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Diversity analysis and seasonal variation of endophytic fungi from *Santalum album*

Basavaraju Sumanth, Balagangadharaswamy Shobha, Krishnappa Rahul, Marappa Rajganesh, Chowdappa Srinivas*
Department of Microbiology and Biotechnology, Jnana Bharathi Campus, Bangalore University, Bangalore, Karnataka, India

Abstract
Given the astonishing number of microbial organisms known to exist, measuring microbial diversity is one of the most difficult tasks in contemporary microbiology. Endophytes make up a significant part of microbial diversity. In the present research, the *Santalum album*, a significant plant with major ethnobotanical uses, was analyzed for the presence of endophytic fungi from the Bisle region, Western Ghats, Karnataka, India, during all three seasons of 2017. Normal isolation procedures were used to separate fungal endophytes from healthy plant sections, including leaves, roots, and petioles. Fungi were isolated and grouped based on colony morphology and identified based on mycelia shape and structure, sexual and asexual Reproductive characters, attachment of spores, and cultural conditions. A total of 54 fungal isolates were obtained from 4 fragments. Leaf fragments had the highest isolation frequency (0.65) during winter, and summer's petiole fragments had the least isolation frequency (0.15). *Cochliobolus* sp. and *Curvularia* sp. commonly appeared in all seasons. Among all endophytic fungi classes, *Deuteromycetes* were leading over other fungal classes. Endophytic fungi were found to have a rich diversity of Shannon-Weiner and Simpson's indices. These indices point to an even and stable distribution of different fungal isolates. The findings contribute to our knowledge of the diversity of endophytic fungi present in host plant tissues.

Keywords: endophytic fungi, microbial diversity, ethnobotanical, isolation frequency

Introduction

The endophytes are microbes that colonize plant tissues in large numbers. The word "endophyte" was coined in the nineteenth century [1]. This term refers to bacteria, fungi, actinomycetes, and algae that live their entire lives or parts of their lives in the symplast or apoplast region of healthy plant tissues without causing disease or clinical symptoms. Endophytes are classified into three categories based on their nature: (1) pathogens from another host that are non-pathogenic in their endophytic relationship; (2) non-pathogenic microbes; and (3) pathogens that have been non-pathogenic but are still capable of invasion through selection methods or genetic alteration [2, 3, 4].

Given the enormous number of microbial organisms known to exist, measuring microbial diversity is one of the most difficult tasks in contemporary microbiology [5]. Based on a 33:1 ratio of fungal to plant organisms, 1,500,000 species of fungi are known to exist [6]. Fungal endophytes are one of the most numerous and widespread classes of organisms [7]. Plants, regardless of their taxonomic affiliation or environmental preferences, have fungal endophytes colonizing their internal tissues. Many influences, including geographic locations, climatic patterns, seasonality, host plant identification, structure and diversity of surrounding plants, morphology, and specificity of the colonized tissues, affect fungal populations' structure and composition [8, 9].

Even though endophytic fungi are omnipresent in plants, the grade of their contribution to fungal biodiversity and their interactions with hosts are still unknown [10]. There have been several investigations into the diversity and colonization of endophytes. Even though tropical and subtropical rainforests have the highest diversity of endophytic microbes, their biodiversity in tropical countries is still poorly understood [11]. It has been suggested that different plants' bioactivity is attributed to the endophytes that live with them [12].

Endophytes, plant parasites, and mycorrhizae colonize plant tissues through various processes that include host identification, spore germination, epidermis penetration, and tissue colonization [13, 14]. Airborne spores and seed transmission, and propagule transmission by insect vectors are commonly thought to be the inoculum basis for fungal endophytes [15]. Colonization of host plants by endophytic fungi contributes to host plant adaptation to biotic and abiotic stress factors [16]. It is of particular interest that host plant immunity to biotic stress has in many cases been associated with fungal natural products [17]. In many cases, these have been implicated in protecting the host plant against phytopathogenic microorganisms [18].

Endophytic fungi found in plant leaves are of particular concern because leaves are organs that perform photosynthesis and transpiration. Endophytic fungi must contend with more biochemical dynamics and environmental changes than endophytic fungi found in woody tissues [19, 20]. The mechanisms of interactions between the host plant and its endophytic microorganisms are still being studied [21]. Variations in the structure, anatomy, and maturity of colonized host tissue and environmental influences influence the proliferation and population composition of endophytic fungi [22]. The endophytic group will vary significantly depending on the leaf's development stage and plant tissue arrangement [23].

The most well-known sandalwood source is the *Santalum album*, or Indian sandalwood, a small tropical tree. Southern India and Southeast Asia are their ancestral lands. The medicinal and aromatic properties of some plants are prized in certain cultures. In certain religions, even now, it is called holy and is utilized in numerous religious rituals. Because of the species' high monetary importance, it has had a

tumultuous history. Since the high importance of species, it has remained over-exploited in the past, putting the wild population at risk of extinction. The tropical belt of peninsular India, eastern Indonesia, and northern Australia is home to *Santalum album* L [25].

The evergreen tree can grow to be 4 to 9 meters tall. They could live to be a hundred years old. The tree's habit is complex, ranging from upright to sprawling, and it can intertwine with other plants. The smooth reddish or brown bark and almost black in young plants crack with a red reveal. As the common name implies, the heartwood is light green to white. The small, opposite and ovate to lanceolate formed leaves. With a pale glaucous opposite, the glabrous color is smooth and bright orange [26].

The study aimed to classify fungal endophytic populations in medicinally valuable plant species' leaves, stems and petioles. The goals were to (i) isolation of endophytic fungi from the *Santalum album*. (ii) Identify the endophytic fungi and their diversity in three different seasons. (iii) Compare the endophytic fungal isolation from three related medicinal plant sections.

Materials and methods

Collection of plant samples

During the winter season, plant parts such as leaves, stems, and petioles were collected from *Santalum album*, a medicinal plant species (Table1) found in the Bisle reserve forest area's natural vegetation in Western Ghats of Karnataka. The natural vegetation consists of evergreen and semi-evergreen trees. Plant samples (leaves, stem, and petioles) were taken from healthy *Santalum album* in different seasons of 2017 (March-June, July-September, and November-February). The samples were collected thrice in each season to study the seasonal variation of endophytic fungi. The samples were packed in polyethylene containers, labeled, and transported to the laboratory, where they were kept at 4°C until required. Within 24 hours of processing, all samples were analyzed [27].

Endophytic fungi isolation:

Plant samples were washed vigorously in distilled water, dried and immersed in 70% ethanol (v/v) for one minute, followed by three minutes in sodium hypochlorite (3.5 percent v/v). To ensure complete drying, sterile filtered water was rinsed three times until it was dried on sterile blotting sheets under laminar airflow. Tissue segments measuring 0.5 × 0.5 cm were excreted using a sterile blade. A total of 135 segments of the Santalum album leaves, stem and petioles were arranged on the PDA complemented by antibiotic tetracycline (250 mg/L) on 9 cm of Petri plates. Five portions of the plate are plated. The plates were coated in parafilm and incubated at 22°C with 12 h of light and dark periods for up to 3 to 4 weeks. Surface sterilization of tissues was investigated by placing aliquots of sterilants on agar plates and monitoring fungal colonies for two weeks [28, 29]. The tissue segments were checked for the presence of a fungal colony regularly. Each colony that emerged from segments was moved to an antibiotic-free potato dextrose agar medium to identify (PDA) [30].

Identification of endophytic fungi by the morphological method

Each colony that emerged from the segments was placed on an antibiotic-free potato dextrose agar medium to identify (PDA). The isolates are inoculated on Petri plates containing PDA (Potato dextrose agar) and incubated at 24±2^{0}C for 12 hr under fluorescent light and 12 hr. darkness. Lactophenol cotton blue staining was used to identify fungi arising out of the explants in their sporulation form. With the aid of regular mycological manuals, endophytic fungal isolates can be described based on morphological characteristics.[31,32] Characters such as colony appearance, mycelium color and structure, the form of anamorph, conidiomata conidia and conidiophore morphology (size, color, shape, ornamentation, etc.) and conidiogenous cells morphology (size, color, shape, ornamentation, etc.) may be used to classify and define morphospecies [28].

Statistical analysis

Isolation rate (IR) and colonization rate (CR)

The number of isolates collected from tissue segments was divided by the total number of tissue segments to determine the endophytic fungi isolation rate (IR). Endophytic fungi colonization rate (CR) was calculated as a percentage of the total number of isolates obtained from various tissue segments divided by the total number of isolates obtained from all tissue segments incubated [33].

The Simpson's diversity index, Simpson's dominance index (D), Species richness (S), Shannon-Wiener index (H) and Evenness (E) were calculated. [34]

i) Simpson's index of diversity was calculated using the formula: 1- D

$$D = \sum n(n-1)/N(N-1)$$

ii) Shannon-Wiener diversity index (H) was calculated using the formula

$$Hs = \sum_{i=1}^{s} (Pi)(Ln\ Pi)$$

H_S - the symbol for the diversity in a sample of S species
S - Number of species in a sample.
P_i - the relative abundance of the i^{th} species
ln - log to base 2

iii) Evenness (E) was expressed by

$$E = H/Log(S)$$

Results and Discussion

Isolation of endophytic fungi from medicinal plants during the winter season

The medicinal plant was collected from the Bisle region of Western Ghats, and the plant authentication certificate was obtained from RARIMD (Figure1A and 1B). The tissue segments of leaf, stem and petiole from the *Santalum album* were collected for endophytic fungal isolation (Figure 2). A total of 54 endophytic fungal isolates were isolated from medicinally important *Santalum album*. Among 54 fungal isolates, 24 from leaves, 21 from stem and 9 from petiole, respectively (Figure 3). Based on morphological characteristics such as color, mycelium growth on PDA, and the presence of reproductive structures, the fungal isolates were classified into different morphotypes (Figure 4, Table 2).

Genus *Curvularia* isolates showed a highest absolute frequency (6) than the 24 other fungal isolates, followed by *Cladosporium* sp. (5) and *Aspergillus* sp. (4). Fungal isolates from leaves showed a greater species richness (18) than that of the stem (16) and Petioles (7) (Table 2). [35]

Seasonal variation

A total of 54 endophytic fungal isolates were isolated from the *Santalum album*, a medicinally important plant belonging to the *Santalaceae* family of Western Ghats of Karnataka. Seasonal variation in the diversity of these endophytic fungi was studied. In which winter season harbored 19 (35.18%) endophytic fungal isolates, which is similar to the monsoon season 19 (35.18%) and then the summer season with 14 (25.09%) endophytic fungal isolates. These endophytic fungal isolates belonging to 25 different genera. *Curvularia sp.* showed the species richness amongst other fungi (Table 2) [36].

Winter season

The colonization rate of endophytic fungi isolated from *Santalum album* during the winter season was calculated based on plants' specific tissues. The colonizing frequency of leaf segments was highest (50%), followed by the stem (32.1%), and petiole segments showed the least colonizing frequency percentage of 17.9% (Figure 5). The Isolation rate of the endophytic fungi from leaves was found to be 0.65, followed by the stem (0.45), and the petiole showed a lower isolation rate of 0.2 (Figure 6).

Summer season

The colonization rate of endophytic fungi isolated from *Santalum album* during the summer season was calculated based on plants' specific tissues. The colonizing frequency of leaf segments was highest (47.41%), followed by the stem (36.8%), and petiole segments showed the least colonizing frequency percentage of 15.8% (Figure 5). The Isolation rate of the endophytic fungi from leaves was 0.35, followed by the stem (0.3), and the petiole showed a lower isolation rate of 0.1(Figure 6).

Monsoon season

The colonization rate of endophytic fungi isolated from the *Santalum album* during the monsoon season was calculated based on plants' specific tissues. The colonizing frequency of leaf segments was equal to that of the stem (37.5%), and petiole segments showed the least colonizing frequency of 25% (Figure 5). The Isolation frequency of the endophytic fungi from leaves was 0.3, followed by the stem (0.2), and the petiole showed a lower isolation rate of 0.15 (Figure 6). Species richness was highest in the summer season (14), followed by the winter season (13) and lowest in the monsoon season (11).

Statistical analysis

Diversity and Distribution of endophytic fungi

Simpson's dominance index and Simpson's diversity index of the *Santalum album* showed less than 1, and the Shannon Wiener index of all the plants was in the range of 2 to 3 (Table 3). This showed that the endophytic fungi from these medicinal plants are highly diversified. Shannon Wiener's evenness of the *Santalum album* is less than 1, which showed the endophytic fungi evenly distributed in the *Santalum album* (Table 3).

Tables and Figures

Table 1: Selected plant for endophytic fungi isolation

Sl.no.	Plant species	Family	Reference number	Medicinal Uses
1.	*Santalum album*	*Santalaceae*	RRCBI-15231	Sandalwood oil has been used to relieve headaches, stomach aches, and gastrointestinal and genital conditions as an antiseptic and astringent. The paste of sandalwood is used in the treatment of inflammatory and eruptive skin diseases. It has diuretic, analgesic, antiseptic, expectorant and stimulant effects.[37]

Table 2: Endophytic fungal taxa isolated from *Santalum album* in different seasons

Sl.no.	Endophytic fungi	Different seasons			Absolute frequency
		Winter	Summer	Monsoon	
1	*Acremonium* sp.	1	0	0	01
2	*Alternaria* sp.	0	1	0	01
3	*Arthrinium* sp.	0	0	1	01
4	*Aspergillus* sp.	3	1	0	04
5	*Bipolaris* sp	0	1	0	01
6	*Botyritis* sp	0	0	1	01
7	*Chaetomium* sp.	1	0	2	03
8	*Cladosporium* sp.	3	2	0	05
9.	*Cochliobolus* sp.	1	1	1	03
10.	*Colletotrichum* sp.	1	0	0	01
11.	*Curvularia* sp.	2	2	2	06
12.	*Cylindrosporium* sp.	1	0	0	01
13.	*Epicoccum* sp.	1	0	0	01
14.	*Fusarium* sp.	1	2	0	03
15.	*Geotrichum* sp.	0	1	0	01
16.	*Helmintosporium* sp.	0	1	0	01
17.	*Macrophomina* sp.	1	0	1	02
18.	*Mucor* sp.	0	0	1	01
19.	*Penicillium* sp.	0	1	0	01
20.	*Phoma* sp.	0	1	0	01
21.	*Phomopsis* sp.	1	0	1	02

22	*Rhizopus* sp.	0	0	1	01
23	Sterile mycelia	1	1	3	05
24.	*Trichoderma* sp.	0	2	0	02
25.	*Xylaria* sp.	0	1	2	03
	Total	19	19	16	54
	Species richness	13	14	11	

Table 3: Diversity analysis by Shannon Weiner and Simpson's Diversity, Dominance and equality indices of endophytic fungi from *Santalum album.*

Different seasons	*Santalum album*			Absolute frequency (f)	Relative frequency (fr)	Simpson's dominance index (D)	Simpson's diversity index (d=1-D)	Shannon-Wiener index (H)	Evenness (H/H_{max})
	Leaf	Stem	Petiole						
Winter	9	8	2	19	0.044	0.076	0.924	2.499	0.946
Summer	9	7	3	19	0.044	0.043	0.957	2.562	0.970
Monsoon	6	6	4	16	0.041	0.109	0.891	2.290	0.958

Fig 1: (A) *Santalum album* tree, (B) *Santalum album* leaves

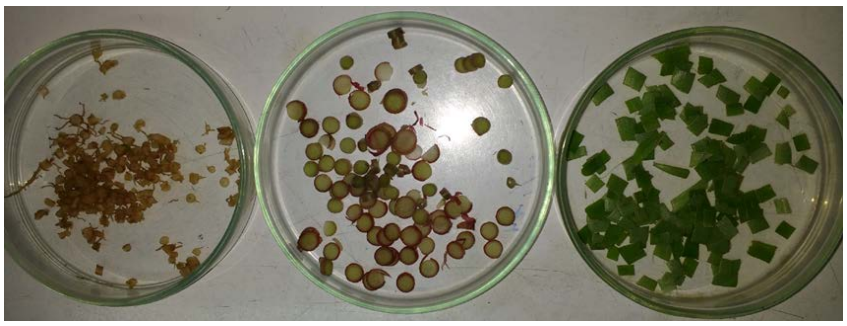

Fig 2: Preparation of explants of petiole, stem and leaf

Fig 3: Petri plate containing different fungal isolates

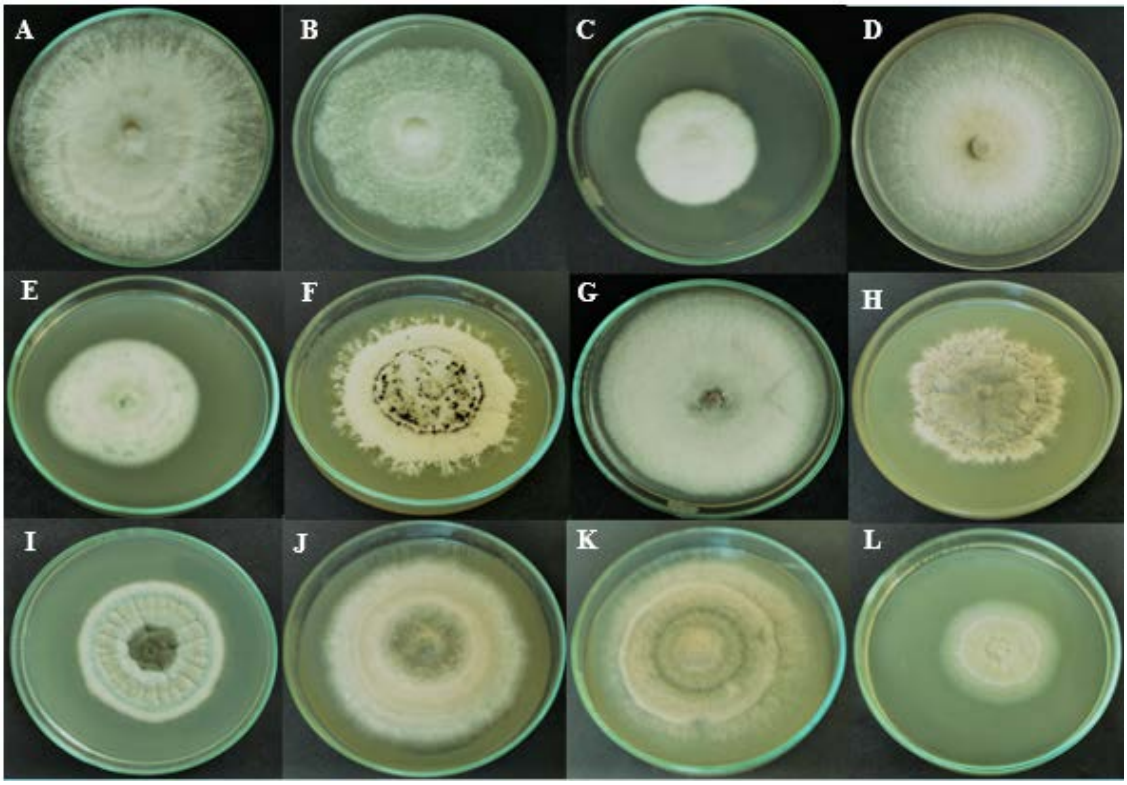

Fig 4: Petri plate containing pure fungal cultures (A) *Trichoderma* sp. (B) *Phomopsis* sp. (C) *Phoma* sp. (D) *Cylindrosporium* sp. (E) *Epicoccum* sp. (F) *Chaetomium* sp. (G) *Arthrinium* sp. (H) *Cladosporium* sp. (I) *Aspergillus* sp. (J) *Fusarium* sp. (K) *Macrophomina* sp. (L) *Geotrichum* sp

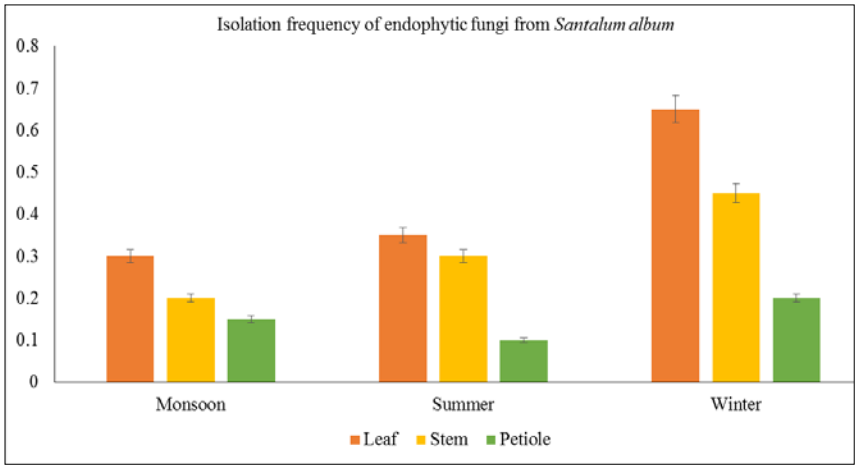

Fig 5: Isolation frequency of endophytic fungi from *Santalum album*

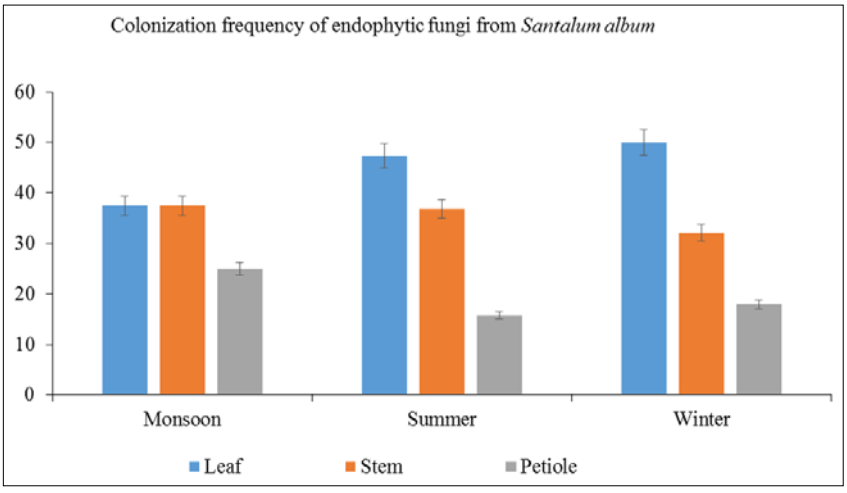

Fig 6: Colonization frequency of endophytic fungi from *Santalum album*

Conclusions

This study aimed to evaluate the abundance, diversity, host affiliations, and local distributions of endophytic fungi associated with the *Santalum album* of the Bisle region in three different seasons of the year. These endophytic fungi' seasonal variations give better knowledge about the host specificity and diversity of endophytic fungi in a host during different environmental conditions. Abiotic factors (e.g., geographic distance, climate, seasonal and spatial variations, microclimates, disturbances) are often described as fungal diversity drivers in tropical environments.

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