement of conidia, and other reproductive structures if any, by using standard manuals and recent research articles (20–29). For microscopic studies, a drop of lactophenol cotton blue stain was added on a clean glass slide, the fungal hyphae with/without reproductive structures were taken on the glass slide containing the stain, a cover slip was placed on the mycelium and the microscopic features were carefully studied. The cultures which failed to sporulate were labelled as sterile mycelia. The fungal cultures were stained using lactophenol cotton blue and studied under a binocular microscope.

Some common fungi that were isolated among these, were chosen for molecular identification by 18S rRNA sequencing-the DNA was extracted by CTAB (cetyltrimethylammonium bromide) method. About 1 g of

the fresh fungal mycelium was taken from the pure cultures grown on PDA media. The fungal mycelium was crushed in a chilled pestle and mortar using the CTAB extraction buffer, the extract was transferred to a tube and the volume was made up to 1 mL using the buffer. DNase-free RNase A (10 mg/mL) was then added and the tubes were incubated for 15 minutes at 37°C then for 30 minutes at 65°C and inverted regularly. 1 mL of phenol:chloroform:isoamylalcohol (25:24:1) was added to the tubes and centrifuged at 10,000 rpm for 10 minutes. The upper layer containing the DNA was transferred to a 2 mL Eppendorf tube containing 2 mL of chloroform:isoamylalcohol (24:1), and then centrifuged at 10,000 rpm for 10 minutes. The upper aqueous layer containing the DNA was then transferred to a fresh Eppendorf tube, equal volumes of 100% isopropyl alcohol and one-tenth 3 M sodium acetate was added to precipitate the DNA. The tubes were then incubated for 30 minutes at room temperature followed by centrifugation for 10 minutes at 10,000 rpm. The DNA pellet was washed by subjecting to centrifugation at 5,000 rpm for 5 minutes using 70% ethanol, air dried and suspended in Tris-EDTA buffer. The DNA was then amplified by Polymerase Chain Reaction (PCR), followed by subjecting the DNA of the PCR product to gel electrophoresis to check its purity (30).

The DNA was sequenced using Sanger's dideoxy method, and lastly the forward and reverse sequences were subjected to NCBI BLAST (National Centre for Biotechnology Information Basic Local Alignment Search Tool) for identifying the fungi (31). The identified sequences were submitted to GenBank in fast-all format and accession numbers were obtained for the same. All isolates were identified and preserved in storage vials in duplicates with 15% glycerol and sterile distilled water for future use.

**Fig. 2.** Endophytic fungi emerging from leaf (A), stem (B) & root bits (C) of *C. forskohlii*.

Statistical analysis

To understand the seasonal diversity of endophytic fungi associated with *C. forskohlii*, the endophyte isolation rate, colonization frequency, relative percentage occurrence and relative species frequency were calculated as per the formulae mentioned below. Shannon-Wiener index, Simpson's Diversity index, Menhinick's index of Evenness, Simpson's Dominance index and Margalef's Richness index were calculated using the formulae below.

Colonization frequency (CF)

The CF% of the endophytic fungi was calculated using the formula given by Hata and Futai (32).

$$CF (\%) = \frac{\text{Number of segments colonized by each endophyte}}{\text{Total number of segments examined}} \times 100 \text{ (Eqn. 1)}$$

Endophytic Isolation Rate (EIR)

The EIR was calculated using the below mentioned formula (33).

$$EIR = \frac{\text{Number of isolates obtained from plant segments}}{\text{Total number of segments incubated}} \text{ (Eqn. 2)}$$

Relative Percentage Occurrence (RPO)

The RPO of the various groups of fungi was calculated using the below formula (34).

$$RPO (\%) = \frac{\text{Number of segments colonized by a group of fungi}}{\text{Total number of segments colonized by all the groups of fungi}} \times 100 \text{ (Eqn. 3)}$$

Relative species frequency (RF)

Relative species frequency of one species in comparison with the other isolated species was calculated using the below mentioned formula (35).

$$RF = \frac{\text{Number of isolates of one species}}{\text{Total number of isolates}} \times 100 \text{ (Eqn. 4)}$$

Fungal diversity index

Simpson's Diversity index: Simpson's diversity index was calculated by using the formula:

$$D = \sum_i n_i(n_i - 1) / N(N - 1) \text{ (Eqn. 5)}$$

where n_i = number of isolates of each species, while N = total number of isolates of all species.

Simpson's Dominance index: It was calculated by using the formula:

$$1 - (\sum_i n_i(n_i - 1) / N(N - 1)) \text{ (Eqn. 6)}$$

where n_i = number of individuals of each species, while N = total number of individuals of all species. Simpson's dominance index is 1-D.

Shannon-Wiener index: Shannon-Wiener index (H) was calculated by using the formula:

$$\sum_i (n_i / N) \cdot \ln (n_i / N) \text{ (Eqn. 7)}$$

where \ln = natural logarithm.

Menhinick's index of Evenness: Species evenness was calculated using the formula:

$$s / \sqrt{\sum_i n_i} \text{ (Eqn. 8)}$$

where S = total number of endophytic species isolated and n_i = sum total of isolates of all the endophytic species.

Margalef's Richness index: Species richness was calculated using the formula:

$$S - 1 / \ln N \text{ (Eqn. 9)}$$

where S = number of species, \ln is natural logarithm, while N = total number of individuals (35).

Results

Isolation and identification of fungal endophytes

About 950 endophytic fungi were isolated from 1,680 plant tissue segments of *Coleus forskohlii* during 2020–2021. Of these, 313, 296, 170 and 171 fungal endophytes were isolated from the old leaves, young leaves, stem (Fig. 2) and roots, respectively. A total of 21 genera of fungal endophytes were obtained. Morphological identification revealed about 55 species of endophytic fungi which are as follows: *Alternaria alternata*, *Amesia cymbiformis*, *Arcopilus flavigenus*, *Aspergillus flavus*, *A. fumigatus*, *A. nidulans*, *A. niger* (Fig. 4), *A. ochraceus*, *A. terreus*, *Chaetomium angustispirale* (Fig. 5), *C. coarctatum*, *C. cochliodes*, *C. globosum*, *C. globosum sensu stricto*, *C. indicum*, *C. madrasense*, *Chaetomium* sp., *C. spinosum*, *C. subspirale*, *C. tectifimeti*, *Cladosporium cladosporioides*, *Cladosporium* sp., *C. sphaerospermum*, *Collariella bostrychodes*, *C. robusta* (Fig. 6), *Colletotrichum endophytica*, *C. falcatum*, *C. fruticola*, *C. gloeosporioides*, *C. siamense*, *Colletotrichum* sp., *C. tropicale*, *C. truncatum*, *C. asianum*, *Epicoccum* sp., *Fusarium chlamydosporum*, *F. equiseti*, *F. solani*, *Fusarium* sp., *F. verticillioides*, *Lasiodiplodia theobromae*, *Mucor circinelloides*, *Nigrospora oryzae*, *Nigrospora* sp., *N. sphaerica*, *Paecilomyces carneus*, *Paecilomyces* sp., *Penicillium chrysogenum*, *P. paradoxum*, *Penicillium* sp., *Phoma herbarum*, *Phoma* sp., *Pseudotorula* sp., *Sarocladium strictum*, *Scopulariopsis brevicaulis*, *Scopulariopsis* sp., *Trichoderma harizianum*, *Trichoderma* sp., *Xylaria acuta*, *X. apoda*, *X. curta*, *X. feejeensis* and *X. grammica* and *Xylaria* sp., (20–29) and the mycelia which did not sporulate were called sterile mycelia. Some of the fungi isolated as a part of this study have been identified by 18S rRNA sequencing and reported in a previous study by Crasta and Raveesha (36) as denoted (*) in Table 4. A phylogenetic tree was also constructed in the above study to understand the phylogenetic affiliations between the fungal endophytes using MEGA X software (37).

Statistical analysis

The total colonization frequency (CF) of fungal endophytes associated with all plant tissues of *C. forskohlii* was found to be 56.54%. The old and young leaves of *C. forskohlii* showed maximum colonization rate of 74.52% and 70.47%, followed by roots with 50.27% and stem showed least CF of 35.41% as indicated in Fig. 7.

The seasons during these 12 months between May 2020–April 2021 were Summer, Monsoon and Winter. Seasons influenced the rate of colonization of endophytic fungi, and it was found that maximum colonization occurred during monsoon with CF of 71%, followed by winter 52.85% and least CF occurred during summer with CF of 40.17% as indicated in Fig. 8.

The RPO of Ascomycota was found to be the highest

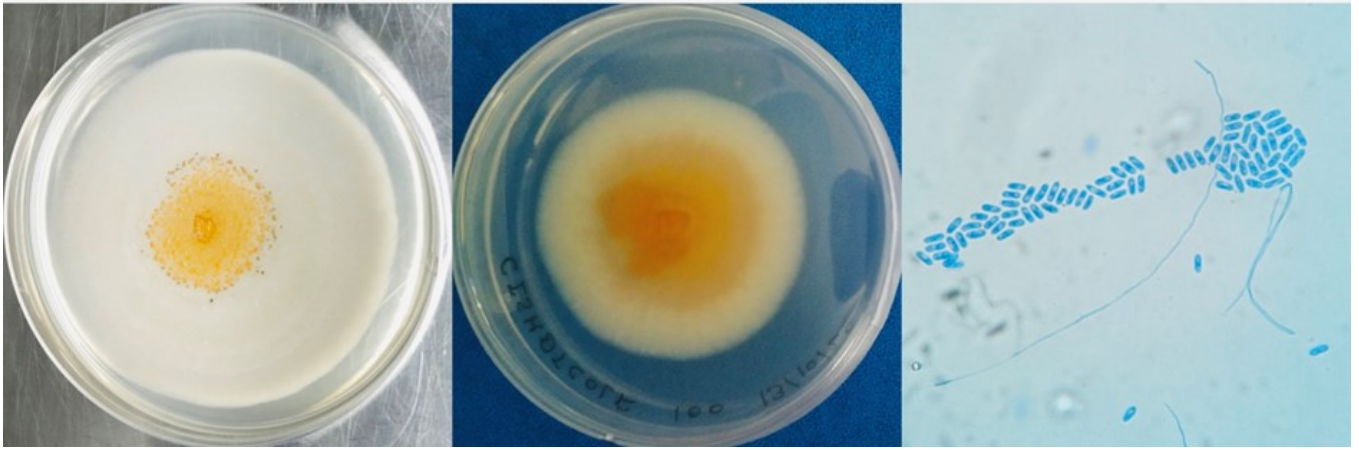


Fig. 3. Endophytic fungus isolated from old leaf-CISHDTCOLF98-*Colletotrichum gloeosporioides*.

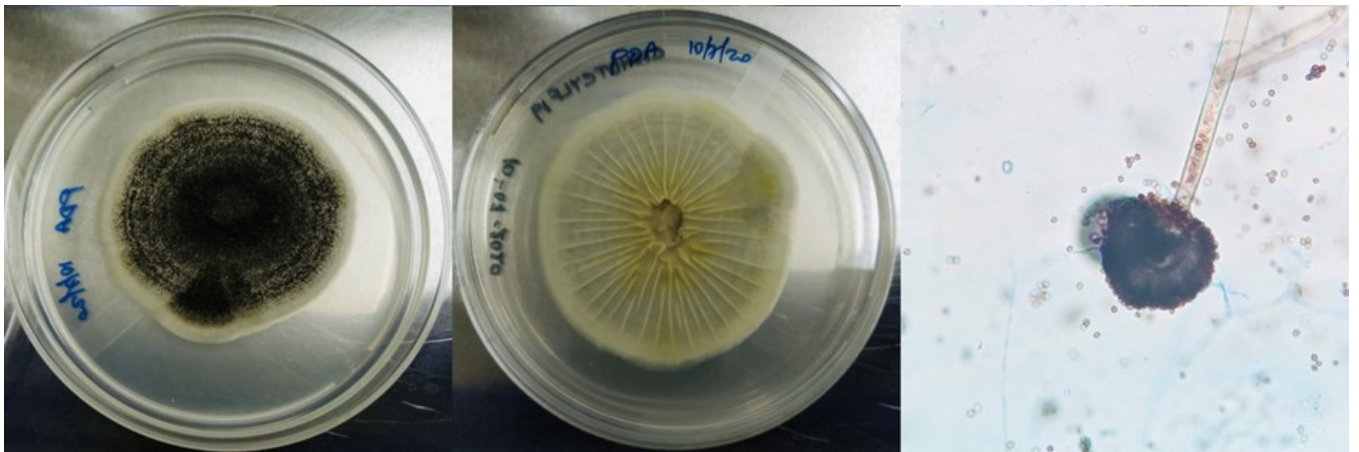


Fig. 4. Endophytic fungus isolated from young leaf-CISHDTCYLF19-*Aspergillus niger*.

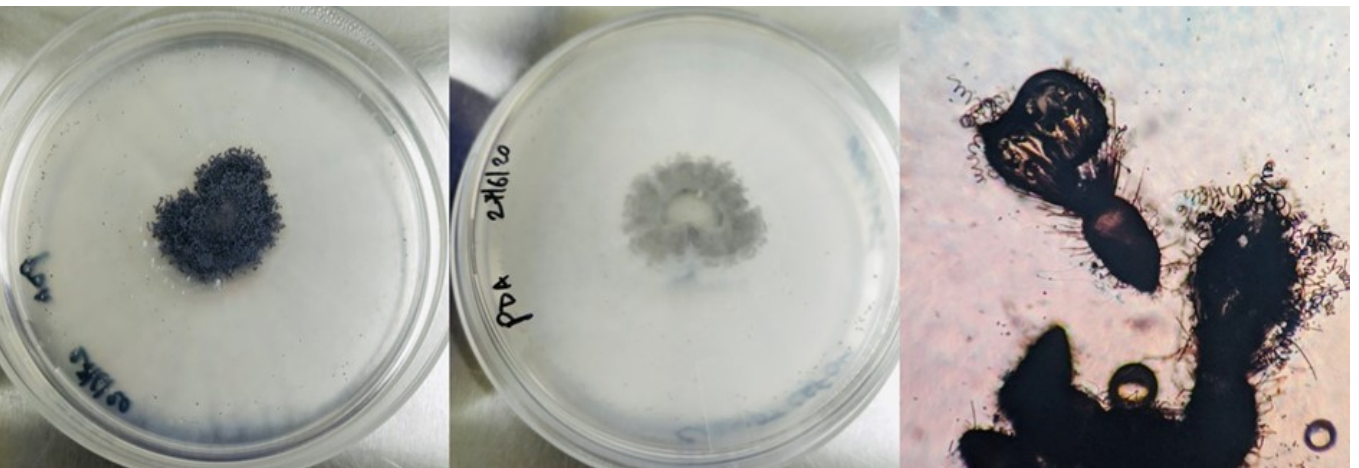


Fig. 5. Endophytic fungus isolated from stem-CISHDTCSF04-*Chaetomium angustispirale*.

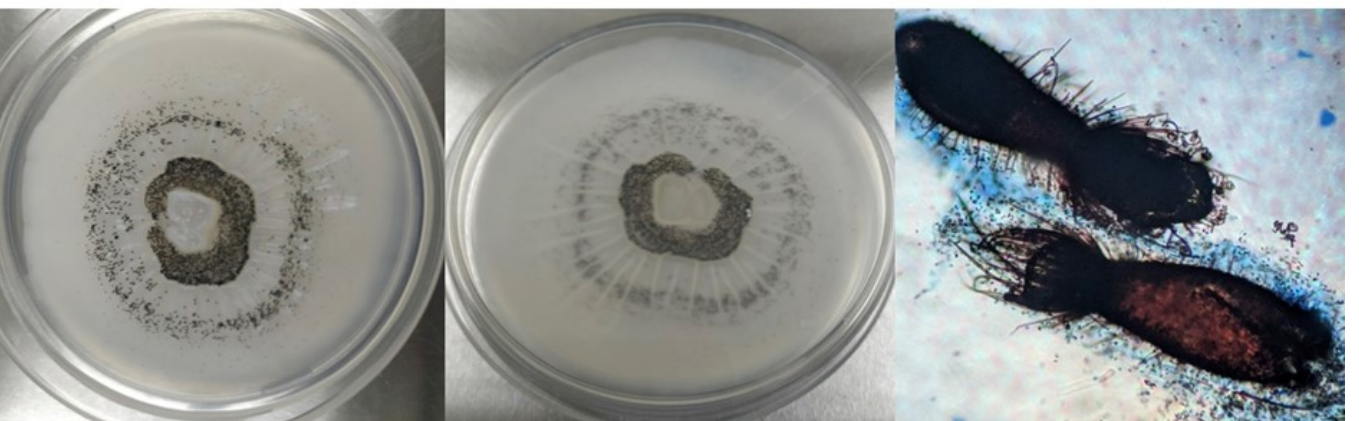


Fig. 6. Endophytic fungus isolated from root-CISHDTCRF22-*Collariella robusta*.

Table 4. Diversity, species richness and evenness of endophytic fungi associated with the different tissues of *C. forskohlii* in different seasons.

Index	By tissue type				By season		
	Old leaf	Young leaf	Stem	Root	Summer	Winter	Monsoon
Simpson's Diversity index	0.049	0.042	0.093	0.0416	0.03	0.036	0.05
Simpson's Dominance index	0.95	0.957	0.906	0.958	0.969	0.963	0.949
Shannon- Wiener index	3.227	3.215	2.651	3.238	3.581	3.494	3.534
Menhinick's index of Evenness	2.035	1.744	1.687	2.447	3.333	3.222	2.826
Margalef's Richness index	6.091	5.096	4.089	6.029	9.047	8.699	9.986
CF%	74.52	70.47	35.41	50.27	40.17	52.85	71

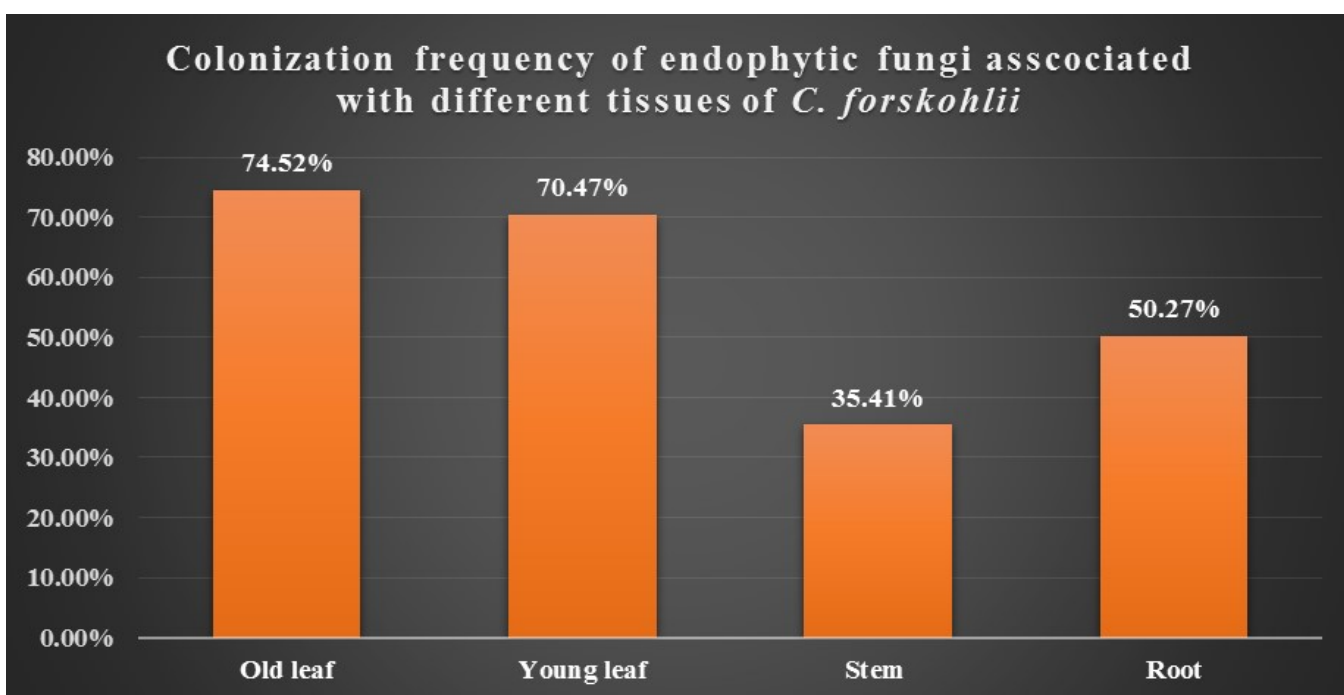


Fig. 7. Colonization frequency of endophytic fungi associated with *C. forskohlii*.

with 81.26%, Sterile mycelia with 16.10% RPO and the RPO of Mucoromycota was found to be 2.63% and is represented in the Fig. 9. The EIR is interpreted in the Table 2. The RF for each species of fungal endophytes is

given in Table 3.

The fungal diversity index of the entire plant was as follows-Simpson's Diversity index was found to be 0.022 and Simpson's Dominance index was found to be 0.977, Shannon-Wiener index was found to be 4.203, Menhinick's

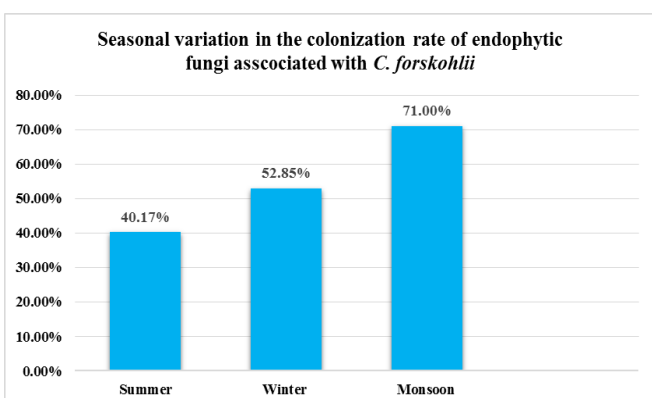


Fig. 8. Seasonal colonization rate of endophytic fungi associated with *C. forskohlii*.

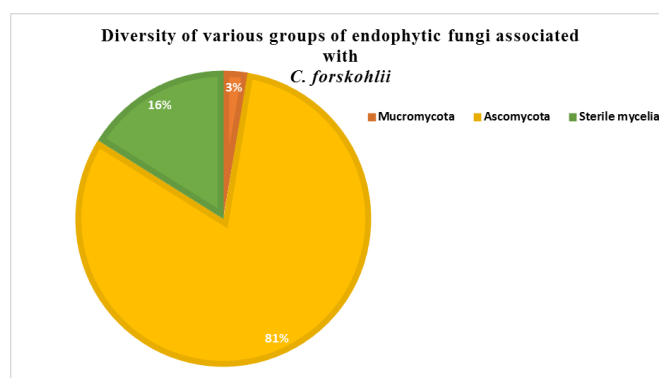


Fig. 9. Relative percentage occurrence of fungal endophytes associated with *C. forskohlii*.

Table 3. The relative species frequency (RF) of endophytic fungi associated with different plant tissues of *C. forskohlii*.

List of endophytic fungi	Old leaf fungi	Young leaf fungi	Root fungi	Stem fungi	Total	RF
<i>Alternaria alternata</i> *MT770930	7				7	0.73
<i>Amesia cymbiformis</i>	9				9	0.9
<i>Arcopilus flavigenus</i>			2		2	0.21
<i>Aspergillus flavus</i> * MT740749		1	8		9	0.9
<i>Aspergillus fumigatus</i>			2	4	6	0.63
<i>Aspergillus nidulans</i>		10			10	1.05
<i>Aspergillus niger</i> * MT750290	11	9	11		31	3.26
<i>Aspergillus ochraceus</i> * MT762907			6		6	0.63
<i>Aspergillus terreus</i>		11			11	1.15
<i>Chaetomium angustispirale</i>				4	4	0.42
<i>Chaetomium cochliodes</i>	14				14	1.47
<i>Chaetomium coarctatum</i>			7	4	11	1.15
<i>Chaetomium globosum</i> * MT770936	15	4	5	32	56	6.31
<i>Chaetomium globosum sensu stricto</i>	2				2	0.21
<i>Chaetomium madrasense</i>	1				1	0.1
<i>Chaetomium</i> sp.	3	7	1	7	18	1.89
<i>Chaetomium spinosum</i>		3	3	7	14	1.36
<i>Chaetomium subspirale</i>		7			7	0.73
<i>Chaetomium tectifimeti</i>			4		4	0.42
<i>Cladosporium cladosporioides</i>				5	5	0.52
<i>Cladosporium</i> sp. * MT770944	1			1	2	0.21
<i>Cladosporium sphaerospermum</i>	1				1	0.1
<i>Collariella bostrychodes</i>	16	10	15	25	66	6.94
<i>Collariella robusta</i>	14	17	5		36	3.78
<i>Colletotrichum coccodes</i> *MT750014				3	3	0.31
<i>Colletotrichum endophytica</i>		15			15	1.57
<i>Colletotrichum falcatum</i>	8				8	0.84
<i>Colletotrichum fruticola</i>	8	11	3		22	2.31
<i>Colletotrichum gloeosporioides</i>	15	10	8	4	37	3.89
<i>Colletotrichum siamense</i>	1				1	0.1
<i>Colletotrichum</i> sp. *MT762814	3	2			5	0.52
<i>Colletotrichum tropicale</i>			4		4	0.42
<i>Colletotrichum truncatum</i> *MT762340			5		5	0.52
<i>Colletotrichum asianum</i>	14				14	1.47
<i>Epicoccum</i> sp.				3	3	0.31
<i>Fusarium chlamydosporum</i>	14	20			34	3.57
<i>Fusarium equiseti</i>				10	10	1.05
<i>Fusarium solani</i>			4		4	0.42
<i>Fusarium</i> sp.	11		3		14	1.47
<i>Fusarium verticillioides</i>	10	12			22	2.31
<i>Lasiodiplodia theobromae</i>	3				3	0.31
<i>Mucor circinelloides</i>		23	2		25	2.63
<i>Nigrospora oryzae</i> *MT762908	10	10		2	22	2.31
<i>Nigrospora</i> sp.		4			4	0.42
<i>Nigrospora sphaerica</i>	13				13	1.36
<i>Paecilomyces carneus</i>		5	1	3	9	0.9
<i>Paecilomyces</i> sp.			3		3	0.31
<i>Penicillium chrysogenum</i>			7		7	0.73
<i>Penicillium paradoxum</i>			1		1	0.1
<i>Penicillium</i> sp. *MT770931	1	9			10	1.05
<i>Phoma herbarum</i>	1				1	0.1
<i>Phoma</i> sp.	4	1		1	6	0.63
<i>Psuedotorula</i> sp.			3		3	0.31
<i>Sarocladium</i> sp.			4		4	0.42
<i>Sarocladium strictum</i>		5	9		14	1.47
<i>Scopulariopsis brevicaulis</i> *MT770935			6		6	0.63
<i>Scopulariopsis</i> sp.			4	7	11	1.15
Sterile hyaline mycelia	49	25	16	20	114	12
Sterile septate mycelia	8	14	13	4	39	4.1
<i>Trichoderma harizianum</i>	6	15			21	2.21
<i>Trichoderma</i> sp.			3		3	0.31
<i>Xylaria acuta</i>	11				11	1.15
<i>Xylaria apoda</i>	3	14			17	1.78
<i>Xylaria curta</i>	11	9	3	6	29	3.05
<i>Xylaria feejeensis</i>	7			4	11	1.15
<i>Xylaria grammica</i>	8	8		11	27	2.84
<i>Xylaria</i> sp.				3	3	0.31
Total	313	296	171	170	950	

Table 2. Colonization frequency (CF) & endophyte isolation rate (EIR) of endophytic fungi associated with the different tissues of *C. forskohlii* in different seasons.

Plant part	Sampling season	No. of tissues studied	No. of isolates obtained	CF%	EIR
Old leaf	Summer	140	77	55	0.55
	Winter	105	75	71.42	0.71
	Monsoon	175	161	92	0.92
Young leaf	Summer	140	77	55	0.55
	Winter	105	71	67.61	0.67
	Monsoon	175	148	84.57	0.84
Stem	Summer	160	30	18.75	0.18
	Winter	120	39	32.5	0.32
	Monsoon	200	101	50	0.50
Root	Summer	120	43	35.83	0.35
	Winter	90	39	43.33	0.43
	Monsoon	150	89	59.33	0.59

index of Evenness was found to be 4.03 and Margalef's Richness index was found to be 18.26. The fungal diversity indexes of the different tissues during different seasons are mentioned in Table 4.

Discussion

A recent estimate of the number of species belonging to the kingdom fungi ranges from 2.2 to 3.8 million species, of which about 150,000 species have been described so far (38). Fungi have established associations with various life forms on Earth, including soil, dead and decaying matter, animals, and plants, demonstrating their ability to survive, absorb nutrients, and reproduce. One of the beneficial symbiotic relationships that fungi exhibit is their association with plants as endophytes. It is reported that fungi have been found in association with almost all groups of plants, and these symbiotic relationships profoundly impact the plant's fitness, ecology, and evolution. Fungi shape the internal communities of plants by producing secondary metabolites and also have significant effects on the diversity of other organisms such as insects, nematodes, and bacteria associated with the host plant (39–41).

Coleus forskohlii, an important aromatic medicinal plant, has been extensively used in folk remedies for centuries across many tropical countries. Due to increased demand, the overharvesting of *C. forskohlii* has led to the endangerment of its population. In recent decades, researchers have turned their attention to plant protection and preservation using biological approaches. Fungal endophytes, known for their ability to protect plants against both abiotic and biotic stresses, play a crucial role in enhancing plant growth and development, safeguarding them from extreme environmental conditions. Understanding the diversity of fungal endophytes associated with different tissues of *C. forskohlii* is essential. This knowledge can aid in isolating some of the most beneficial fungal endophytes capable of producing

bioactive molecules similar to those found in the host plant. By doing so, we can harness these bioactive compounds without further endangering the plant through overharvesting. As a first step, this study was conducted over a period of one year to comprehensively understand the diversity and spatiotemporal distribution of endophytic fungi associated with *C. forskohlii*. This research marks the inaugural report on the seasonal diversity of fungal endophytes associated with *C. forskohlii*.

A total of 950 endophytic fungi were isolated from 1680 segments of *C. forskohlii*, indicating a colonization frequency of 56.54%. These results align with the percentage of fungal communities isolated and reported in studies on *Pinus taeda* (42). When considering the seasonal diversity of fungal endophytes associated with *C. forskohlii*, it was observed that the maximum isolates were obtained during the monsoon season, with a CF of 71%. In the winter, the CF was 52.85%, while the summer season exhibited the lowest CF of 40.17%. This trend is consistent with earlier studies (43), suggesting that fungal endophytes spore tend to germinate and colonize host tissues more readily when the humidity is moderate to high, and temperature range between 20–30°C. Moreover, the majority of isolates were found in the old/mature leaves, with a CF of 74.52%. This finding supports the results by Hilarino *et al.* and Arnold *et al.* (44, 45).

Fungal endophytes were identified using both morphology and 18S rRNA gene sequencing. In total, 21 genera and 55 species were identified, aligning with similar studies conducted on medicinal plants such as *Salvia multicaulis* (46), *Citrus reticulata* (35), *etc.* Notably, 81.26% of the isolates belonged to Ascomycota, a consistent trend found in many other studies (47), while 2.63% were classified under Mucoromycota. These endophytes fell into 10 different orders spanning three classes: Sordariomycetes (Hypocreales, Xylariales, Microascales, Trichosphaeriales, Glomerellales and Sordariales), Dothiomycetes (Pleosporales, Capnodiales, Botryosphaeriales) and Eurotiomycetes (Eurotiales).

Among these, Sordariomycetes were predominant, constituting 52% of the identified endophytes, followed by Eurotiomycetes at 9.6%, and Dothiomycetes (8.8%).

Simpson's Diversity index of 0.022 and Simpson's Dominance index of 0.977 highlights the high diversity of fungal endophytes in this location. Additionally, the Shannon-Wiener index of 4.203 and Margalef's Richness index of 18.26 underscores the extensive species richness and diversity among the fungal endophytes. Some of the predominant genera of fungal endophytes associated with *C. forskohlii* include *Chaetomium*, *Collariella*, *Colletotrichum*, *Aspergillus*, *Fusarium*, *Nigrospora* and *Xylaria*, aligns with finding from other researchers (48, 49). The predominant species include *Sterile mycelia* (narrow width), *Chaetomium globosum*, *Collariella bostrychodes*, *C. robusta*, *Colletotrichum gloeosporioides*, *Fusarium chlamydosporum*, *Aspergillus niger*, *Xylaria curta*, *X. grammica*, and *Mucor circinelloides*. These finding suggests the potential for sourcing bioactive compounds of pharmaceutical value from these fungi. Among the isolated species, *Trichoderma harizianum*, *C. globosum*, *C. bostrychodes*, *C. gloeosporioides*, sterile hyaline mycelia and *X. curta* were found distributed in all parts of *C. forskohlii*, indicating their ecological and physiological adaptations within the tissues of *C. forskohlii* during different seasons. Moreover, species like *C. globosum*, *T. harizianum* and *X. curta*, identified in our study, have been recognized as plant growth promoting endophytes (50). These endophytes are also employed as potential biocontrol agents in agriculture, safeguarding plants against pathogenic microorganisms such as *Rhizoctonia solani*, *Trichoderma* spp., *Gliocladium* spp., *Chaetomium* spp., *Bacillus* spp., *Pseudomonas* spp. and *Aspergillus* spp. These findings underscore the potential benefits that the fungal endophytes could offer *C. forskohlii*. They not only contribute to its growth and development, but maintain a healthy internal environment, augmenting its medicinal value.

Conclusion

In conclusion, isolating endophytic fungi from the healthy tissues of the *Coleus forskohlii* could help minimize the overharvesting of this medicinally important plant. We found that fungi belonging to Mucoromycota and Ascomycota predominantly colonize the healthy tissues of *C. forskohlii*. Moreover, changes in environmental conditions during different seasons can affect the rate of isolation of the fungal endophytes. This study has also identified the optimal season for isolating endophytes from *C. forskohlii*, which could facilitate the examination and discovery of bioactive compounds with pharmaceutical potential. Notably, sterile hyaline mycelium, *Chaetomium globosum*, *Collariella bostrychodes* and *Fusarium chlamydosporum* were the most dominant species of endophytic fungi associated with *C. forskohlii*. This suggests that these isolates may contribute to the medicinal properties of *C. forskohlii* by producing essential secondary metabolites. Further studies on these isolated fungal endophytes could decipher their antagonistic

potential, enhancing the antimicrobial properties of the secondary metabolites they produce.

Acknowledgements

We wish to thank Dr. N. Praveen Kumar (Department of Botany, SJU) for his help in identifying fungi, Dr. Grace Prabhakar (Department of Biotechnology, SJU), Mr. Cyril (Department of Commerce, SJU) and Mr. Leo Richard Crasta for helping analyse the data, and St. Joseph's University for its constant support.

Authors' contributions

KAR designed the experiment, shared his expertise, provided guidance and support. GLC carried out the research, wrote the article, analysed and interpreted the results.

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

Ethical issues: None.

References

- Li WC, Zhou J, Guo SY, Guo LD. Endophytic fungi associated with lichens in Baihua mountain of Beijing, China. *Fungal Divers.* 2007 Apr 30;25:69-80.
- Zhang T, Zhang YQ, Liu HY, Wei YZ, Li HL, Su J, Zhao LX, Yu LY. Diversity and cold adaptation of culturable endophytic fungi from bryophytes in the Fildes Region, King George Island, maritime Antarctica. *FEMS Microbiology Letters.* 2013 Apr 1;341(1):52-61. <https://doi.org/10.1111/1574-6968.12090>
- Kumaresan V, Veeramohan R, Bhat MM, Sruthi K, Ravindran CP. Fungal endophyte assemblages of some Pteridophytes from Mahe, India. *World Journal of Science and Technology.* 2013;3(1):7-10.
- Arnold AE, Lutzoni F. Diversity and host range of foliar fungal endophytes: Are tropical leaves biodiversity hotspots? *Ecology.* 2007 Mar;88(3):541-49. <https://doi.org/10.1890/05-1459>
- Chetia H, Kabiraj D, Bharali B, Ojha S, Barkataki MP, Saikia D, Singh T, Mosahari PV, Sharma P, Bora U. Exploring the benefits of endophytic fungi via omics. *Advances in Endophytic Fungal Research: Present Status and Future Challenges.* 2019;51-81. https://doi.org/10.1007/978-3-030-03589-1_4
- Hardoim PR, Van Overbeek LS, Berg G, Pirttilä AM, Compant S, Campisano A, Döring M, Sessitsch A. The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiology and Molecular Biology Reviews.* 2015 Sep;79(3):293-320. <https://doi.org/10.1128/MMBR.00050-14>
- De Bary A. Morphologie und physiologie der pilze, flechten und myxomyceten. Engelmann; 1866. <https://doi.org/10.5962/bhl.title.120970>
- Nalini MS, Sunayana N, Prakash HS. Endophytic fungal diversity in medicinal plants of Western Ghats, India. *International Journal of Biodiversity.* 2014 Oct; 2014:1-9. <https://doi.org/10.1155/2014/494213>

9. Bezerra JD, Nascimento CC, Barbosa RD, da Silva DC, Svedese VM, Silva-Nogueira EB, Gomes BS, Paiva LM, Souza-Motta CM. Endophytic fungi from medicinal plant *Bauhinia forficata*: Diversity and biotechnological potential. *Brazilian Journal of Microbiology*. 2015 Mar 31;46:49-57. <https://doi.org/10.1590/S1517-838246120130657>
10. Chutulo EC, Chalannavar RK. Endophytic mycoflora and their bioactive compounds from *Azadirachta indica*: A comprehensive review. *Journal of Fungi*. 2018 Mar 24;4(2):42. <https://doi.org/10.3390/jof4020042>
11. Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW. Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*. 1997 Oct 1;43(10):895-914. <https://doi.org/10.1139/m97-131>
12. Suman A, Yadav AN, Verma P. Endophytic microbes in crops: Diversity and beneficial impact for sustainable agriculture. *Microbial Inoculants in Sustainable Agricultural Productivity: Vol. 1: Research Perspectives*. 2016;117-43. DOI:10.1007/978-81-322-2647-5_7
13. Wu H, Yang HY, You XL, Li YH. Diversity of endophytic fungi from roots of *Panax ginseng* and their saponin yield capacities. *SpringerPlus*. 2013 Dec;2(1):1-9. <https://doi.org/10.1186/2193-1801-2-107>
14. Juybari HZ, Tajick Ghanbary MA, Rahimian H, Karimi K, Arzanlou M. Seasonal, tissue and age influences on frequency and biodiversity of endophytic fungi of *Citrus sinensis* in Iran. *Forest Pathology*. 2019 Dec;49(6):e12559. <https://doi.org/10.1111/efp.12559>
15. Lukhoba CW, Simmonds MS, Paton AJ. *Plectranthus*: A review of ethnobotanical uses. *Journal of Ethnopharmacology*. 2006 Jan 3;103(1):1-24. <https://doi.org/10.1016/j.jep.2005.09.011>
16. Srivastava SK, Chaubey M, Khatoon S, Rawat AK, Mehrotra S. Pharmacognostic evaluation of *Coleus forskohlii*. *Pharmaceutical Biology*. 2002 Jan 1;40(2):129-34. <https://doi.org/10.1076/phbi.40.2.129.5842>
17. Alasbahi RH, Melzig MF. *Plectranthus barbatus*: A review of phytochemistry, ethnobotanical uses and pharmacology—part 1. *Planta Medica*. 2010 May;76(07):653-61. <https://doi.org/10.1055/s-0029-1240898>
18. Suryanarayanan TS, Murali TS, Venkatesan G. Occurrence and distribution of fungal endophytes in tropical forests across a rainfall gradient. *Canadian Journal of Botany*. 2002 Aug 1;80(8):818-26. <https://doi.org/10.1139/b02-06919>
19. Srivastava AP, Anandrao RK. Antimicrobial potential of fungal endophytes isolated from leaves of *Prosopis juliflora* (SW.) DC. an important weed. *International Journal of Pharmacy and Pharmaceutical Sciences*. 2015;7(12):128-36.
20. Wang XW, Houbraken J, Groenewald JZ, Meijer M, Andersen B, Nielsen KF, Crous PW, Samson RA. Diversity and taxonomy of *Chaetomium* and *Chaetomium*-like fungi from indoor environments. *Studies in Mycology*. 2016 Jun 1;84:145-224. <https://doi.org/10.1016/j.simyco.2016.11.005>
21. Hao Y, Aluthmuhandiram JV, Chethana KT, Manawasinghe IS, Li X, Liu M, Hyde KD, Phillips AJ, Zhang W. *Nigrospora* species associated with various hosts from Shandong Peninsula, China. *Mycobiology*. 2020 May 3;48(3):169-83. <https://doi.org/10.1080/12298093.2020.1761747>
22. Costa MM, Silva BA, Moreira GM, Pfenning LH. *Colletotrichum falcatum* and *Fusarium* species induce symptoms of red rot in sugarcane in Brazil. *Plant Pathology*. 2021 Oct;70(8):1807-18. <https://doi.org/10.1111/ppa.13423>
23. Wang L, Wang N, Yu J, Wu J, Liu H, Lin K, Zhang Y. Identification of pathogens causing alfalfa *Fusarium* root rot in Inner Mongolia, China. *Agronomy*. 2023 Feb 3;13(2):456. <https://doi.org/10.3390/agronomy13020456>
24. Chen J, Zhang LC, Xing YM, Wang YQ, Xing XK, Zhang DW, Liang HQ, Guo SX. Diversity and taxonomy of endophytic xylariaceous fungi from medicinal plants of *Dendrobium* (Orchidaceae). *PloS one*. 2013 Mar 5;8(3):e58268. <https://doi.org/10.1371/journal.pone.0058268>
25. Rahman M, Borah SM, Borah PK, Bora P, Sarmah BK, Lal MK, Tiwari RK, Kumar R. Deciphering the antimicrobial activity of multifaceted rhizospheric biocontrol agents of solanaceous crops viz., *Trichoderma harzianum* MC2 and *Trichoderma harzianum* NBG. *Frontiers in Plant Science*. 2023 Mar 3;14:1141506. <https://doi.org/10.3389/fpls.2023.1141506>
26. Wang XW, Lombard L, Groenewald JZ, Li J, Videira SI, Samson RA, Liu XZ, Crous PW. Phylogenetic reassessment of the *Chaetomium globosum* species complex. *Persoonia-Molecular Phylogeny and Evolution of Fungi*. 2016 Jun 30;36(1):83-133. <https://doi.org/10.3767/003158516X689657>
27. Ma X, Chomnunti P, Doilom M, Daranagama DA, Kang J. Multigene phylogeny reveals endophytic xylariales novelties from *Dendrobium* species from Southwestern China and Northern Thailand. *Journal of Fungi*. 2022 Feb 28;8(3):248. <https://doi.org/10.3390/jof8030248>
28. Weir BS, Johnston PR, Damm U. The *Colletotrichum gloeosporioides* species complex. *Studies in Mycology*. 2012 Sep 1;73:115-80. <https://doi.org/10.3114/sim0011>
29. Manoharachary C, Atri NS, Prameela Devi T, Deeba Kamil S, Singh K, Singh AP. *Bilgrami's Fungi of India List and References (1988-2020)*. New Delhi: Today and Tomorrow's Printers and Publishers; 2022.
30. Cubero OF, Crespo AN, Fatehi J, Bridge PD. DNA extraction and PCR amplification method suitable for fresh, herbarium-stored, lichenized and other fungi. *Plant Systematics and Evolution*. 1999 Sep;216:243-49. <https://doi.org/10.1007/BF01084401>
31. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. *Proceedings of the National Academy of Sciences*. 1977 Dec;74(12):5463-67. <https://doi.org/10.1073/pnas.74.12.5463>
32. Hata K, Futai K. Endophytic fungi associated with healthy pine needles and needles infested by the pine needle gall midge, *Thecodiplosis japonensis*. *Canadian Journal of Botany*. 1995 Mar 1;73(3):384-90. <https://doi.org/10.1139/b95-040>
33. Petrini O, Stone J, Carroll FE. Endophytic fungi in evergreen shrubs in western Oregon: A preliminary study. *Canadian Journal of Botany*. 1982 Jun 1;60(6):789-96. <https://doi.org/10.1139/b82-102>
34. Suryanarayanan TS, Thennarasan S. Temporal variation in endophyte assemblages of *Plumeria rubra* leaves. *Fungal Diversity*. 2004 Feb 1;15:197-204.
35. Sadeghi F, Samsampour D, Seyahooei MA, Bagheri A, Soltani J. Diversity and spatiotemporal distribution of fungal endophytes associated with *Citrus reticulata* cv. Siyahoo. *Current Microbiology*. 2019 Mar 15;76:279-89. <https://doi.org/10.1007/s00284-019-01632-9>
36. Crasta GL, Raveesha KA. Molecular identification of endophytic fungi associated with *Coleus forskohlii* (Willd.) Briq. *Journal of Applied Biology and Biotechnology*. 2021 Nov 10;9(6):162-72. <https://doi.org/10.7324/JABB.2021.96021>
37. Kumar S, Stecher G, Li M, Knyaz C, Tamura K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution*. 2018 Jun;35(6):1547. <https://doi.org/10.1093/molbev/msy096>
38. Hyde KD. The numbers of fungi. *Fungal Diversity*. 2022 May;114(1):1-. <https://doi.org/10.1007/s13225-022-00507-y>
39. Arnold AE, Henk DA, Eells RL, Lutzoni F, Vilgalys R. Diversity

- and phylogenetic affinities of foliar fungal endophytes in loblolly pine inferred by culturing and environmental PCR. *Mycologia*. 2007 Mar 1;99(2):185-206. <https://doi.org/10.1080/15572536.2007.11832578>
40. U'Ren JM, Lutzoni F, Miadlikowska J, Arnold AE. Community analysis reveals close affinities between endophytic and endolichenic fungi in mosses and lichens. *Microbial Ecology*. 2010 Aug;60:340-53. <https://doi.org/10.1007/s00248-010-9698-2>
 41. Rodriguez RJ, White Jr JF, Arnold AE, Redman AR. Fungal endophytes: Diversity and functional roles. *New Phytologist*. 2009 Apr;182(2):314-30. <https://doi.org/10.1111/j.1469-8137.2009.02773.x>
 42. Wang L, Ren L, Li C, Gao C, Liu X, Wang M, Luo Y. Effects of endophytic fungi diversity in different coniferous species on the colonization of *Sirex noctilio* (Hymenoptera: Siricidae). *Scientific Reports*. 2019 Mar 25;9(1):5077. <https://doi.org/10.1038/s41598-019-41419-3>
 43. Naik BS, Shashikala J, Krishnamurthy YL. Diversity of fungal endophytes in shrubby medicinal plants of Malnad region, Western Ghats, Southern India. *Fungal Ecology*. 2008 May 1;1(2-3):89-93. <https://doi.org/10.1016/j.funeco.2008.05.001>
 44. Hilarino MP, Silveira FA, Oki Y, Rodrigues L, Santos JC, Corrêa Junior A, Fernandes GW, Rosa CA. Distribution of the endophytic fungi community in leaves of *Bauhinia brevipes* (Fabaceae). *Acta Botanica Brasiliica*. 2011;25:815-21. <https://doi.org/10.1590/S0102-33062011000400008>
 45. Arnold AE, Maynard Z, Gilbert GS. Fungal endophytes in dicotyledonous neotropical trees: Patterns of abundance and diversity. *Mycological Research*. 2001 Dec;105(12):1502-07. <https://doi.org/10.1017/S095375620100495646>
 46. Jahromi MS, Azizi A, Soltani J. Diversity and spatiotemporal distribution of fungal endophytes associated with *Salvia multicaulis*. *Current Microbiology*. 2021 Apr; 78:1432-47. <https://doi.org/10.1007/s00284-021-02430-y>
 47. Sakayaroj J, Preedanon S, Supaphon O, Jones EB, Phongpaichit S. Phylogenetic diversity of endophyte assemblages associated with the tropical seagrass *Enhalus acoroides* in Thailand. *Fungal Diversity*. 2010 May;42:27-45. <https://doi.org/10.1007/s13225-009-0013-9>
 48. Hamzah TN, Lee SY, Hidayat A, Terhem R, Faridah-Hanum I, Mohamed R. Diversity and characterization of endophytic fungi isolated from the tropical mangrove species, *Rhizophora mucronata* and identification of potential antagonists against the soil-borne fungus, *Fusarium solani*. *Frontiers in Microbiology*. 2018 Jul 25;9:1707. <https://doi.org/10.3389/fmicb.2018.01707>
 49. Khalil AM, Hassan SE, Alsharif SM, Eid AM, Ewais EE, Azab E, Gobouri AA, Elkelish A, Fouda A. Isolation and characterization of fungal endophytes isolated from medicinal plant *Ephedra pachyclada* as plant growth-promoting. *Biomolecules*. 2021 Jan 22;11(2):140. <https://doi.org/10.3390/biom1102014050>
 50. Rashmi A. *Chaetomium globosum*: A potential biocontrol agent and its mechanism of action. *Indian Phytopathology*. 2015;68(1):8-24.