Seasonal variation and tissues specificity of endophytic fungi of *Dillenia indica* L. and their extracellular enzymatic activity

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Received: 13 September 2021 / Revised: 17 April 2022 / Accepted: 19 April 2022 / Published online: 20 May 2022
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Abstract
Endophytes are microbes that live inside the tissues of plants without causing any disease. Many of these belonging to fungi have been exploited earlier for their biological activities. This study focused on the exploration and characterization of culturable endophytic fungi inhabiting the medicinal plant *Dillenia indica* L. during four different seasons (summer, monsoon, autumn, and winter) from 2018 to 2019. A total of 2360 segments from different parts (leaves, fruits, and stem) were screened to isolate endophytic fungi. During the study, 25 species of fungi belonging to 20 genera were isolated from the selected plant. The identification of these fungi was validated at morphological, microscopic, and molecular levels. Results indicate the plant has the highest affinity for *Daldinia eschscholtzii*, followed by *Colletotrichum gloeosporioides* and *Cladosporium cladosporioides*. Further, the percent frequency was highest in leaves, followed by stem and fruits. The results were further supported by a similar trend of colonization rate for different plant parts. The monsoon season had the highest number of isolates (312), followed by summer (208), winter (164), and autumn (114). Species diversity was highest during the monsoon season and lowest during the winter. These fungi also produce amylase, lipase, protease, asparaginase, cellulase, and ligninolytic enzymes. This study focused only on culturable fungal endophytes, yet the scope can be extended for other non-culturable microbes and their interaction by using high-throughput genomics and novel next-generation sequencing (NGS) tools. The results indicate that *Dillenia indica* L. harbors novel endophytic fungi having industrial applications.

Keywords Endophytes · *Dillenia indica* · Seasonal variation · Extracellular enzymes · Bioactive molecules

Introduction
Human beings have been dependent on plants for their food and medicines since human civilization. It is estimated that more than 400,000 different plant species exist worldwide, and the majority of them are used to treat various diseases. It is also noted that relatively 80% of the world’s population depends on herbal remedies to address their initial health problems (Joppa et al. 2010). *Dillenia indica* L. is an evergreen flowering plant that belongs to the family Dilleniaceae and is commonly known as elephant apple. It occurs in Bangladesh, China, Indonesia, Nepal, and India. It is an ethnomedical plant having various medicinal properties such as antibacterial, antioxidant, antidiabetic, anticancer, antidiarrheal, and anti-inflammatory. It generally appears in forests, but nowadays people grow it in their gardens because of its lovely blossoms and plump fruits. The mature fruits are plentiful in nutrients, and their extracts have antioxidant properties. The fruit possesses tonic and laxative properties, is eaten up as chutney, made into jam, and relieves abdominal pain. The juice of fruits is used to treat heart complications, fever, cough, diarrhea, and cancer (Singh and Saha 2019; Kumar and Prasher 2021).

Fungi are a large group of microorganisms, having an estimated about 1.5 million; from them, only 75,000 are known species. It is feasible to find fungi in moderate and highly severe climatic conditions in the Earth’s utmost parts. They live in soils, on seas, on the surface of rocks, etc., either as individuals, colonies, or parasites (Blackwell 2011). Adapting to varied habitats enables them to make many secondary metabolites with diverse structures. Approximately,
97,000 secondary metabolites have been isolated from fungal sources (Brakhage and Schroechl 2011). Some of these secondary metabolites have demonstrated biological activities such as antimicrobial, antifungal, antiviral, anti-protozoal, anticancer, and immunosuppressant (Kumar and Prasher 2022). 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). Endophytic fungi are among the poorly recognized groups of fungi, even though they are effective in plant populations and communities (Rodriguez et al. 2009). They reside asymptotically within living tissues of plants studied to date (Li et al. 2007). The diversity and composition of endophytic fungi are influenced by factors such as host plant, area, time, topography, host physiology, tissues, and organs of the host plant (Naik et al. 2009). Endophytes degrade dead and decomposing tissues of plants, which are vital for nutrient cycling in an ecosystem (Saikkonen et al. 2015). Endophytes were reported from plants thriving in various environmental conditions like tropical, temperate, xerophytic, and aquatic habitats (Rather et al. 2018). The abundance and distribution of fungal endophytes generally differ among tree individuals. They are influenced by the host plant’s conditions, sampling time, and the precise location of sampling units (Unterrecher et al. 2007). Therefore, reliable figures of species abundance and composition for a given host tree species require repeated sampling of numerous individuals throughout vegetation seasons (Hoffman and Arnold 2008). It is estimated that there might be at least one million endophytic fungal species. They support their host against insect pests, pathogens, and even domestic herbivores (Malinowski and Belesky 2006). Almost all the plant species (~400,000) harbor one or more endophytic organisms (Tan and Zou 2001). Endophytes protect their host from toxic agents and provide adaptability to live in adverse environmental conditions (Martinez-Arias et al. 2020). To date, merely a few plants have been extensively explored for their endophytic biodiversity.

Several reports are available on the diversity of endophytic fungi derived from medicinal plants. Still, as per the literature survey, no data are available on fungal diversity inhabited in Dillenia indica. 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 fungi for their different extracellular enzymatic activity.

### Materials and methods

#### Study area

The chosen study site was 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.

#### Sample collection and isolation of endophytic fungi

Healthy parts (leaves, stem, and fruits) of *Dillenia indica* were collected from the Botanical Gardens of Panjab University, Chandigarh, India. The plant parts were randomly excised and brought in sterilized plastic bags to the laboratory. The samples were surface sterilized before the isolation of endophytic fungi. The samples were immersed in water for 10–15 min and then washed with distilled water to remove dust and debris. The leaves, fruits, and stem samples were excised into 0.5–1.0 cm segments with surgical blades. For surface sterilization, the cut pieces were soaked in 70% ethanol for 2 min and then disinfected with 3% sodium hypochlorite (NaOCl) solution for 2–3 min and rinsed three times with sterile distilled water, and dried in a laminar airflow chamber (Hallmann et al. 2006). The sterilized samples were plated on Potato Dextrose Agar (PDA) medium supplemented with chloramphenicol (100 μg/mL concentration). 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 could not sporulate. Pure strains were maintained in PDA slant tubes and deposited in the Panjab University Herbarium culture collection (PAN).

#### Identification

##### Morphological identification

To identify the isolated fungi, culture characteristics such as colour and colony appearance were examined after 10 days of incubation at 24 ± 1 °C. 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, spore 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 fungal endophytes do not sporulate when sub-cultured in vitro. Fungal endophytes that fail to sporulate were...
described as sterile fungal endophytes and were subjected to molecular characterization to identify the genus/species.

**Molecular identification of endophytic fungi**

To ascertain identification and phylogenetic link among isolated fungi, the internal transcribed spacer (ITS) regions were used as marks for all fungal species. The purified cultures of fungal isolates were grown on PDA for 10–12 days. 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 deoxyribonucleic acid (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 polymerase chain reaction (PCR). The PCR 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 (Ferreira et al. 2020). 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.

**Estimation and quantification of fungal diversity**

**Percentage frequency (Suryanarayanan et al. 2003)**

\[
PF = \frac{\text{No. of endophytes isolated from a plant tissue}}{\text{Total number of endophytes isolated from that plant}} \times 100.
\]

**Percentage colonization frequency (Suryanarayanan et al. 2003)**

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

**Colonization rate (Sunayana et al. 2014)**

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

**Isolation rate (Sunayana et al. 2014)**

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

**Shannon–wiener (H') index (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.

**Simpson’s (Ds) index (Kusari et al. 2013)**

\[
Ds = 1 - \sum P_i^2.
\]

**Evenness index (E): (Jin et al. 2017)**

\[
E = \frac{H'}{\ln(S)}.
\]

\( S \) = Total no. of the taxa present within each sample.

**Phylogenetic analysis**

Consensus sequences were established for all the isolated fungi and were exposed to the Basic Local Alignment Search Tool (BLAST) on http://www.ncbi.nlm.gov/BLAST in the National Center for Biotechnology Information (NCBI) database. Fungal names were generated by analysing sequences with those already submitted in Genbank. The evolutionary relationship among the isolates was created with the help of MEGA 7 software using the Neighbour-joining method (Nishad et al. 2021). The consensus sequences were incurred from the internal transcribed spacer (ITS) to frame the phylogenetic tree with 500 replications bootstrap.

**Enzymatic screening**

The isolated fungi were screened for cellulase, ligninolytic, amylase, lipase, protease, and asparaginase activity by the plate assay technique. The activity was evaluated by inoculating fungal discs on solid media enriched with a substrate specific to different enzymes: starch to test amylase, gelatine to test protease, Tween 80 to test lipase, carboxymethylcellulose to test cellulase, Azure B to test ligninolytic, and \( \tau \)-asparagine to test asparaginase. The plates were incubated for 7 days at room temperature, and the enzyme activity was measured after that time (Archibald 1992; Dhouib et al. 2005; Patil et al. 2012). The experiment was carried out in triplicate.

**Influence of seasons on fungal colonization**

The effect of seasons on the colonization of endophytic fungi was studied by collecting samples in 2018 and 2019. The endophytic fungi were isolated, identified, and the data was recorded.
Results and discussion

As per our literature survey, the present study about the diversity and seasonal variation of endophytic fungi from *Dillenia indica* l. is the first of its kind. A high diversity of endophytic fungi is associated with different parts of *Dillenia indica* l. A total of 798 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. Only those isolates that differed morphologically were identified as different endophytic fungal isolates. As a result of this, only 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 commune*, *Phomopsis* sp., *Colletotrichum gigasporum*, *Fomitopsis meliae*, *Fusarium breygibbosum*, *Pseudofusicoccum adansoniae*, *Daldinia eschscholtzii*, *Nigrospora sphaerica*, *Xylaria longipes*, *Neopestalotiopsis clavispora*, *Alternaria tenuissima*, *Aspergillus fumigatus*, *Colletotrichum musae*, and *Colletotrichum boninense* from fresh tissues. *Colletotrichum gloeosporioides*, *Cladosporium cladosporioides*, and *Daldinia eschscholtzii* were the most frequently occurring species (Fig. 1). All the isolated fungi belong to four classes, i.e. Sordariomycetes, Dothideomycetes, Agaricomycetes, and Eurotiomycetes. In the current study, some genera (*Curvularia*, *Fusarium*, and *Alternaria*), which are cosmopolitan, are also obtained as endophytes. Various researchers have already reported these taxa from tropical plants (Ferreira et al. 2015). Isolation of endophytic fungi from medicinal plants gives an idea about their distribution in different climatic conditions, geographic locations, and tissue. Various studies were carried out by other scientists from different parts of the world, which provide information on the distribution of endophytic fungi in different medicinal plants. Similar to our studies, 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 24 culturable endophytic fungi from *Oryza rufipogon* Griff and screened for their antagonistic activity against phytopathogens. One hundred fungal endophytes were isolated from black cumin seeds (*Nigella sativa* l.), and *Penicillium*, *Alternaria*, and *Cladosporium* were predominant genera. Most of 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 203 endophytic fungi belonging to 29 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 plant tissues 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 obtained ITS sequences of different

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**Fig. 1** Colonization frequency of endophytic fungi isolated from different parts of *Dillenia indica* l.
### Table 1  Morphological characteristics of some edaphic fungi isolated from *Dillenia indica* L.

| Endophytic Fungus | Cultural characteristics | Microscopic characteristics |
|-------------------|--------------------------|-----------------------------|
| **Curvularia lunata** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline ranges from 3 to 13 μm in width. Conidiophores, simple to branched, septate, arising singly, or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Cladosporium cladosporioides** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Alternaria alternata** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Colletotrichum gloeosporioides** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Bipolaris crotonis** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Chaetomium globosum** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| **Fusarium oxysporum** | Colonies were fast growing, having feathery to cottony black mycelium | Hyphae, branching, septate, hyaline to dark brown, smooth, 3–6.4 μm in width. Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. | Conidiophores, simple or branched, septate, usually simple at the base, becoming dichotomously or irregularly branched at the apex, arising singly or in clusters of up to 20 or more on each apex of hyphae or from intercalary positions on hyphae. Conidia were 15–26 × 0.4–2.1 μm. |
| Endophytic fungi          | Cultural characteristics                                                                 | Microscopic characteristics                                                                                                                                 |
|--------------------------|-------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------|
| **Clonostachys rosea**   | Colonies were powdery; initially, yellowish-white, later turned to citrine green           | Conidiophores were dimorphic. Primary conidiophores verticillum-like, formed throughout the colony, dominating towards the margin; stipes 26–120 × 2–3.5 μm. Secondary conidiophores formed frequently and penicillate-like. Phialides hyaline, mostly in whorls of 3–5, divergent, tapering towards the tip (1–2 μm), with or without a visible collarette, 11–34 × 1.5–3 μm. Conidia globose to subglobose, hyaline, smooth walled, 3–8 × 2–4 μm |
| **Alternaria tenuissima** | Colonies on PDA were olivaceous grey to dark grey                                          | Conidia are dilute tan to brown with dark smooth walls, 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 μm wide |
| **Aspergillus fumigatus** | Colonies spread rapidly, dull blue-green colour, velvety to floccose; reverse colorless to varying shades | Conidial heads columnar, compact, often densely crowded, up to 395 × 50 μm; conidiophores short, smooth, light green, up to 300 μm in breadth, septate, gradually enlarging into a flask-shaped vesicle; vesicles fertile on the upper 1/2 to 2/3, 20–30 μm in diameter, bearing a single series of phialides; phialides closely packed, 6–8 × 2–3 μm; conidia globose to sub globose, green in mass, echinulate, 2.5–3.0 μm |
| **Trichoderma viride**   | Colonies on PDA were fast growing initially creamish-white, turning light green to dark bluish-green due to sporulation | Phialides are straight or sinuous, sometimes hooked, the terminal phialide of whorl and solitary phialides often cylindrical and constricted only below the tip to form a narrow neck. Phialides were 7.6–14 μm long, 2–2.3 μm wide at the base, whereas 3.0–3.8 μm at the widest point. Conidia broadly subglobose to obvoid, mostly 3.0–3.8 × 2.8–3.5 μm, smooth walled, light green to dark green |
| **Neopestalotiopsis clavispora** | Colonies whitish and cottony, undulate edge, wavy surface, circular growth appearance, and reverse of culture pale luteous | Conidia were smooth, fusiform to clavate, five celled (20.1–24.8 × 5.9–7.1 μm), and wider at the middle than at the apex and base (n = 100). Cells at the apex and base of the conidia were hyaline, whereas the third and fourth cells from the base were darker (brown) than the second cell from the base (pale brown). Two to four (three being the most frequently observed) straight hyaline appendages were apparent at the apical cells and one basal appendage |
isolates 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 the phylogenetic tree was constructed (Fig. 2).

There was a significant effect of host tissues on the colonization of fungal endophytes. A total of 498 isolates belonging to 24 taxa were isolated from the leaves, 228 isolates having 16 species from the stem, and 72 isolates belonging to 6 species from fruits. The isolation rate was 5.85, 3.90, and 1.21% for leaves, stem, and fruits. Colonization rate

### Table 2: The endophytic fungi identified based on molecular basis along with their accession number and identity percentage

| S.No | Fungal identification | Code   | NCBI accession no | Identity %
|------|-----------------------|--------|-------------------|-----------
| 1    | Diaporthe phaseolorum | DLP21S4a1 | MK757169.1       | 97%       |
| 2    | Schizophyllum commune | DSP22S3a1 | MK756215.1       | 99%       |
| 3    | Phomopsis sp.         | DLP24S2a1 | MK757156.1       | 98%       |
| 4    | Fomitopsis meliae     | DLP30S2a1 | MK757195.1       | 98%       |
| 5    | Nigrospora sphaerica  | DLP46S2a1 | MK757157.1       | 100%      |
| 6    | Lasiodiplodia theobromae | DSP25S4a1 | MK644105.1       | 99%       |
| 7    | Fusarium brachygibbosum | DLP41S3a1 | MK757199.1       | 99%       |
| 8    | Colletotrichum gigasporum | DSP26S2a1 | MK756322.1       | 99%       |
| 9    | Xylaria longipes      | DLP41S2a1 | MK756123.1       | 99%       |
| 10   | Pseudofussicoccum adansoniae | DSP42S2a1 | MK757196.1       | 99%       |
| 11   | Daldinia eschscholtzii | DSP40S2a1 | MN854982.1       | 99.6%     |
| 12   | Colletotrichum gloeosporioides | DLP31S1a1 | MN855105.1       | 99.6%     |
| 13   | Colletotrichum boninense | DSP32S3a3 | MW521131.1       | 99.87%    |

Fig. 2: Evolutionary relationships between fungal taxa isolated from *Dillenia indica*. The phylogenetic tree is based on the internal transcribed spacer (ITS)–rDNA gene sequences of isolated fungi and their related ITS sequences procured from the NCBI GenBank. The evolutionary distances were computed using the neighbour-joining method and are in the units of the number of base substitutions per site. Evolutionary analyses were conducted in MEGA7.
was found to be 55.6, 25.6, and 08.8% for leaves, stem, and fruits. Similarly, the values of percentage frequency were 96% for leaves, 64% for the stem, and 20% for fruits. Thus, results clearly show that fungal endophytes are more in leaves than stem and fruits. The colonization frequency was 61.2, 28.9, and 9.01 for leaves, stem, and fruits. The values of diversity indices, i.e. Simpson’s, Shannon–Wiener, and evenness index, indicate that the tissues are highly diverse. The highest value of the Simpson’s index was observed in leaves, i.e. 2.6, whereas the lowest value was 1.07 and was recorded in fruits. The present data also revealed the highest Shannon–wiener index in the leaves, suggesting that the leaves have a more diverse endophytic fungal community than the fruits and stem (Fig. 3).

The study showed that the seasons influenced the composition of fungal endophytes in *Dillenia indica* L. Monsoon (rainy season) had the highest number of isolates (312), followed by summer (208), winter (164), and autumn (115). During the monsoon season, the dominating taxa were *Colletotrichum gloeosporioides*, *Neopostalotiopsis clavispora*, *Fusarium brachygibbosum*, and *Daldinia eschscholtzii* followed by *Alternaria alternata*, *Fusarium oxysporum*, *Chaetomium globosum*, and *Alternaria tenuissima*. Species diversity was highest during the monsoon season (19 species) and lowest during the winter (12 species). The highest colonization frequency was in the monsoon season (37.89%), followed by autumn (32.5%), summer (30.3%), and winter (25%). However, species diversity data somewhat correlate 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. Similarly, the colonization rate ranges from 25.0 to 38.4% for endophytic fungi obtained in the present study during different seasons. It is important to note that the seasons directly affect the composition of fungal communities (Fig. 4). The numbers of fungal endophytes obtained during different months of the years also varied significantly. The maximum isolates were obtained during September, followed by August, March, and April, and the minimum during July. 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. *Schizophyllum commune* was found in the monsoon and winter seasons. The seasonal variation in colonization patterns may be due to the seasonal activities of fungal endophytes. Similar to our study, during the monsoon (rainy) season, greater endophytic fungal diversity was also observed in Terminalia arjuna twigs and bark (Tejesvi et al. 2005). The rainy (monsoon) and summer seasons, as previously stated, support high endophytic fungi expression. 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 than in the rainy and summer months. The seasonal variation in fungal endophytic diversity may be due to secondary metabolite levels fluctuating throughout the year (Fang et al. 2013). Seasons influence endophytic actinobacterial communities in medicinal plants (Barman and Dkhar 2020). The composition and richness of fungal endophytes in the tropics are designed by seasons and climate (Oita et al. 2021). Plant age and season timing enhanced the growth of endophytes and alkaloids production by the host plant (Fuchs et al. 2017). Tissue type, location, and seasons directly influence the assemblage of fungal endophytes in Tectona grandis. Leaves and monsoon season host more endophytes than stem and bark during different seasons. Location 5 had more endophytes than other sites (Singh et al. 2017). The endophytic fungal community in Citrus sinensis was affected by the seasons, tissues, and age. The maximum number of isolates were obtained from leaves followed by bark and xylem. Higher diversity of fungi obtained in winter followed by summer, autumn and spring (Juybari et al. 2019). The colonization of endophytic fungi in crucifers was influenced by seasons, plant tissues, plant identity, and environmental factors. Stems harbor more endophytes than leaves and roots. Higher diversity was found in autumn and lowest during the winter. Fungal communities inside the Camellia sinensis were affected by spatial and temporal variations (Wu et al. 2020). There was higher diversity of endophytic fungi in twigs than in leaves (Win et al. 2018). The older leaves harbor more endophytes than the younger leaves (Taufiq and Darah 2018).

The majority of the isolated fungi belong 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. The Simpson index for leaves, fruits, and stem were 2.60, 1.598, and 1.077, while Shannon–weiner index was 0.919, 0.776, and 0.892 for leaves, stem, and fruits. The evenness index was 0.937, 0.892, and 0.980 for leaves, stem, and fruits. It was seen that only one or a few species dominated the endophytic community of the host, whereas the majority of them were rare. Numerous factors such as biotic, abiotic, chemical composition, and architecture of host tissues are involved in the colonization of endophytic fungi in specific tissues (Liu et al. 2012).

**Extracellular enzyme productions**

The endophytes produce various bioactive molecules, including 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 against biotic and abiotic stress. 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).

The isolated endophytic fungi were screened for 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, and 40% of the isolates were positive for amylase, lipase, protease, asparaginase, cellulase, and ligninolytic enzymes, respectively [Table 3 and (Fig. 5)]. *Fomitopsis meliae* exhibited maximum amylase activity, whereas *Phomopsis* sp., *Curvularia lunata*, *Schizophyllum commune*, *Daldinia eschscholtzii* exhibited significant activity. The highest activity for cellulase and lignin was shown by *Schizophyllum commune*, *Daldinia eschscholtzii*, *Colletotrichum gigasporum*, and *Schizophyllum commune*. 

![Fig. 4](image-url) Seasonal variations: (A) Isolation rate. B Colonization rate. C Number of isolates. D Percentage frequency. E Colonization frequency. F Diversity indexes of endophytic fungi isolated from *Dillenia indica* during different seasons of 2018 and 2019.
showed the highest activity for protease and lipase. Most of the isolates showed asparaginase activity. The results indicate that the fungi from *Dillenia indica* are a promising source of extracellular enzymes with immense value in pharmaceutical and industrial applications. Similarly, Uzma et al. 2016, screened 112 fungal endophytes for cellulase, laccase, pectinase, amylase, and asparaginase enzymes. Of them, 29, 28, 18, and 40% of isolates showed positive activity for cellulase, laccase, pectinase, and amylase activity. In contrast, these strains did not demonstrate asparaginase activity. Thus, these enzymes help to invade the plant tissues. These enzymes also suppress plant pathogens. Elango et al. 2020, evaluated the extracellular enzyme activity of *Aspegillus sojae* isolated from *Plectranthus amboinicus*. The fungus was screened for amylase, cellulase, lipase, and laccase. The strain produces all the enzymes studied. Endophytic fungi isolated by Sopalun and Iamtham 2020 from five different orchids such as *Doritis pulcherrima, Dendrobium phyllum, Dendrobium anosmum, Ascocentrum curvifolium*, and *Aerides falcata* were screened for amylase, protease, cellulase, pectinase, and lipase. The results showed that 67, 56, 49, 39, and 25% of the isolates produce lipase, cellulase, pectinase, protease, and amylase.

**Conclusion**

The study showed that *Dillenia indica* is inhabited by diverse endophytic fungi. The endophytic fungi obtained in this study were only culturable. Many endophytic fungi are obligatory symbionts that cannot 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* during different seasons. The
majority of the isolated endophytic fungi belong to the class Sordariomycetes of division Ascomycota. To summarise, tissue type and season influenced the fungal endophyte composition of *Dillenia indica*. The enzymes produced by the isolated fungi can be used on an industrial scale to produce valuable enzymes commercially. These fungi need to explore further for their potential bioactive molecules.

Acknowledgements The authors acknowledge the Department of Botany, Panjab University Chandigarh, India, for providing infrastructure and instrumentation. Vijay Kumar is also thankful for the Senior Research Fellowship (File No. 09/135(0854)/2019-EMR-I) by the Council of Scientific and Industrial Research (CSIR), India, during research work.

Author contribution VK carried out experimental work and prepared the manuscript. IBP supervised the work and refined the manuscript.

Funding The research work was not funded by any agency.

Data availability Data are included in this article.

Code availability Not applicable.

Declarations

Conflict of interest The authors declare no conflict of interest.

Ethics approval and consent to participate Not applicable.

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

Table 3 Extracellular enzymatic activity of isolated endophytic fungi

| S.No | Fungus                  | Amylase | Lipase | Protease | Asparaginase | Cellulase | Ligninolytic |
|------|-------------------------|---------|--------|----------|--------------|-----------|--------------|
| 1    | *Curvularia lunata*     | −       | −      | −        | +            | −         | −            |
| 2    | *Daldinia eschscholtzii*| + +     | + + +  | + +      | + + +        | + +       | + +          |
| 3    | *Lasiodiplodia theobromae* | + + +  | + +    | + + +    | +            | +         | −            |
| 4    | *Schizophyllum commune*  | + + +   | −      | −        | −            | + +       | + +          |
| 5    | *Cladosporium cladosporioides* | −      | +      | +        | −            | −         | −            |
| 6    | *Colletotrichum gigasporum* | +      | −      | +        | + +          | + + +     | + +          |
| 7    | *Diaporthe phaseolorum*  | + +     | −      | −        | + + +        | + +       | + +          |
| 8    | *Colletotrichum gloesporioides* | +      | −      | + + +    | + + +        | + +       | −            |
| 9    | *Xylaria longipes*       | +       | −      | + + +    | +            | + + +     | +            |
| 10   | *Trichoderma viride*     | + +     | + +    | +        | +            | + +       | −            |
| 11   | *Fomitopsis meliae*      | + +     | +      | + + +    | +            | + +       | +            |
| 12   | *Fusarium oxysporum*     | −       | −      | −        | −            | −         | −            |
| 13   | *Neopestalotiopsis clavispora* | + +    | −      | −        | + +          | +         | −            |
| 14   | *Chaetomium globosum*    | −       | −      | −        | + + +        | + +       | +            |
| 15   | *Nigrospora sphaerica*   | + +     | + + +  | + + +    | −            | −         | −            |

− No activity, + less activity, + + good activity. + + + Significant activity

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