

# Seasonal Variation and Tissues Specificity of Endophytic Fungi of *Dillenia Indica* L. and their Extracellular Enzymatic Activity.

Vijay Kumar (✉ [vijaysharmasanmotra100@gmail.com](mailto:vijaysharmasanmotra100@gmail.com))

Panjab University <https://orcid.org/0000-0001-7935-3711>

I. B. Prasher

Panjab University

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## Research Article

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# Abstract

Endophytes are an unexplored group of microbes that live inside the living tissues of healthy plants without any visible symptoms of the disease. This study focused on the exploration and characterisation of culturable endophytic fungi inhabiting in different parts the medicinal plant *Dillenia indica* L during different seasons. A total of 2360 segments from different parts like leaves (820), stems (820) and fruits (720) were screened to isolate endophytic fungi from the plants growing in Botanical Gardens of Panjab University, Chandigarh (India), during different seasons i.e., Summer, Spring, Autumn and Winter of the years 2018 and 2019. A total of twenty-five (25) species of fungi belonging to twenty (20) genera were isolated from the selected plant during different seasons. The plant has the highest affinity for *Lasiodiplodia theobromae* followed by *Colletotrichum gloeosporioides* and *Cladosporium cladosporides*. The percentage frequency was found to be 96%, 64% and 20% for leaves, stems and fruits. The colonization rate for leaves, stems and fruits was 55.6%, 25.6% and 8.8%. The isolated fungi were identified by morphological, microscopic and molecular characteristics. Monsoon (Rainy season) had the highest number of isolates (312 isolates), followed by summer (208 isolates), Winter (164 isolates) and Autumn (114 isolates). Species diversity was highest during the rainy season (19 species) and lowest during the Winter (12 species). The isolated fungi also produce extracellular enzymes such as amylase, lipase, protease, asparaginase, cellulase and ligninolytic enzymes. The results indicate that *Dillenia indica* L. harbors novel endophytic fungi having agricultural, medical and industrial applications.

## 1. Introduction

Human beings depend on plants for their food and medicines since human civilization (Manganyi et al., 2018). It is estimated that more than 400,000 different plant species exist worldwide and the majority of them are utilized in treating various diseases. ( Bussmann et al., 2008, Abbasi et al., 2010, Joppa et al., 2010). It is also noted that relatively 80% of the world's population and those who live in developing countries depend on herbal remedies to address their initial health problems (World Health Organization 2003). *Dillenia indica* L. is an evergreen flowering plant that belongs to the Dilleniaceae family and is commonly known as Elephant apple. It occurs in Bangladesh, China, Indonesia, Nepal and India. It is an ethnomedicinal plant used to relieve several diseases such as fever, cancer, diarrhea, mouth ailments, nausea, weakness, etc. (Singh et al., 2019). It generally appears in forests, but nowadays, people are growing in their gardens because of their lovely blossoms and plump fruits. The mature fruits are plentiful in nutrients and their extracts have got antioxidant properties. The fruit possesses tonic and laxative properties, is eaten up as chutney, made into jam, and relieves abdominal pain by traditional Assam communities. The juice of fruits is utilized to treat heart complications, fever, cough, diarrhea and cancer (Kumar et al., 2011). Fungi are a large group of microorganisms, having an estimated number of about 1.5 million; from them, only 75,000 are known species (Blackwell 2011). It is feasible to find fungi in moderate and extremely severe climatic conditions in the earth's utmost parts. They live in soils, at seas, on the surface of rocks, etc., either as individuals, colony or parasites (Hawksworth 2001). The capability of adaptation to varied habitats enables fungi to make many secondary metabolites with diverse structures. According to Brakhage and Schroeckh (2011), approximately 97,000 secondary metabolites have been isolated from fungal sources. Some of these secondary metabolites have

demonstrated biological actions such as antimicrobial, antifungal, antiviral, antiprotozoal, anticancer, immunosuppressant etc. (Nascimento et al., 2015).

Finding out the role of various fungal species in ecosystems has been hampered because of limited sampling and scarcity of information on fungal diversity (Hyde et al., 2007). Endophytes are among the poorly recognized groups of fungi, even though they are considered to be effective in plant populations and communities (Arnold et al., 2003, Arnold and Engelbrecht, 2007, Arnold, 2007, Arnold et al., 2007, Saikkonen, 2007, Rodriguez et al., 2009).

Endophytes reside asymptotically within most living tissues of plants studied to date (Schulz et al., 2002, Li et al., 2007, Tao et al., 2008). Endophytic fungi are non-pathogenic fungi found ubiquitously in distinct parts of plants. They live in intercellular or intracellular spaces or inside vascular tissues cells (Hallmann et al., 1997). The majority of these fungi belong to ascomycetes and basidiomycetes. The diversity and composition of endophytic fungi are influenced by factors such as host plant, area, time, topography, physiology of host, tissues and organs of the host plant (Arnold and Lutzoni, 2007; Naik et al., 2009). Endophytes help in the degradation of plants dead and decomposing tissues, which is vital for nutrient cycling in an ecosystem (Strobel, 2000). Endophytes were examined from plants thriving in various environmental conditions like tropical (Mohali et al., 2005), temperate (Ganley et al., 2004), xerophytic (Suryanarayanan et al., 2005). and aquatic habitat (Sraj-Krzic et al., 2006).

Endophytic species abundance and distribution generally differ among tree individuals. They are further influenced by the host plant's conditions (Sieber and Hugentobler 1987), by the time of sampling and by the precise location of sampling units (Unterseher et al. 2007). Therefore, reliable figures of species abundance and composition for a given host tree species requires repeated sampling of numerous individuals throughout an entire vegetation season (Bills and Polishook 1992; Hoffman and Arnold 2008). Dreyfuss and Chapela (1994) estimated that there might be at least one million endophytic fungal species. They support their host against insect pests, pathogens and even domestic herbivores (Weber, 1981, Malinowski and Belesky, 2006). Almost all the plant species (~ 400,000) harbor one or more endophytic organisms (Tan and Zou, 2001). Medicinal plants are recorded to harbor endophytes (Strobel, 2002), which provide security to their host from toxic agents and further provide adaptability to live in adverse environmental conditions. Endophytes protect the host plant from many biotic and abiotic threats (Martinez-Arias, 2020). To date, merely a few plants have been extensively explored for their endophytic biodiversity.

There are many reports on the diversity of endophytic fungi associated with various medicinal plants. Still, as per literature survey, no data available on fungal diversity inhabited in *Dillenia indica* L. The rationale of the present study was to find out the effect of different seasons and tissues on the colonization of culturable endophytic fungi within this plant. The study was also insight into the potential of the isolated endophytic fungi for their different extracellular enzymatic activity.

## **2. Materials And Methods**

### **2.1. Study area:**

The study site was chosen is the Panjab University, Chandigarh, India (30.74°N 76.79°E). The annual rainfall is 1110.7 mm, and there is a considerable variation in temperature ranging from 2°C to 45°C.

## **2.2. Sample collection and isolation of endophytic fungi:**

Healthy plant parts (leaves, stems and fruits) of *Dillenia indica* L were collected from the Botanical Gardens of Panjab University, Chandigarh, India. The plant parts were randomly excised and brought to the laboratory in sterilized plastic bags. The samples were surface sterilized before the isolation of endophytic fungi. The samples were immersed in water for 10–15 minutes and then washed with distilled water to remove dust and debris. The leaves, fruits and stems samples were excised into 0.5- 1.0 cm segments with surgical blades. For surface sterilization, the samples were soaked in 70% ethanol for 2 minutes and then disinfected with 4% sodium hypochlorite solution for 4 minutes and rinsed three times with sterile distilled water and dried in a laminar airflow chamber (Kuklinsky-Sobral et al., 2005, Hallmann, Berg, and Schulz 2007). The sterilized samples were plated on Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol (100µg/mL concentration) to inhibit bacterial growth. The plates were then incubated at 25 ± 1°C for 8–15 days. Purification of the fungal isolates was done by the single-spore method. The hyphal-tip method was used for those species which cannot sporulate. Pure cultures of strains were maintained in PDA slant tubes and 20% glycerol stock solution and deposited in the Panjab University Herbarium culture collection (PAN).

## **2.3. Identification:**

### **2.3.1. Morphological identification**

To identify the isolated fungi, culture characteristics such as colour and colony appearance were examined after ten days of incubated cultures. For microscopic observation, slides were prepared and stained with 2% Congo-red or lactophenol cotton blue reagent and examined using a transmission microscope at 10X, 40X and 100X times magnification. The identification was based on the hyphal morphology, spores characteristics and reproductive structures. The fungal isolates were identified only up to the genus/species level based on their microscopic characters viz. type of conidiophores, the shape of conidia, presence or absence of septa etc. Many of the fungal endophytes do not sporulate when sub-cultured *in-vitro* (White and Cole, 1986). Non-sporulating strains of fungal

endophytes that fail to sporulate were described as sterile fungal endophytes and were subjected to molecular characterization to identify the genus/species.

### **2.3.2. Molecular identification of endophytic fungi:**

To ascertain the identification and phylogenetic link among the isolated fungi, the Internal Transcribed Spacer (ITS) regions were used as marks for all fungal species (Khorasani, 2013). The purified cultures of fungal isolates were grown on PDA for 10–12 days. The mycelium of fully grown cultures was scraped from the plates using an inoculation needle under aseptic conditions in the laminar airflow and is used for DNA isolation. The Qiagen DNeasy Mini Plant Kit was used to isolate DNA from the mycelium of fungi. DNA was assessed using a Nanodrop and stored at - 20°C for PCR analysis. The polymerase chain reaction was performed to amplify the internal transcribed spacer (ITS) region of fungal isolates. PCR amplifications were done using ITS1 and ITS4 primer sequences to target fungal isolates (Khorasani, 2013). Amplifications were

done in a Thermal Cycler. The 1% (w/v) agarose gel containing 0.1 µg/mL ethidium bromide was used to resolve the PCR products by electrophoresis. The Amplified products were visualized under UV light and were purified and sequenced.

## 2.4. Statistical analysis:

$$2.4.1. \text{Percentage frequency} = \frac{\text{No. of endophytes isolated from a plant tissue}}{\text{Total number of endophytes isolated from that plant}} \times 100$$

### 2.4.2. % Colonization frequency (Suryanarayanan et al. 2003)

$$CF = \frac{\text{No. of segments colonized by each endophytes}}{\text{Total number of segments inoculated}} \times 100$$

### 2.4.3. Colonization rate: (Sunayana et al. 2014)

$$CR = \frac{\text{No. of segments colonized by endophytes}}{\text{Total number of segments inoculated}} \times 100$$

### 2.4.4. Isolation rate: (Sunayana et al. 2014) and Sharma et al. (2015).

$$IR = \frac{\text{No. of endophytes isolated}}{\text{Total number of segments inoculated}} \times 100$$

### 2.4.5. Shannon-Wiener (H'): (Yuan et al. 2010)

$$(H') = -\sum P_i \times \ln(P_i),$$

Where,  $P_i = n_i / N$ , is the relative abundance of the endophytic fungal species,  $n_i$  is the number of isolates of one species, and  $N$  is the total species number of isolates present within each sample.

### 2.4.6. Simpson's (Ds) diversity index: (Kusari et al. 2013)

$$D_s = 1 - \sum P_i^2$$

### 2.4.7. Evenness index (E): (Jin et al. 2017)

$$E = H' / L_n(S)$$

$S$  = Total no. of the taxa present within each sample.

## 2.5. Phylogenetic analysis:

Consensus sequences were established for all isolated fungi and were exposed to the Basic Local Alignment Search Tool (BLAST) on <http://www.ncbi.nlm.nih.gov/BLAST> in the NCBI database. Fungal names were generated by analyzing sequences with those already submitted in Gene bank. The evolutionary relationship among the isolates was created with the help of MEGA 7 software using the UPGMA method. The consensus sequences were incurred from the Internal Transcribed Spacer (ITS) to frame the phylogenetic tree with 500 replications bootstrap.

## 2.6. Enzymatic screening:

The fungi isolated from the selected medicinal plant were screened for different extracellular enzymes such as Cellulase, Ligninolytic, Amylase, Lipase, Protease, Laccase and Asparaginase activity by plate assay technique. The extracellular activity was evaluated by inoculating fungal disc on solid media enriched with a substrate-specific to different enzymes. Starch to test amylase, gelatine to protease, tween 80 to lipase, carboxymethylcellulose to test cellulase, Azure B to test ligninolytic and L-Asparagine to test for asparaginase. The plates were incubated for seven days at room temperature, and the enzyme activity was measured after that time. (Archibald F. S. 1992, Patn, L. and Anagnostakis S.L. 1975, Patil et al., 2012). The experiment was carried out in triplicate.

## 2.7. Influence of seasons on fungal colonization:

To study the effect of seasons on colonization of endophytic fungi, samples were collected during different seasons of the years 2018 and 2019, and the endophytic fungi were isolated, identified and data was recorded.

## 3. Results And Discussion

### 3.1. Diversity of Endophytic Fungi in *Dillenia indica* L.:

The present study about the diversity of endophytic fungi from *Dillenia indica* L. is the first of its kind, as per our literature survey. There is a high diversity of endophytic fungi associated with different plant parts of *Dillenia indica* L. A total of three hundred and ninety-nine (399) fungal endophytes were isolated from the different surface-sterilized explants of *Dillenia indica* L. Some of these isolates were morphologically similar after macroscopic and microscopic examinations. Fungal endophytes with identical characteristics were marked as repeats, with only those isolates that differed morphologically being identified as different endophytic fungal isolates. As a result of this, only twenty-five (25) different fungal endophytes were isolated from *Dillenia indica* L., which comprises 25 different species, namely *Curvularia lunata*, *Cladosporium cladosporioides*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Bipolaris crotonis*, *Fusarium oxysporum*, *Chaetomium globosum*, *Trichoderma viride*, *Clonostachys rosea*, *Diaporthe phaseolorum*, *Lasiodiplodia theobromae*, *Schizophyllum cummne*, *Phomopsis* sp., *Colletotrichum gigasporum*, *Fomitopsis meliae*, *Fusarium brchygibbosum*, *Pseudofusicoccum adensoniae*, *Daldinia eschscholtzii*, *Nigrospora sphaerica*, *Xylaria longipes*, *Neopestalotiopsis clavispora*, *Alternaria tenuissima*, *Aspergillus fumigatus*, *Colletotrichum musae*, and *Colletotrichum boninense* from fresh tissues of plant. *Colletotrichum gloeosporioides*, *Cladosporium cladosporioides*, *Lasiodiplodia theobromae* were the most frequently occurring species during the study (Fig. 1). All the isolated fungi belong to four classes i.e., Sordariomycetes, Dothideomycetes, Agaricomycetes and Eurotiomycetes (Fig. 2). Some genera (*Curvularia*, *Fusarium* and *Alternaria*), which are cosmopolitan, are also obtained as endophytes in the current study. These taxa have already been reported by various researchers from tropical plants (Ferreira et al., 2015). Similarly, Arora et al., 2019 isolated fungal endophytes from *Glycyrrhiza*

*glabra* L. and found that the genus *Phoma* had a higher affinity than *Fusarium*. Wang et al., 2015 isolated twenty-four culturable endophytic fungi from *Oryza rufipogon* Griff and screened for their antagonistic activity against phytopathogens. One hundred fungal endophytes were isolated from black seed (*Nigella sativa* L.),

and *Penicillium*, *Alternaria* and *Cladosporium* were predominant genera. All the isolated fungi belong to the phylum Ascomycota (Gopane et al., 2021). Endophytic fungi from *Gentiana rigescens* exhibited good cytotoxicity activity against different cell lines (Xu et al. 2020). Jagannath et al. 2020 isolated two hundred and three endophytic fungi belonging to twenty-nine species from *Baliospermum montanum* and found that the colonization and isolation rate was higher in stem followed by seed, root, leaf and flower. These fungi were also screened for amylase, lipase, protease, cellulase and phosphatase activity.

Fungal endophytes isolated from various tissues of the plant were identified morphologically to generic/species level. The fungal endophytes which could not be identified morphologically were subjected to molecular characterization (Tables 1–2). The ITS sequences of different isolates obtained were subjected to BLAST analysis, which revealed their homology with similar species. The ITS sequences have been deposited in the GenBank database, for which accession numbers have been provided and phylogenetic tree was constructed (Fig. 16–17).

Table 1

The endophytic fungi identified based on morphological characteristics, isolated different parts of *Dillenia indica* L.

S.No.	Endophytic fungi	Spore characteristics
1.	<i>Curvularia lunata</i>	Conidia 14–26 × 8.5–13 μm, 3–5 celled, asymmetrical to more or less curved at the third cell from the base, intermediate cells dark brown and usually smooth-walled.
2.	<i>Cladosporium cladosporioides</i> .	Conidia solitary or catenate, in unbranched or branched acropetal chains, usually obovoid, ellipsoid, fusiform, 3.0–6.0 × 2–4 μm.
3.	<i>Alternaria alternata</i> .	Conidia are pale to brown in colour, smooth or verruculose, usually 2–8 transverse and several longitudinal or oblique septa, 15–58 × 7.5–16 μm.
4.	<i>Colletotrichum gloeosporioides</i>	Conidiophores cylindrical, hyaline to subhyaline, up to 30 μm long. Conidia were cylindrical, straight oval borne on hyaline conidiophores having 14–16 μm in length and 5–6 μm diameter.
5.	<i>Bipolaris crotonis</i>	Conidia olivaceous brown to pale brown, smooth uniformly pigmented, broadly ellipsoidal, 6–11 (mostly 7–9) distoseptate, (48) 80–110 × 18–29 μm.
6.	<i>Chaetomium globosum</i>	Asci fasciculate, fusiform or clavate, spore-bearing part 25–40 × 5–10 μm, stalks 15–20 μm long. Each ascus consists of eight biserial to irregularly-arranged ascospores, which are olivaceous brown when mature, limoniform, usually biapiculate, bilaterally flattened, 7–8.5 × 5–8 μm, with an apical germ pore.
7.	<i>Fusarium oxysporum</i>	Asci fasciculate, fusiform or clavate, spore-bearing part 25–40 × 5–10 μm, stalks 15–20 μm long. Each ascus consists of eight biserial to irregularly-arranged ascospores, which are olivaceous brown when mature, limoniform, usually biapiculate, bilaterally flattened, 7–8.5 × 5–8 μm, with an apical germ pore.
8.	<i>Clonostachys rosea</i>	Conidiophore dimorphic i.e. verticillium-like and penicillate-like. Conidia globose to sub globose having size 3–8 × 2–4 μm.
9.	<i>Alternaria tenuissima</i> .	Conidia are dilute tan to brown with dark walls mostly smooth, occasionally verruculose, 4–7 transverse and several longitudinal or oblique septa, overall length 22–75 × 8–15 μm thick in the broadest part swollen apex 4–5 μ wide.
10.	<i>Aspergillus niger</i>	Conidiophore smooth, non-septate, thick walled, globose vesicle. Conidia globose to sub globose, black, 4–5 μm.
11.	<i>Trichoderma viridae</i> .	Colonies are initially white later turn light green in colour. Conidiophore arise from undifferentiated aerial mycelium. Phialides are straight or sinus or hooked. The terminal phialide are whorl or solitary phialide are cylindrical. Conidia sub-globose to obovoid having 2.9 – 3.8 × 2.8–3.4.
12.	<i>Neopestalotiopsis clavispora</i>	Colonies are white to pale yellow in colour, raised and fluffy, conidia 5 celled fusiform, basal cell is hyaline, 2–3 unbranched arise from the apical cell.

Table 2  
The endophytic fungi identified based on morphological characteristics, isolated different parts of *Dillenia indica* L.

S.No.	Fungal identification	Code	NCBI Accession no	Identity %age
1.	<i>Diporthe phaseoli</i>	DLP21S4a1	MK757156	97%
2.	<i>Schizophyllum communeae</i>	DSP22S3a1	MK756215	99%
3.	<i>Phomopsis</i> sp.	DLP24S2a1	MK757169	98%
4.	<i>Fomitopsis meliae</i>	DLP30S2a1	MK757195	98%
5.	<i>Nigrospora sphaerica</i>	DLP46S2a1	MK757157	100%
6.	<i>Lasiodiplodia theobromae</i>	DSP22S4a1	MK644105	99%
7.	<i>Fusarium brachygibbosum</i>	DLP41S3a1	MK757199	99%
8.	<i>Colletotrichum gigasporum</i>	DSP26S2a1	MK756322	99%
9.	<i>Xylaria longipilus</i>	DLP41S2a1	MK756123	99%
10.	<i>Pseudofusicoccum adansoniae</i>	DSP42S2a1	MK757196	99%
11.	<i>Daldinia eschscholtzii</i>	DSP40S2a1	MN854982	99.6%
12.	<i>Colletotrichum gleosporides</i>	DLP31S1a1	MN855105	99.6%
13.	<i>Colletotrichum boninense</i>	DSP32S3a3	MW521131	99.87%

There was a significant effect of host tissues on the colonization of fungal endophytes. A total of two hundred forty-nine (498) isolates belonging to 24 taxa were isolated from the leaves, one hundred fourteen (228) isolates having 16 species from the stem and thirty-six (72) isolates belongs to 6 species from fruits. The isolation rate was 5.85 %, 3.90 % and 1.21 found for leaves, stems and fruits, as shown below in Fig. 2. Colonization rate was found 55.6%, 25.6% and 08.8% for leaves, stems and fruits. The values of percentage frequency were 96% for leaves, 64% for stems and 20% for fruits. Thus, our results clearly show that fungal endophytes are more in number in leaves than stems and fruits. The colonization frequency was 61.2, 28.9 and 9.01 for leaves, stems and fruits. The values of diversity indices i.e., Simpson Index, Shannon Index and evenness index, indicates that the tissues are highly diverse. The highest value of the Shannon index was observed in leaves i.e., 2.77, whereas the lowest value was 1.07 and was recorded in fruits. The present data also revealed the highest Shannon- Wiener diversity index in the leaves, which suggests that the leaves have a more diverse endophytic fungal community in contrast to the fruits and bark.

The study showed that the composition of fungal endophytes in *Dillenia indica* L. was influenced by the seasons. Monsoon (Rainy season) had the highest number of isolates (312 isolates), followed by summer (208 isolates), Winter (164 isolates) and Autumn (115 isolates). During the rainy season, the dominating taxa were *Colletotrichum gloeosporioides*, *Neopestalotiopsis clavispora*, *Fusarium brchygibbosum* and *Daldinia eschscholtzii* followed by *Alemaria alternata*, *Fusarium oxysporum*, *Chaetomium globosum* and *Alternaria tenuissima*. Species diversity was highest during the rainy season (19 species) and lowest during the Winter (12 species). Different numbers of fungal endophytes were obtained during different months of the year. The

maximum isolates were obtained during September, followed by August, March and April month and the minimum during July month. Some taxa of the endophytic fungi were reported in only one or two seasons. For example, *Colletotrichum boninense* was exclusively reported during Winter, whereas the *Colletotrichum gloeosporioides* was reported in all the seasons. *Schizopyllum commune* was found in the Rainy and Winter seasons.

The highest colonization frequency was in the rainy season (37.89%), followed by Autumn (32.5), summer (30.3%) and Winter (25%). However, species diversity data show a somewhat correlation with the number of isolates observed during different seasons. The isolation rate ranges from 4.0 to 6.0%, and the percentage frequency varies from 48% to 76 % during different seasons. The colonization rate from 25.0–38.4% of endophytic fungi was obtained from in the present study (Fig. 3–9). It is important to note that the seasons have a direct effect on the composition of fungal communities. The seasonal variation in colonization patterns may be due to the seasonal activities of fungal endophytes. During rainy seasons, greater endophytic fungal diversity was observed in *Terminalia arjuna* twigs and bark (Tejesvi et al., 2005) and *Centella asiatica* leaves (Gupta and Chaturvedi, 2017). The rainy and summer seasons, as previously stated, support high endophytic fungi expression (Kim et al. 2013, Singh et al. 2016, Schulthess et al. 1998, Higgins et al., 2011). It has been reported that during the rainy seasons, high moisture and temperature support the growth and dispersal of fungal endophyte spores (Mishra et al., 2012). However, several studies have found that endophytic fungal diversity is substantially higher in the Winter months than in the rainy and summer months (Naik et al., 2008; Fang et al., 2013). The seasonal variation in fungal endophytic diversity may be due to secondary metabolite levels fluctuate throughout the year (Fang et al., 2013). Seasons influence endophytic antibacterial communities in medicinal plants (Barman and Dkhar 2020).

The majority of the isolated fungi belonged to Ascomycota (83.33%), which is identical to the findings of Goveas et al. (2011) from the threatened plant *Coscinium fenestratum*. Only 2% belonged to Basidiomycota. Sordariomycetes and Dothideomycetes were the main classes of Ascomycota in the endophytic assemblage, as revealed from the results of Li et al. (2016). The Shannon-wiener index for leaves, fruits and stems were 2.60, 1.598 and 1.077, while Simpson's diversity index was 0.919, 0.776 and 0.892 for leaves, stems and fruits. The evenness index was 0.937, 0.892 and 0.980 for leaves, bark and fruits (Fig. 10–15). It was seen that only one or a few species dominated the endophytic community of the host, whereas the majority of them were rare (Petrini et al. 1992). Numerous factors such as biotic, abiotic, chemical composition and architecture of host tissues are involved in the deviation of colonization of endophytic fungi in specific tissues (Sanchez–Azofeifa 2012, Liu et al. 2012).

### **Extracellular enzymes productions:**

The endophytes produce a variety of bioactive molecules, which include enzymes; microbial enzymes help in hydrolysis and biodegradation processes and facilitate their colonization inside the host plant tissues. Endophyte obtains nutrition from plant tissues and helps plants against biotic and abiotic stress tolerance. (Sunitha et al., 2013).

The isolated endophytic fungi were screened for extracellular enzymes like amylase, lipase, protease, asparaginase, cellulase and ligninolytic enzymes by the agar plug method. All the fungi can produce one or

other extracellular enzymes. The results showed that 73%, 40%, 33%, 73%, 46%, 40% of the isolates were positive for amylase, lipase, protease, Asparaginase, cellulase and ligninolytic enzymes respectively (Table 3 and Fig. 18). *Fomitopsis meliae* exhibited maximum amylase activity whereas *Phomopsis* sp., *Curvularia lunata*, *Schizophyllum commune*, *Daldinia eschschozia* exhibited significant activity. The highest activity for cellulase and lignin was shown by *Schizophyllum commune*, *Daldinia eschschozia*, *Colletotrichum gigasporum* and *Schizophyllum commune* showed significant activity. The highest activity for protease and lipase was shown by *Lasiodiplodia theobromae* and *Schizophyllum commune*. Most of the isolates showed asparaginase activity. The results indicate that the fungi from *Dillenia indica* L. are a promising source of extracellular enzymes having an immense value in pharmaceutical and industrial applications. Similarly, Uzma et al. (2016) screened 112 fungal endophytes for different enzymes such as cellulase, laccase, pectinase, amylase and asparaginase. From them, 29%, 28%, 18%, 40% and 0% were showed positive activity for cellulase, laccase, pectinase, amylase and asparaginase activity. Thus, these enzymes help to invade the plant tissues. These enzymes also suppress plant pathogens. Endophytes might behave as latent saprophytes; when the host dies, they use these enzymes to degrade the plant tissues to obtain nutrients (Vazquez de Aldana et al., 2013). *Aspergillus* sp. isolated from corneal ulcers/ keratitis produces protease, lipase, DNase, elastase, and keratinase (Sangeetha 2020).

Table 3  
Extracellular enzymatic activity of isolated endophytic fungi.

S.No.	FUNGUS	ENZYMES					
		Amylase	Lipase	Protease	Asparaginase	Cellulase	Ligninolytic
1.	<i>Curvularia lunata</i>	-	-	-	++	-	-
2.	<i>Daldinia eschscholtzii</i>	++	+++	++	+++	++	++
3.	<i>Lasiodiplodia theobromae</i>	+++	++	+++	+	+	-
4.	<i>Schizophyllum commune</i>	+++	-	-	-	+++	+++
5.	<i>Cladosporium cladosporioides</i>	-	+	+	++	-	-
6.	<i>Colletotrichum gigasporum</i>	+		-	+	++	++
7.	<i>Diaporthe phaseolorum</i>	++	-	-	+	+++	+++
8.	<i>Colletotrichum gleosporides</i>	+	-	+++	+++	+	-
9.	<i>Xylaria longipilus</i>	+	-	++	++	++	+
10.	<i>Trichoderma viride</i>	++	++	+	++	+	-
11.	<i>Fomitopsis meliae</i>	++	+	+	+++	++	+
12.	<i>Fusarium oxysporum</i>	-	-	-	-	-	-
13.	<i>Neopestalotiopsis clavispora</i>	++	-	-	++	+	-
14.	<i>Chaetomium globosum</i>	-	+	-	++	+	-
15.	<i>Nigrospora sphaerica</i>	++	+++	++	+++	-	-

- No activity, + less activity, ++ good activity. +++ significant activity.

## 4. Conclusion

The study showed that *Dillenia indica* L. is inhabited by diverse endophytic fungi. The endophytic fungi obtained in this study were only culturable. Many endophytic fungi are obligatory symbiont that could not be isolated and cultured using the conventional isolation approach. The study gives an insight into the diversity of endophytic fungi inhabiting the tissues of *Dillenia indica* L during different seasons. The majority of the isolated endophytic fungi belonging to the class Sordariomycetes of division Ascomycota. To summarise,

tissue type and season influenced the fungal endophyte composition of *Dillenia indica* L. The enzymes produced by the isolated fungi can be used in an industrial scale for the commercial production of valuable enzymes. These fungi need to explore further for their potential bioactive molecules.

## Declarations

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**Conflict of interest:** The authors declare that they have no conflict of interest.

**Availability of data and material:** Data included in this article.

**Code availability:** Not applicable.

**Author's contribution:** Vijay Kumar, carried out experimental work and prepared manuscript. I.B. Prasher supervised the work and refined manuscript.

**Ethics approval and consent to participates:** Not applicable.

**Consent for publication:** The work is original; there is no plagiarism, and it has not been published anywhere.

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## Figures

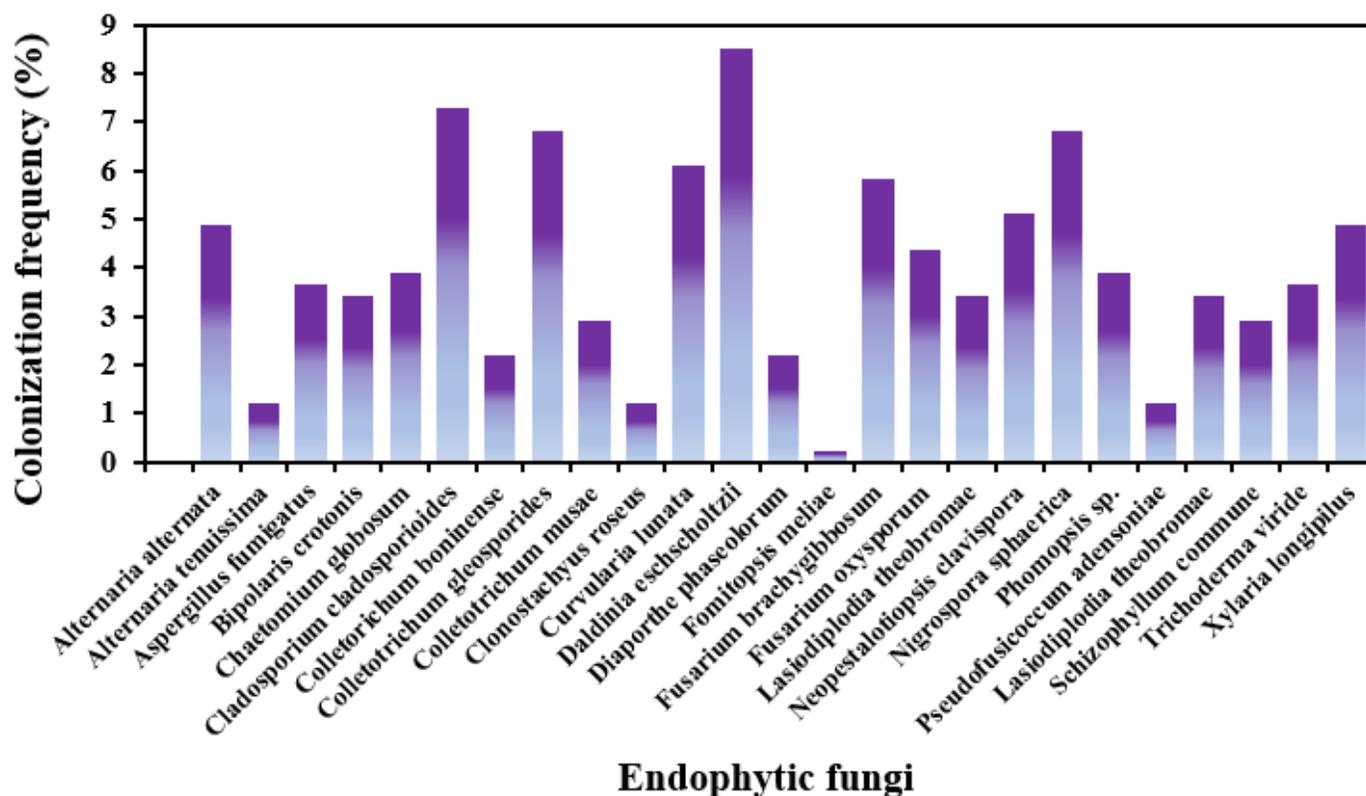


Figure 1

Colonization frequency of endophytic fungi isolated from different parts of *Dillenia indica* L.

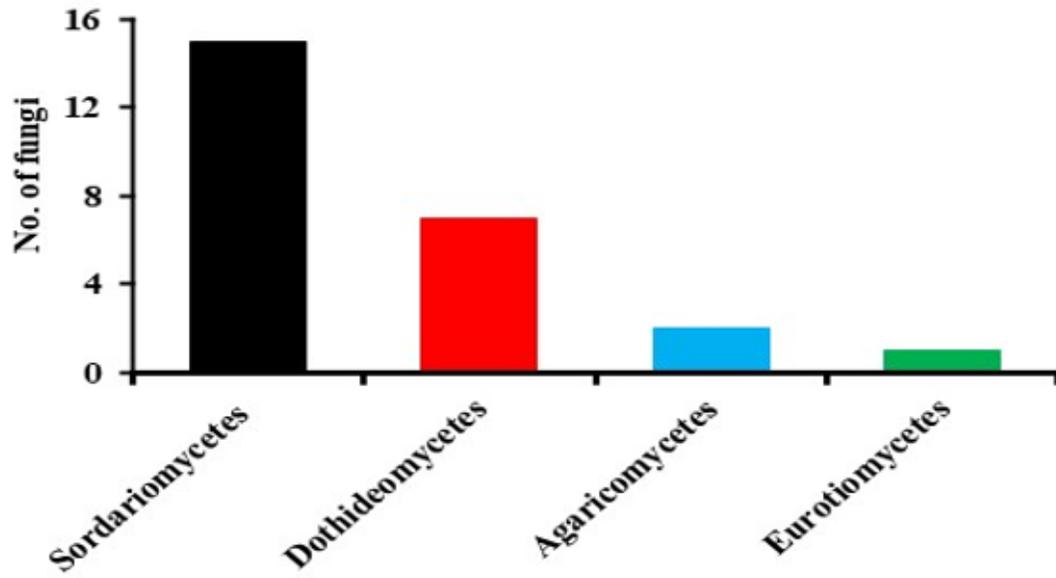


Figure 2

Class-wise distribution (in number) of fungal isolates recovered from *Dillenia indica* L.

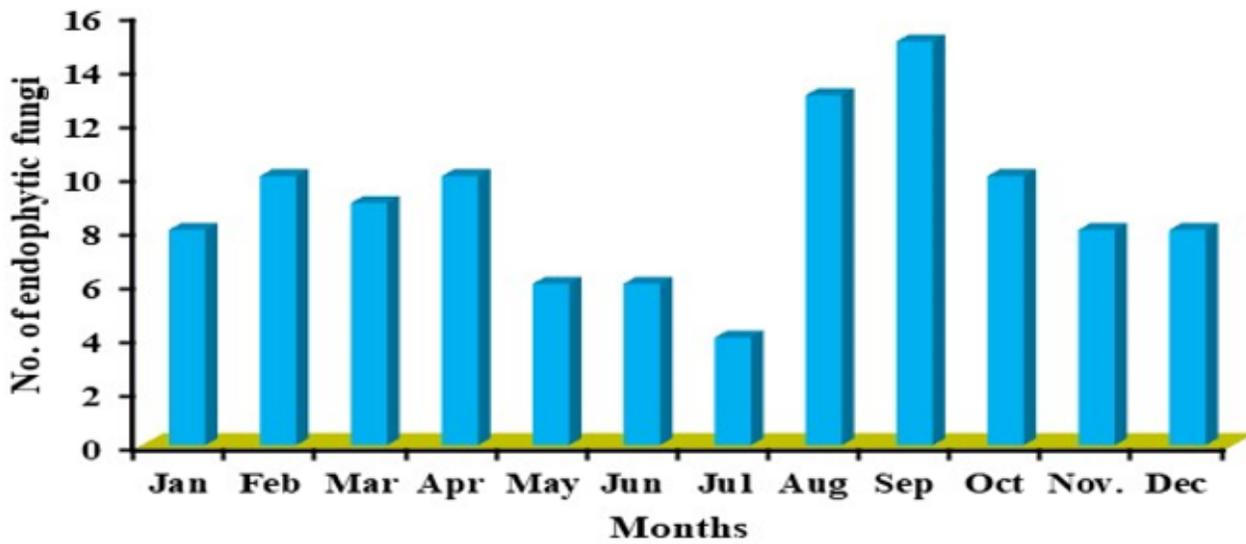
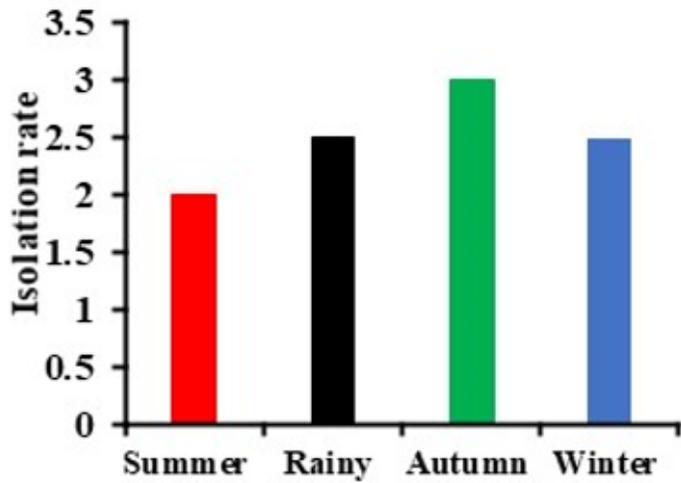


Figure 3

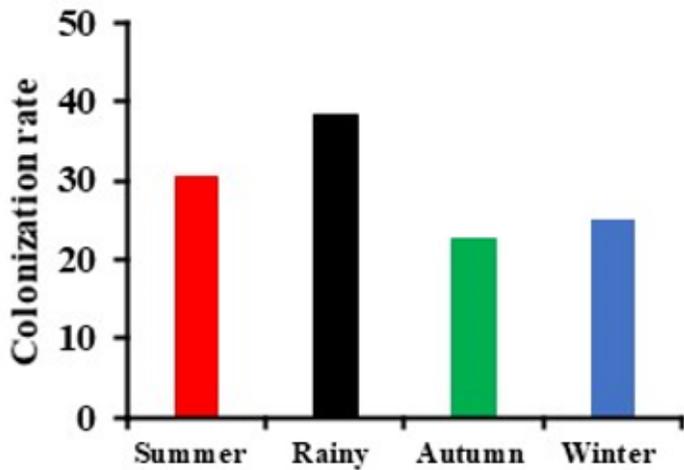
Monthly variations in the number of endophytes isolated during the study.



**Fig.4:** Isolation rate of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 4

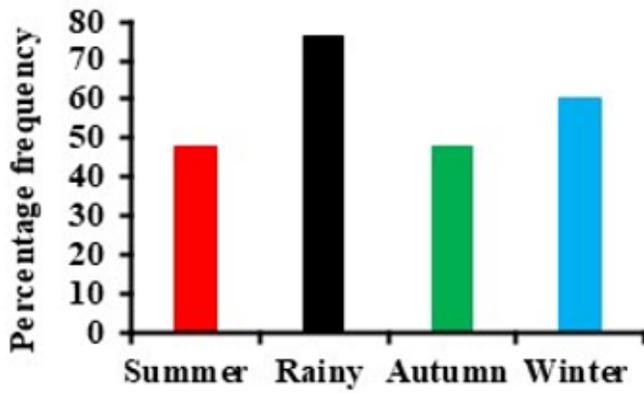
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**Fig.5:** Colonization rate of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 5

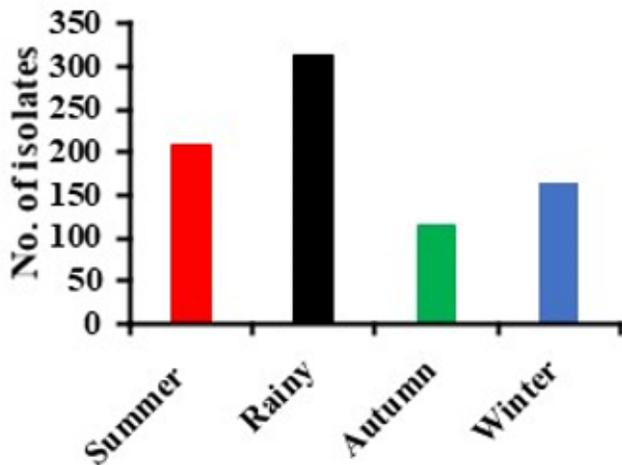
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**Fig.6:** Percentage frequency of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 6

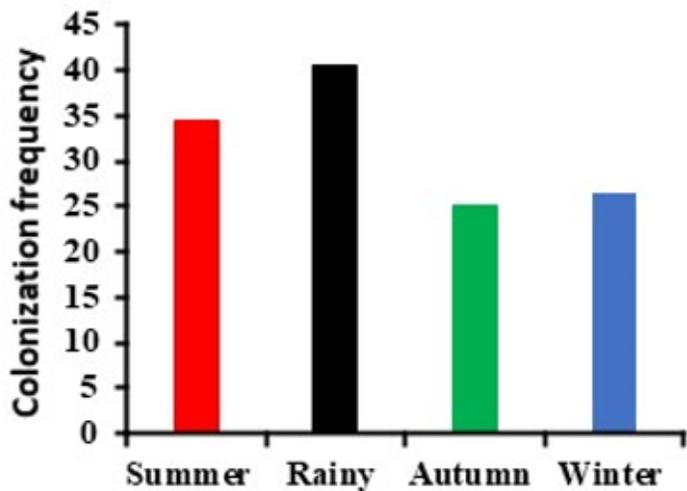
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**Fig.7:** Tissues wise distribution of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 7

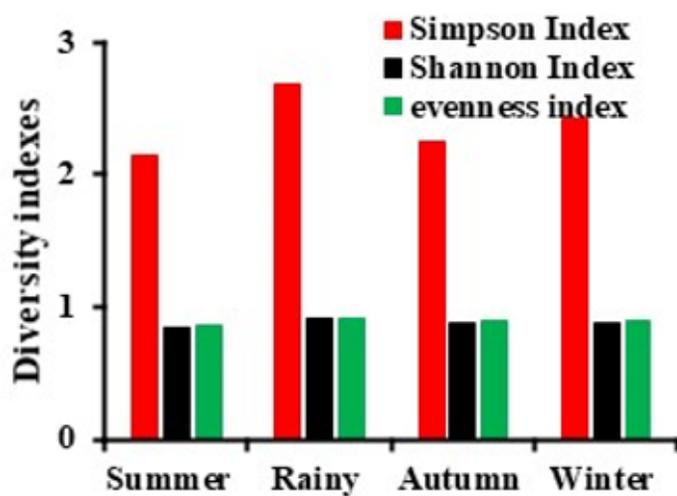
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**Fig.8:** Colonization frequency of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 8

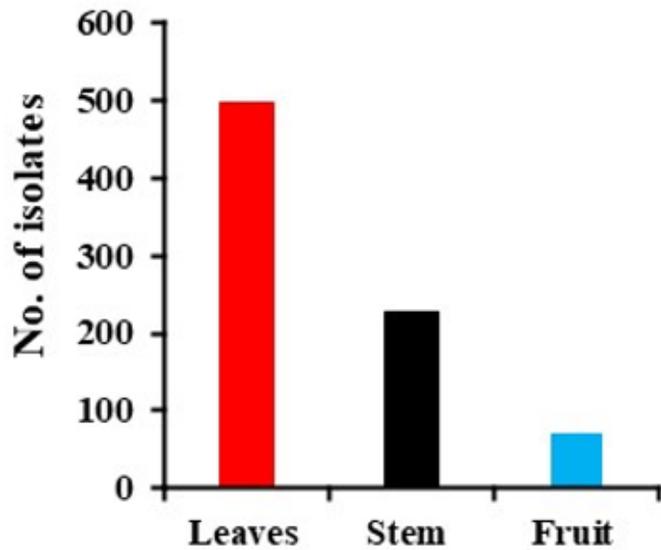
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**Fig.9:** Shanon-Wiener ( $H'$ ), Simpson's ( $D_s$ ) diversity index and Evenness index ( $E$ ) of isolated endophytic fungi from *Dillenia indica* L. during different seasons.

Figure 9

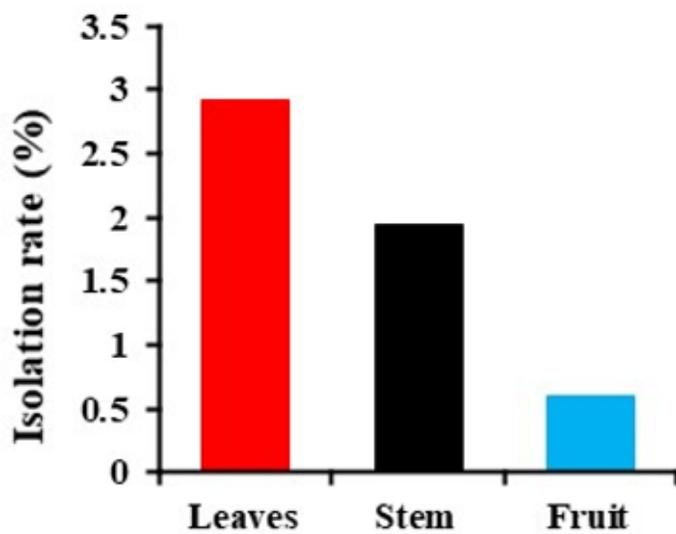
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**Fig 10. Tissue wise distribution of isolated fungal endophytes from *Dillenia indica* L.**

Figure 10

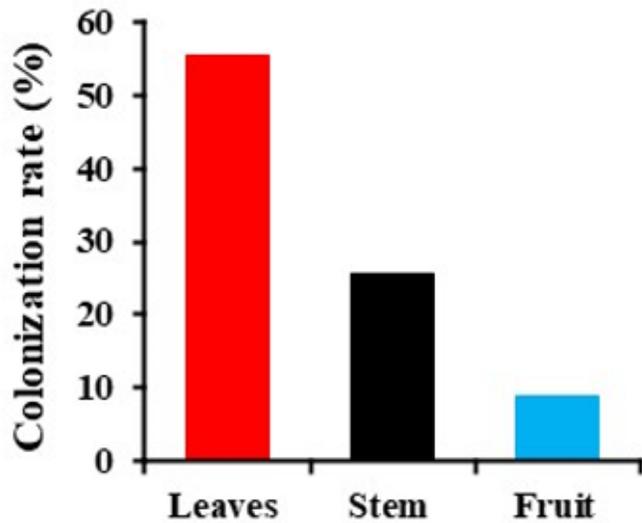
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**Fig 11. Isolation rate of isolated endophytic fungi from *Dillenia indica* L. from different tissues.**

Figure 11

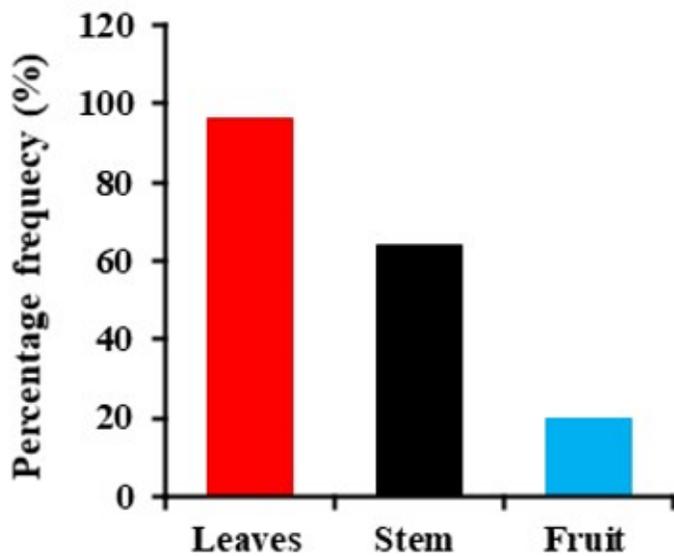
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**Fig 12. Colonization rate of endophytic fungi isolated from *Dillenia indica* L. from different tissues.**

Figure 12

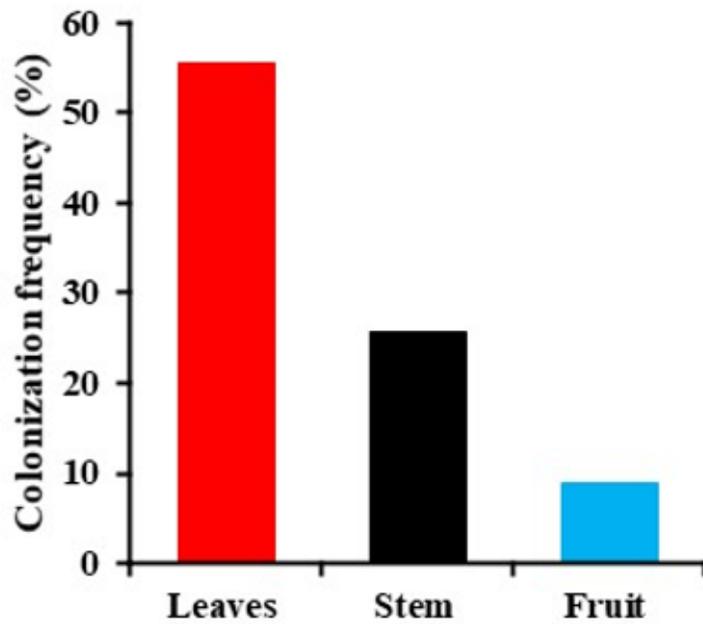
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**Fig 13. Percentage frequency endophytic fungi isolated from *Dillenia indica* L. from different tissues.**

Figure 13

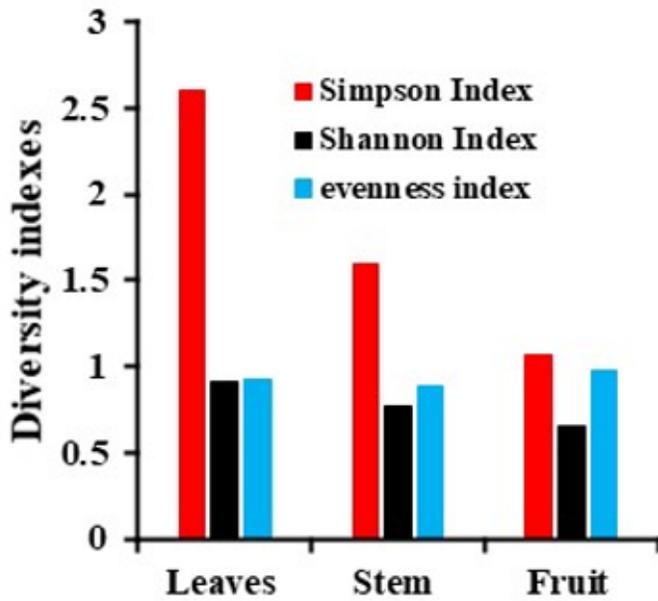
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**Fig 14. Colonization frequency of endophytic fungi isolated from *Dillenia indica* L. from different tissues**

Figure 14

See image above for figure legend.



**Fig 15.** Shannon-Wiener ( $H'$ ), Simpson's ( $D_s$ ) diversity index and Evenness index ( $E$ ) of endophytic fungi isolated from *Dillenia indica* L. from different tissues.

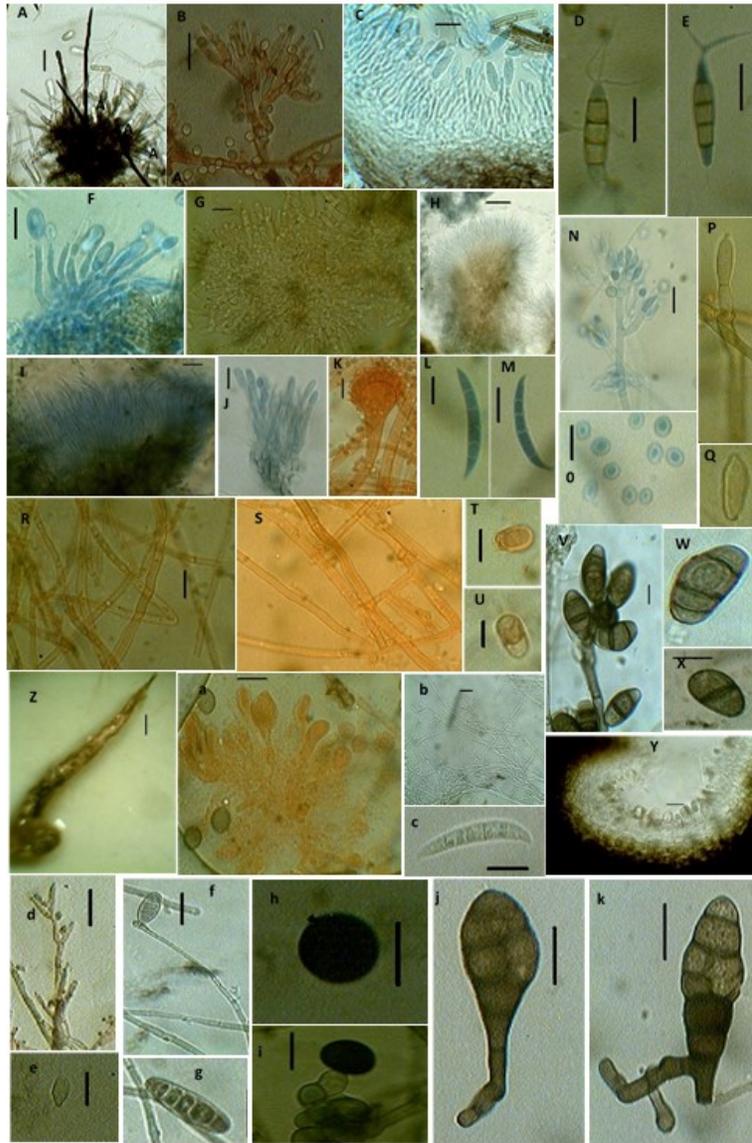
Figure 15

See image above for figure legend.

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Figure 16

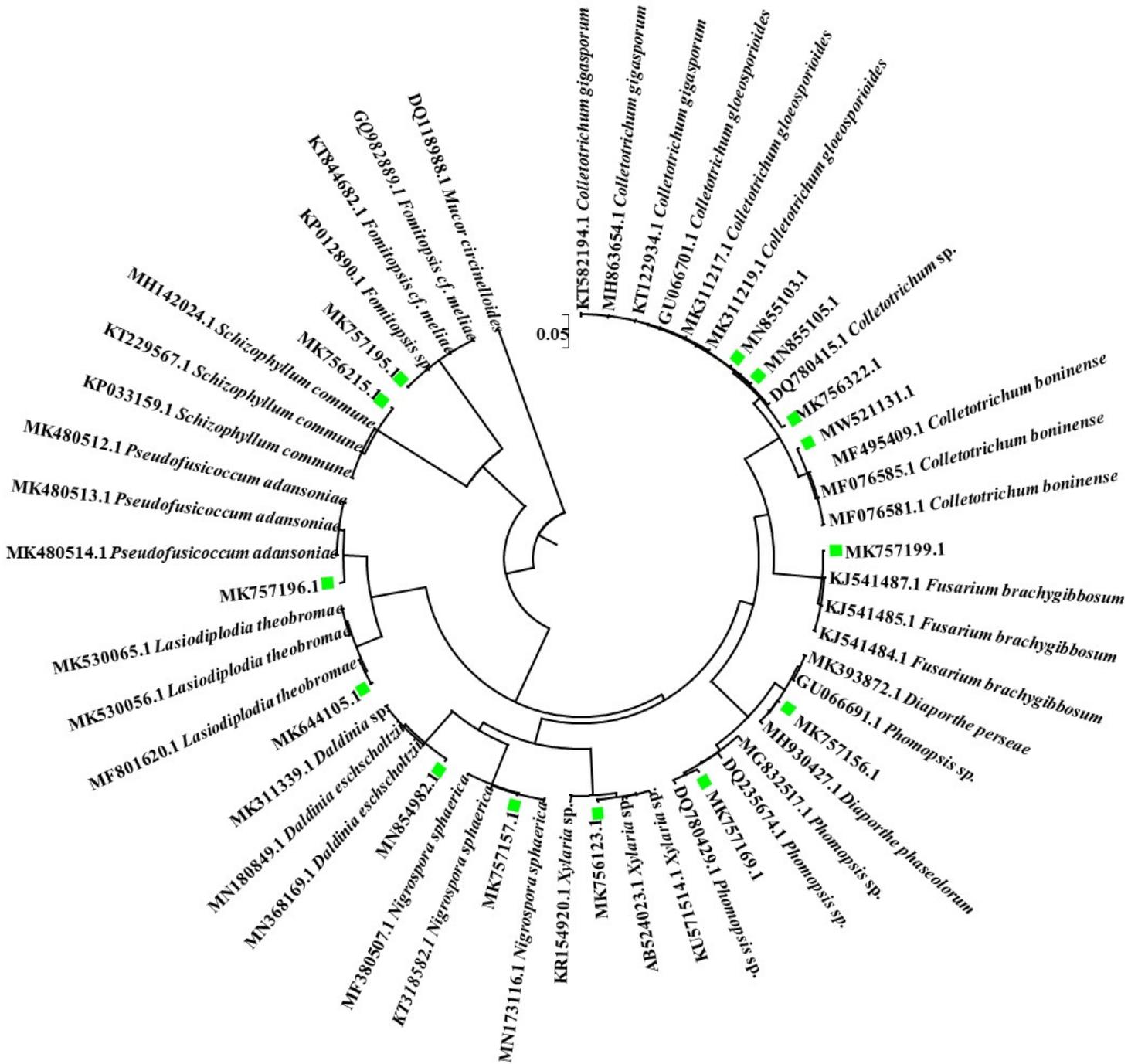
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**Fig 17.** **A:** *Colletotrichum gigasporum*, **B:** *Clanostachyus roseus*, **C:** *Pseudofusicoccum adensoniae*, **D&E:** *Neopestalotiopsis clavispora*, **F:** *Colletotrichum musae*, **G:** *Colletotrichum boninense* **H:** *Diaporthe phaseoli*, **I:** *Phomopsis* sp., **J:** *Colletotrichum gloeosporioides*, **K:** *Aspergillus fumigatus*, **L&M:** *Fusarium brchygibbosum*, **N&O:** *Trichoderma viride*, **P&Q:** *Cladosporium cladosporioides*, **R:** *Schizophyllum cummne*, **S,T&U:** *Fomitopsis meliae*, **V&W:** *Curvularia lunata*, **X&Y:** *Lasiodiplodia theobromae*, **Z:** *Xylaria longipes*, **a:** *Chaetomium globosum*, **b&c:** *Fusarium oxysporum*, **d&e:** *Daldinia eschscholtzii*, **f&g:** *Bipolaris crotonis*, **h&i:** *Nigrospora oryzae* and **j&k:** *Alernaria alternata*.

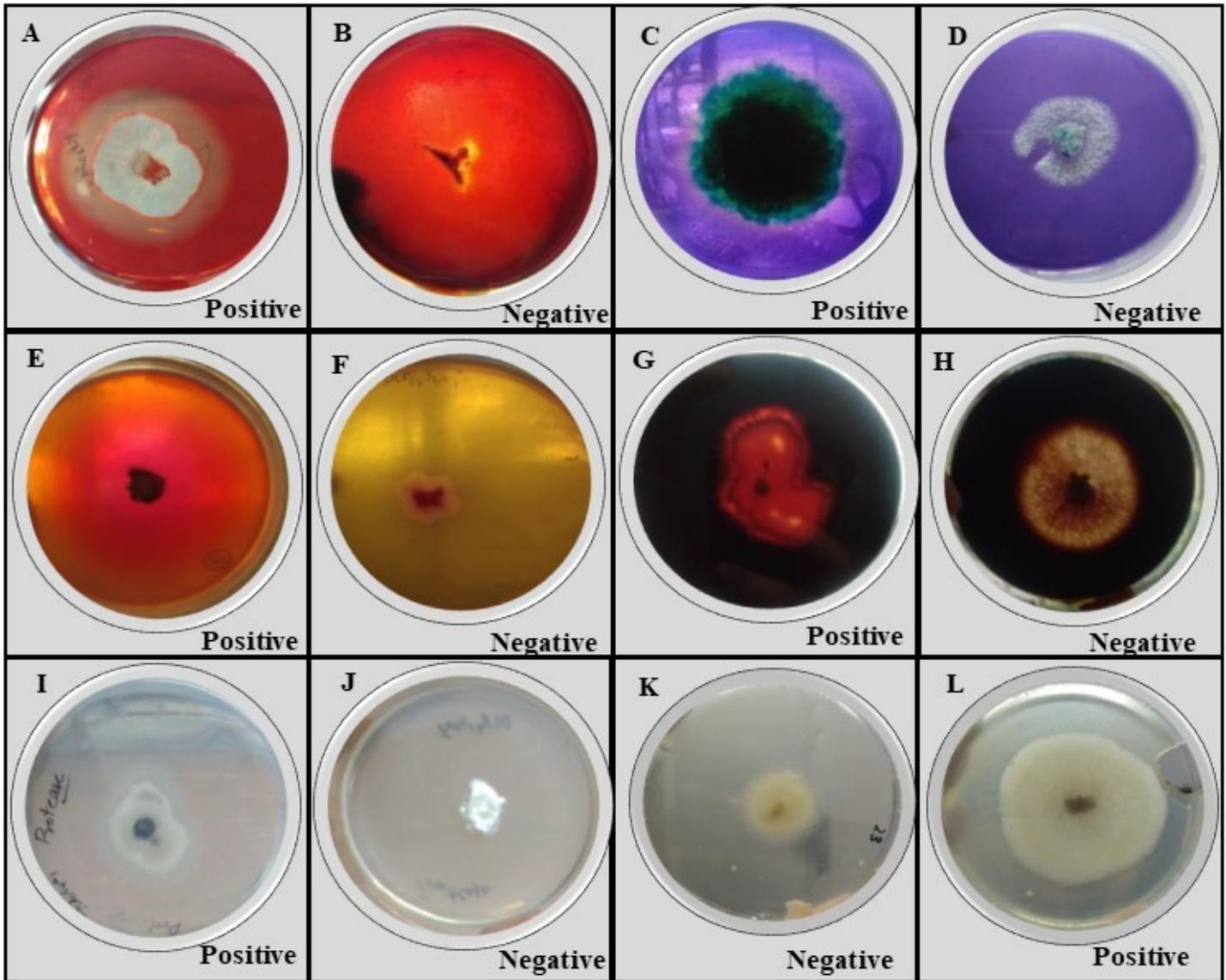
## Figure 17

See image above for figure legend.



**Figure 18**

Evolutionary relationships between fungal taxa isolated from *Dillenia indica* L. The phylogenetic tree based on the internal transcribed spacer (ITS)–rDNA gene sequences of isolated fungi and their related ITS sequences procured from NCBI genebank. The optimal tree with the sum of branch length = 1.79911421 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. There were a total of 342 positions in the final dataset. Evolutionary analyses were conducted in MEGA7



**Fig 19. A-B) Cellulase activity, C-D) Ligninolytic activity, E-F) Asparaginase activity, G-H) Amylase activity, I-J) Protease activity, K-L) Lipase activity.**

**Figure 19**

See image above for figure legend.