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Diversity of endophytic fungi associated with *Dillenia indica* L., an ethnomedicinal plant

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Abstract

Endophytic fungi are an important source of novel bioactive molecules having broad applications in agriculture, pharmaceutical and medical industries. In the present study, endophytic fungi were isolated from *Dillenia indica* L. and characterized on the basis of morphological and molecular approaches. Twenty-five different endophytic fungi belonging to twenty genera i.e., *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 brachygibbosum, Pseudofusicoccum adansoniae, Daldinia eschscholtzii, Nigrospora sphaerica, Xylaria longipes, Neopestalotiopsis clavispora, Alternaria tenuissima, Aspergillus fumigatus, Colletotrichum musae* and *Colletotrichum boninense* were isolated and identified from different parts (leaves, fruits and stems) of *Dillenia indica.* To the best of our knowledge, *Colletotrichum gigasporum* is being reported for the first time from northwestern India and this is the first report on endophytic fungal diversity from *Dillenia indica* L.

Keywords – Agricultural – Industries – Knowledge – Morphological – Pharmaceutical – *Pseudofusicoccum adansoniae*

Introduction

Endophytes are microbes (bacteria, fungi, or actinomycetes) that reside inside the tissues of plants for the whole or part of their life cycle without causing any disease to the host (Strobel 2012). These are ubiquitous microorganisms, reported in almost all the vascular plants studied to date (Hardoim et al. 2015). They are found in every part of plants, i.e., leaves, fruits, flowers, stems, roots and seeds and are transmitted by horizontal or vertical means (Bacon & White 2000, Hartley & Gange 2009). These microbes have potential applications in agricultural, pharmaceutical and other industries.

Fungi are a large group of microorganisms, having an estimated number of about 1.5 million, out of which approximately 75,000 species are known (Hawksworth & Lucking 2017). Endophytic fungi play a crucial role in the life cycle of plants by facilitating the uptake of nutrients for their growth and protecting them against biotic and abiotic stresses (Dias et al. 2012, Bilal et al. 2018, Rana et al. 2020). Moreover, these fungi also increase the growth and development of plants by

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enhancing resistance against abiotic stresses (extremely high or low temperature, drought, low or high pH, heavy metals and salinity) and biotic stresses (pathogens and herbivores) (Hallmann et al. 2007). They are an essential source of novel bioactive compounds (Tiwari et al. 2015, Qader et al. 2017). Therefore, isolation of fungal endophytes from different plant species will help in discovering new fungal species which can be used in an industrial progress (Aly et al. 2011).

In India, huge plant diversity offers scope for exploring different fungi (Kumar & Kaushik 2013). From the last few decades, endophytic fungi have attracted scientists and it has become the center of attraction for research in the present time. Various scientists have studied endophytic fungi from different medicinal plants (Kharwar et al. 2012, Gond et al. 2102, Ginting et al. 2013, Huang et al. 2015, Manganyi et al. 2018, Arora et al. 2019, Al-Rashdi et al. 2020, Kumar & Prasher 2021). *Dillenia indica* L. is an important medicinal plant having antimicrobial, antioxidant, analgesic, anti-inflammatory, dysentery, anti-diabetic, and antileukemic properties (Chowdhury et al. 2013, Singh et al. 2016). Gogoi et al. (2008) studied the antimicrobial activity of the endophytic fungus *Hypocrea* spp. isolated from *Dillenia indica* L. and optimized the cultural conditions for the production of bioactive metabolites. As per the literature survey, very limited work has been done related to the diversity of endophytes from the genus *Dillenia.* The present study aimed to isolate culturable endophytic fungi from *Dillenia indica* L. and their phylogenetic relationship.

Materials & Methods

Study area and samples collection

Fresh, healthy and disease-free samples of *Dillenia indica* L. were collected from Botanical Gardens of Panjab University Chandigarh (Latitude 30.760618 and longitude 76.765388), India. The samples were collected during different seasons of the years 2018 and 2019. The samples were collected in sterile polythene bags and brought to the laboratory to isolate fungal endophytes. A total of 2360 segments from different parts like leaves (820), stems (820) and fruits (720) were screened to isolate endophytic fungi. The plant specimen was authenticated on botanical characteristics and submitted to the Herbarium of Panjab University Chandigarh, India (PAN: 22063).

Isolation of endophytic fungi

The collected samples were first washed with tap water and allowed to dry for 1-2 hrs. The explants were sterilized with ethanol, sodium hypochlorite (NaOCl) solution and distilled water. The leaves, fruits and stems were first cut into segments of about 0.5-1.0 cm in length with the help of a sterilized surgical blade. The chopped pieces were first rinsed with 80% ethanol for 2 minutes, then 3% sodium hypochlorite for 2-3 minutes. Afterwards, the segments were again immersed in 90% ethanol for 2 minutes and then rinsed thrice with sterilized distilled water and allowed to dry in the laminar airflow (Hallmann et al. 2007). The sterilized explants were transferred to the petri plates containing Potato Dextrose Agar (PDA) supplemented with chloramphenicol (100 µg/mL). The plates were incubated at 24ºC for 15 days and observed regularly. The fungal mycelium emerging from the tissue segments was transferred to fresh PDA plates. The pure cultures were obtained by the single hyphal tip isolation method after repeated sub-culturing. The effectiveness of the surface sterilization procedure was confirmed by spreading out last rinse water on plates containing PDA. Afterwards, the plates were incubated at 24°C±1 for 10 days (Schulz et al.1993).

Morphological identification of isolated endophytic fungi

Preliminary identification was done by studying the cultural characteristics of the fungi, i.e., colony growth, colour, shape, etc. The morphological characters were examined by growing cultures on PDA plates for 30 days at 24°C±1. Microscopic observations (Conidiophore, conidia and mycelial characters) were carried out by preparing slides stained with cotton blue and congo red and observed under the microscope (Matrix VRS-2f).

Molecular identification of isolated endophytic fungi

For molecular analysis, DNA was isolated from the mycelium of fully-grown cultures by using a fungal DNA extraction kit (Qiagen DNeasy Mini Plant Kit). The isolated DNA was quantified on 2% gel electrophoresis. The Internal Transcribed Spacer (ITS) region was amplified by PCR (BioRad, Hercules, CA, United States) using ITS1 & ITS4 primers (White et al. 1990). The amplified regions were sequenced by PGIMER Chandigarh, India. The obtained ITS sequences were used to find the matching sequences from the NCBI GenBank sequence database using the nBLAST software in NCBI. The obtained ITS sequences were submitted to the NCBI GenBank database for accession number.

Phylogenetic analysis

The obtained ITS sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) in the NCBI database and compared with the sequences found in the NCBI GenBank database. Multiple sequence alignment was performed on the sequences using CLUSTAL W, and gaps were eliminated from the sequences. The MEGA 7 software was used to create the phylogenetic tree using highly relevant sequences and the maximum parsimony method. *Mucor circinelloides* was considered as an outgroup.

Statistical analysis

The diversity and richness of fungal endophytes isolated from different tissues of *Dillenia indica* L. were quantified using various indices such as Percentage frequency, Colonization frequency, Colonization rate, Shannon-Wiener, Simpson's and Evenness index.

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

% Colonization frequency (Suryanarayanan et al. 2003)

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

Colonization rate (Sunayana et al. 2014)

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

Isolation rate: (Sunayana et al. 2014)

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

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

$$\textbf{(H')} = -\sum P_i \times \text{Ln}\,(P_i),$$

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

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

$$Ds = 1 - \Sigma Pi^2$$

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

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

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

Relative abundance was calculated by numbering the total number of isolates of each taxon.

Results

A total of 798 fungal isolates belonging to 20 genera comprising 25 different taxa were isolated from 2360 tissue segments of *Dillenia indica* L. The isolated fungi were identified on morphological and molecular basis. The isolated taxa were *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 brachygibbosum, Pseudofusicoccum adansoniae, Daldinia eschscholtzii, Nigrospora sphaerica, Xylaria longipes, Neopestalotiopsis clavispora, Alternaria tenuissima, Aspergillus fumigatus*, *Colletotrichum musae*, and *Colletotrichum boninense*. The morphological characteristics and the microphotographs of isolated endophytic fungi are given below in Table 1 and Figs. 1-25. The isolated fungi were identified on the molecular level by comparing the obtained ITS sequences to the sequences available in the GenBank database. Table 2 lists the identified fungi along with their GenBank accession numbers and identity percentage. Phylogenetic tree was constructed using ITS 1 / 5.8S rDNA / ITS 2 sequences with the help of MEGA 7 software. The evolutionary link between the several endophytic fungi under study is shown in the phylogenetic tree (Fig. 26). The highest number of fungal species were isolated from leaves (24 species), followed by stems (16 species) and fruits (5 species). It has been observed that leaves harbor more endophytes than stems and fruits. The results indicate that the distribution of endophytic fungi inside the host plant differs among different tissues. The majority of the isolated fungi, i.e., approximately 88% of the fungi belong to Ascomycota, followed by Basidiomycota (8.0%) and Zygomycota (4.0%) (Fig. 27).

Table 1 Morphological characteristics of endophytic fungi isolated from *Dillenia indica* L.

S. No.	Endophytic fungi	Morphological characteristics
1.	*Alternaria alternata*	Colonies grey or olivaceous black, Conidia long, obclavate, obpyriform, ovoid or ellipsoid, often with a short conical or cylindrical beak, pale to mid golden brown, smooth or verruculose, with up to 8 transverse and usually several longitudinal or oblique septa, 15–58 × 7.5–16 µm. Conidiophores simple or branched, arise singly or in groups, up to 47 µm long, 3–6 µm thick.
2.	*Alternaria tenuissima*	Colonies olivaceous grey to dark grey. Conidia solitary or in short chains, straight or curved, obclavate, generally with 4–7 transverse and several longitudinal or oblique septa, overall length 22–75 × 8–15 µm thick. Conidiophores solitary or in groups, simple or branched, septate, pale or mid pale brown, up to 115 µm long, 3– 6 µm thick.
3.	*Aspergillus fumigatus*	Colonies dull blue-green colour. Conidia globose to sub globose, green in mass, echinulate, 2.5–3 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.

Table 1 Continued.

S. No.	Endophytic fungi	Morphological characteristics
4.	*Bipolaris crotonis*	Colonies dark grey-black in colour. Conidia olivaceous brown to pale brown, smooth uniformly pigmented, broadly ellipsoidal, 6-10 (mostly 6-9) distoseptate, 80–108 × 18–29 µm. Conidiophores arising terminally or laterally on hyphae, simple sometimes branched, pale olivaceous to brown, septate, straight to flexuous, geniculate above, pale toward apex 50–250 × 6.5– 7.0 µm.
5.	*Cladosporium cladosporioides*	Colonies were mostly olivaceous-brown. Conidia solitary or catenate, in unbranched or branched acropetal chains, usually obovoid, ellipsoid, fusiform, 3–6 × 2–3.5 µm. Conidiophores mononematous or macronematous, solitary, fasciculate, usually erect, branched or unbranched subhyaline, smooth to verruculose, usually sympodial.
6.	*Chaetomium globosum*	Colonies were initially white, becoming citrine green to yellow-greenish. Asci fasciculate, fusiform or clavate, spore-bearing part 25–40 × 5–10 µm, stalks 15–20 µm long. Each ascus consists of eight biseriate 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.	*Clonostachys rosea*	Colonies powdery, initially yellowish-white later turned citrine green. Conidia globose to subglobose, hyaline, smooth-walled, 3–8 × 2–4 µm. Primary conidiophores verticillium-like, formed throughout the colony, dominating towards the margin; stipes 26–120 × 2–3.5 µm. Secondary conidiophores formed frequently and penicillate. 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.
8.	*Colletotrichum boninense*	Colonies white to black. Conidia aseptate oval, cylindrical with rounded and slightly tapered ends, ranging from 10–15 × 4–6 µm. Conidiophores cylindrical hyaline to subhyaline branched or unbranched, aseptate or septate, up to 35 µm long.
9.	*Colletotrichum gigasporum*	Colonies white turned black at maturity. Conidia large in size ranging from 19.5–29 × 5.04–7.84 µm. Conidiophore elongated, conidia abundant and are hyaline, aseptate, straight, cylindrical having obtuse apices.
10.	*Colletotrichum gloeosporioides*	The colonies initially white turned greyish as mature. Conidia were cylindrical, having 14–20 µm in length and 5–6 µm diameter. Conidiophores cylindrical, hyaline to subhyaline, tapered toward the apex; up to 32 µm long.
11.	*Colletotrichum musae*	Colonies white to grey. Conidia were hyaline, aseptate, guttulate, oval, elliptical or and cylindrical with obtuse to slightly round ends, ranging from 8.0–15.5 × 2.5–5 µm. Conidiophores cylindrical, hyaline to subhyaline toward the base, tapered toward the apex; up to 30 µm long.
12.	*Curvularia lunata*	Colonies black. Conidia 15.5–26 × 9–13 µm, 3–5 celled, asymmetrical to more or less curved at the third cell from the base. Conidiophores simple to branched, septate, arising singly from hyphae, subhyaline to dark brown, 90.0–196.0 × 4.0–6.0 µm.

Table 1 Continued.

S. No.	Endophytic fungi	Morphological characteristics
13.	*Daldinia eschscholtzii*	Colonies initially white, turned smokey gray to olivaceous grey with age. Conidia ellipsoid, solitary, aseptate, hyaline, with attenuated base ranging from 4.7–7.5 × 2.2–3.8 µm. Conidiophores were hyaline, septate, irregularly branched.
14.	*Diaporthe phaseolorum*	Colonies white dark brown. Conidia unicellular, hyaline, ellipsoidal to cylindrical, rounded at both ends, 5–8 × 1.8–2 µm.
15.	*Fusarium brachygibbosum*	Colonies white, orange-yellow to medium red. Macroconidia 25.92 × 4.43 µm with 3–4 septa; microconidia were slightly curved, ovoid, and fusiform, 11.05 × 3.97 µm.
16.	*Fusarium oxysporum*	Colonies pinkish to peach. Microconidia are oval to ellipsoid, 0–1 septate, 10–25 × 2-5 µm. Macroconidia were oval with tapering ends to spindle and septate in 3–4 cells, 28–46 × 3–5 µm.
17.	*Fomitopsis meliae*	Colony on PDA were white in colour, slow growing, hyphae were single walled dimitic, generative hyphae with clamps, Cystidia lacking, basidiospores are cylindrical, hyaline having 6–8 × 2–3 µm.
18.	*Lasiodiplodia theobromae*	Conidia subovoid to ellipsoid-ovoid, apex broadly rounded, tapering to truncate base, widest in middle to upper third, thick-walled, one-septate, 21-31 × 13-15.5 µm.
19.	*Nigrospora sphaerica*	Colonies initially white, becoming black with time. Conidia solitary, globose or subglobose, black, shiny, smooth, aseptate, 16–21 µm diameter. Conidiophores micronematous or semi-macronematous, multiseptated, hyaline to pale brown flexuous or straight, 4–7 µm thick.
20.	*Neopestalotiopsis clavispora*	Colonies cottony, whitish to pale yellow. 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. Two to four (three being the most frequently observed) straight hyaline appendages were apparent at the apical cells and one basal appendage.
21.	*Phomopsis* sp.	Colonies white to beige. Conidiophores subcylindrical, hyaline, branched, 13–34 × 2–3 µm. Alpha conidia ovoid-ellipsoid, mostly with acute ends, guttulate, measuring 4.5–6.5 × 1.7–3.0 µm. Beta conidia not seen.
22.	*Pseudofusicoccum adansoniae*	Colonies white, dark olivaceous grey to olivaceous black. Conidia 18–25.9 × 3.6–5.6 µm, ellipsoid and straight, occasionally slightly bent or irregularly shaped, apices rounded, smooth with fine granular content, hyaline, thin- walled, unicellular, covered with persistent mucous layer.
23.	*Trichoderma viride*	Colonies creamish-white, light green to dark bluish. Phialides were 7.6-14 µm long, 2–2.3 µm wide at base whereas 3.0–3.8 µm at widest point. Conidia broadly sub globose to obovoid, mostly 3.0–3.8 × 2.8–3.5 µm, smooth-walled, light green to dark green.
24.	*Schizophyllum commune*	Colonies on PDA were cottony, white in colour, irregular margin and yellow brown reverse side. Hyphae were hyaline, septate, branched with clamp connection.

Table 1 Continued.

S. No.	Endophytic fungi	Morphological characteristics
25.	*Xylaria longipes*	Stromata clavate consist of long stipes which ended into round fertile apices measuring 2–8 cm tall; up to 2 cm wide. The fruiting body was club-shaped with a rounded tip, externally grayish to brownish when young, becoming black with maturity. The surface of the fruiting body became cracked and scaly with maturity.

Table 2 Fungal endophytes identified based on molecular characteristics, isolated from leaves, fruits and stems of *Dillenia indica* L.

S. No.	Fungal identification	Code	NCBI Accession no	Identity %age
1.	*Phomopsis* sp.	DLP21S4a1	MK757156.1	97%
2.	*Schizophyllum commune*	DSP22S3a1	MK756215.1	99%
3.	*Diaporthe phaseolorum*	DLP24S2a1	MK757169.1	98%
4.	*Fomitopsis meliae*	DLP30S2a1	MK757195.1	98%
5.	*Nigrospora sphaerica*	DLP46S2a1	MK757157.1	100%
6.	*Lasiodiplodia theobromae*	DSP22S4a1	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 eschscholzii*	DSP40S2a1	MN854982.1	99.6%
12.	*Colletotrichum gloeosporioides*	DLP31S1a1	MN855105.1	99.6%
13.	*Colletotrichum boninense*	DSP32S3a3	MW521131.1	99.87%

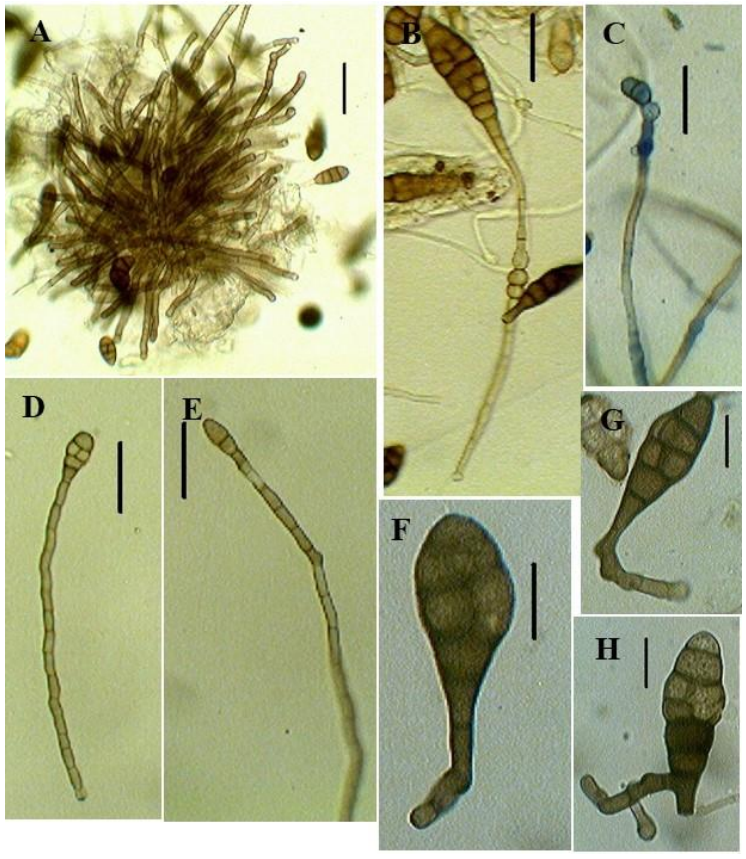

Fig. 1 – *Alternaria alternata*. A–E Conidiophores bearing conidia. F–H Conidia. Scale bars: A–E = 20 µm, F–H = 10 µm

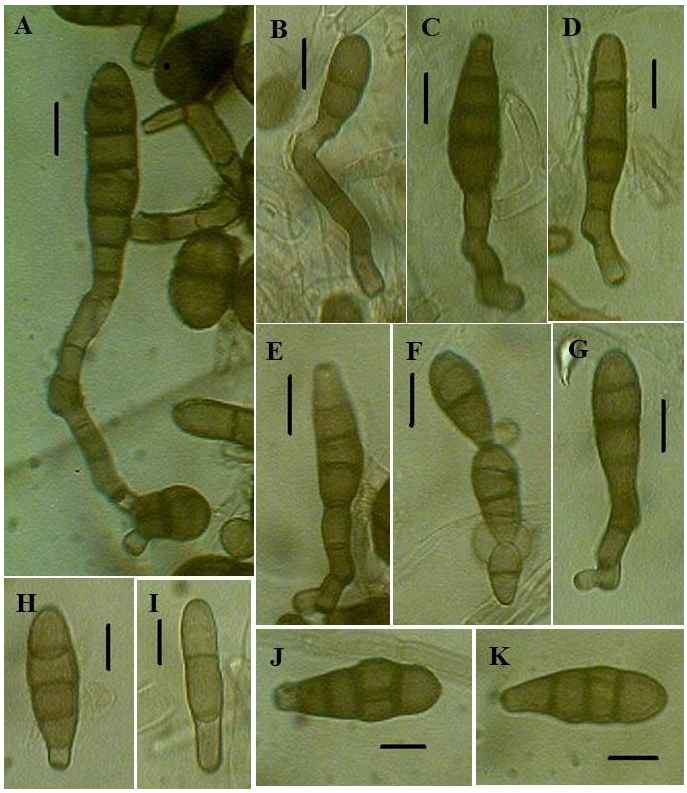

Fig. 2 – *Alternaria tenuissima*. A–E Conidiophore bearing conidia. F–K Conidia. Scale bars = 10 μm.

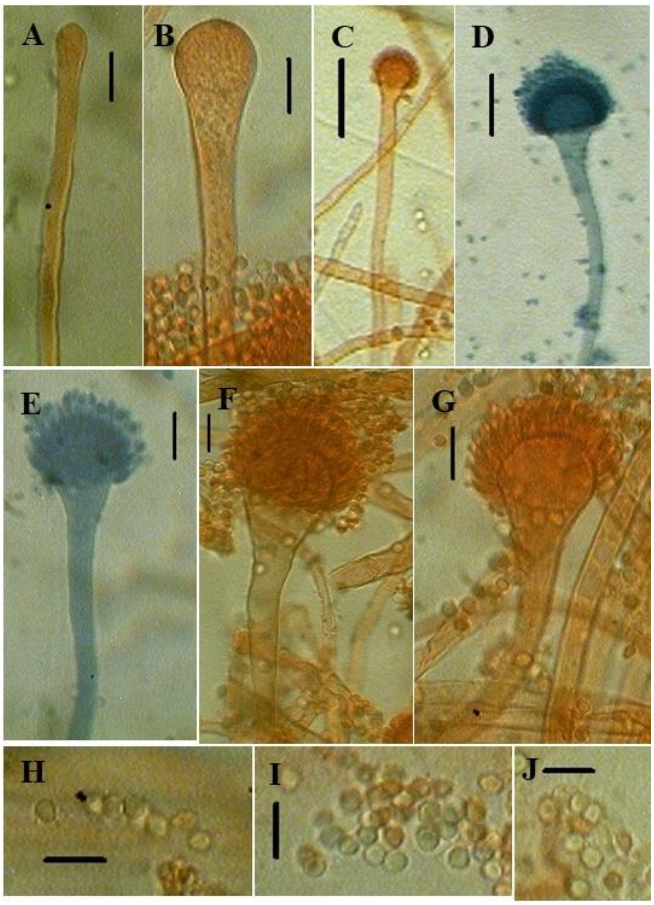

Fig. 3 – *Aspergillus fumigatus.* A–B Developing conidiophores. C–G Conidiophore bearing sterigmata and conidia. H–J Conidia. Scale bars = 10 μm.

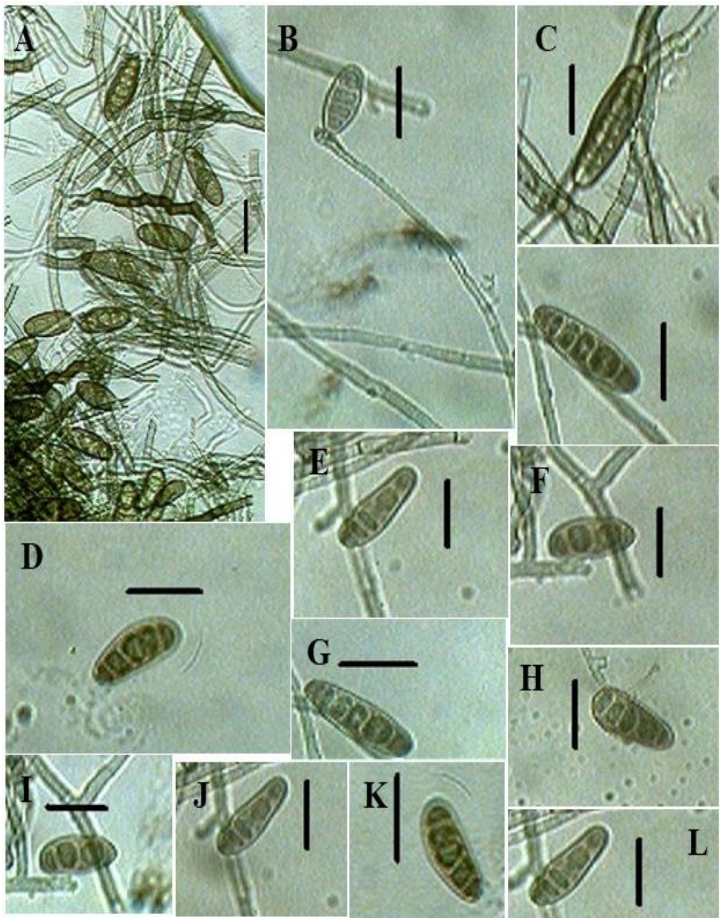

Fig. 4 – *Bipolaris crotonis*. A–B Conidiophores bearing conidia. C–L Conidia. Scale bars = 20 μm.

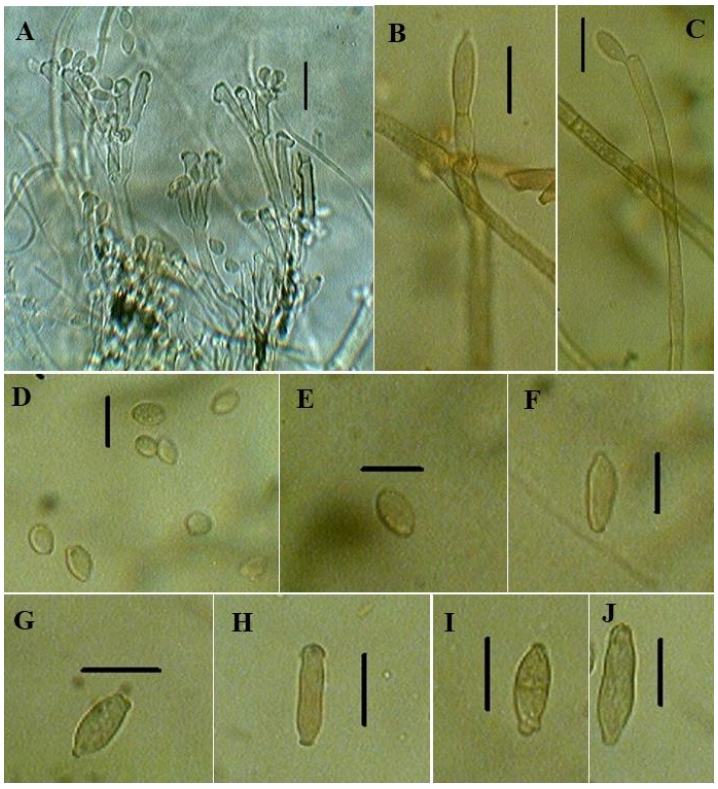

Fig. 5 – *Cladosporium cladosporioides* A–C Conidiophores bearing conidia. D–J Different types of conidia. Scale bars A = 20 μm, B–J = 10 μm.

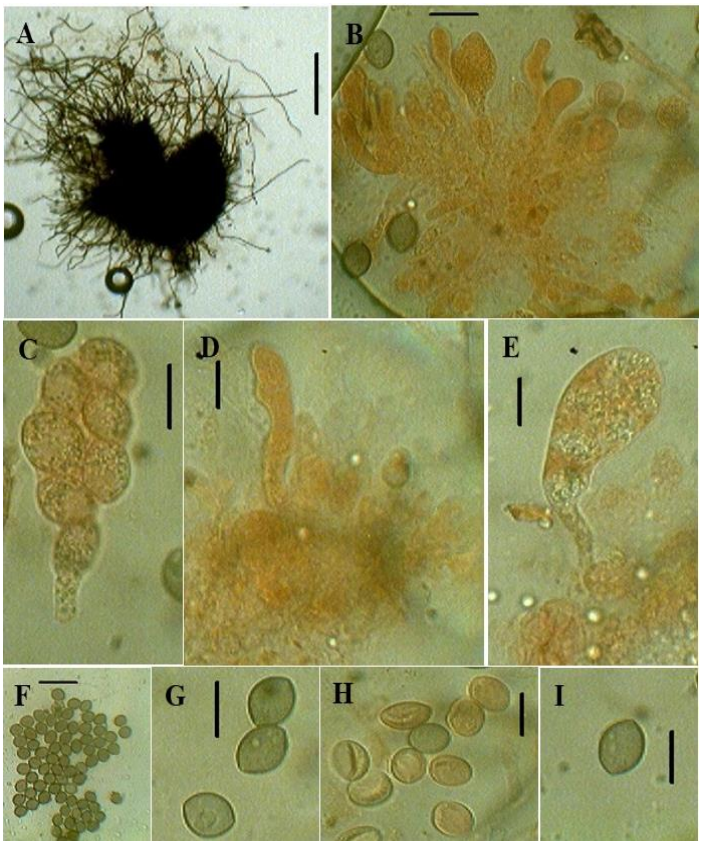

Fig. 6 – *Chaetomium globosum*. A Perithecium B Ascomata bearing asci. C–E Asci developing ascospores. F–I Ascospores. Scale bars: A, F = 20 µm, B–E, G–H = 20 µm.

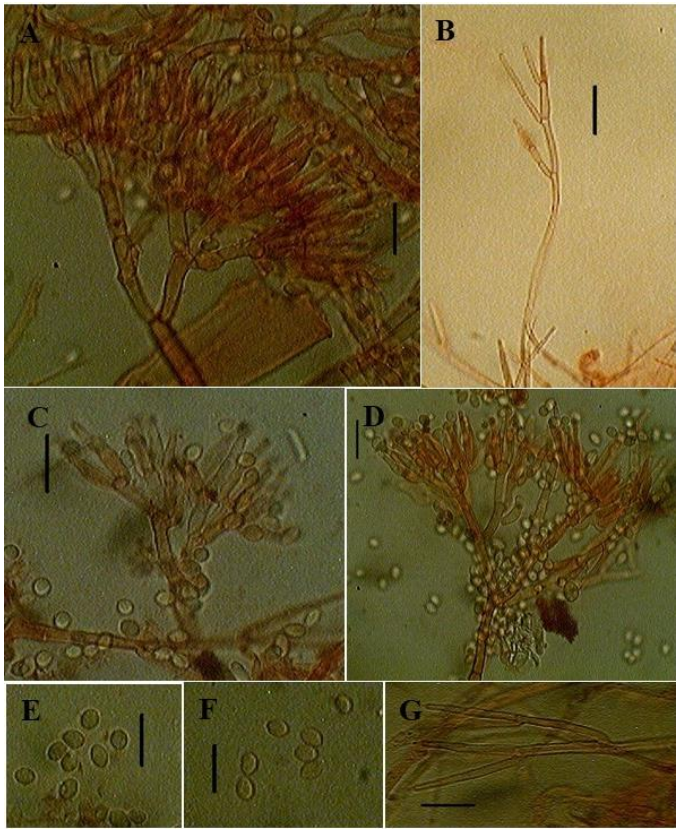

Fig. 7 – *Clonostachys rosea*. A–D Conidiophore bearing conidia. E–F Conidia. G Conidiophore. Scale bars = 10 µm.

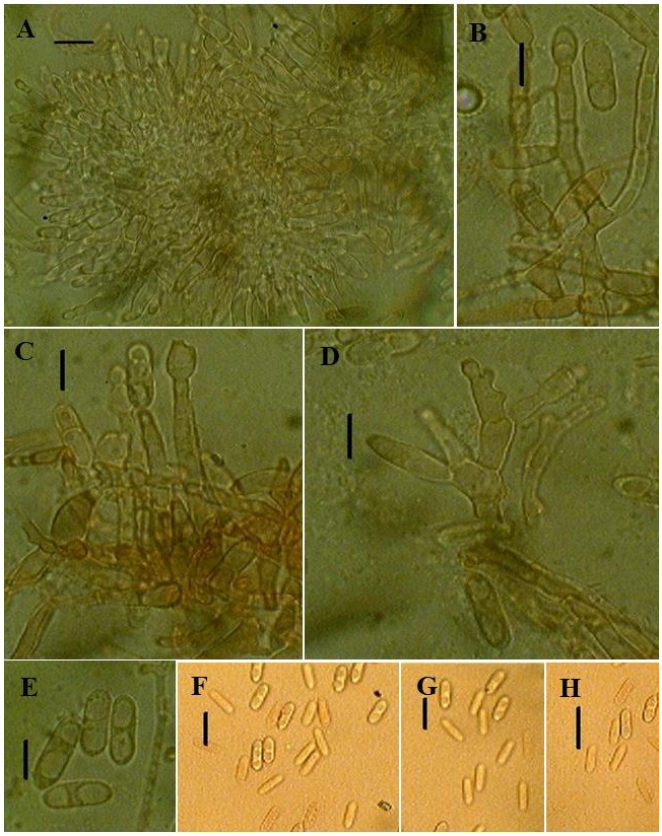

Fig. 8 – *Colletotrichum boninense.* A–D Conidiophores bearing conidia on conidiogenous cell. E–H Conidia. Scale bars: A–E = 10 µm, F–H = 20 µm.

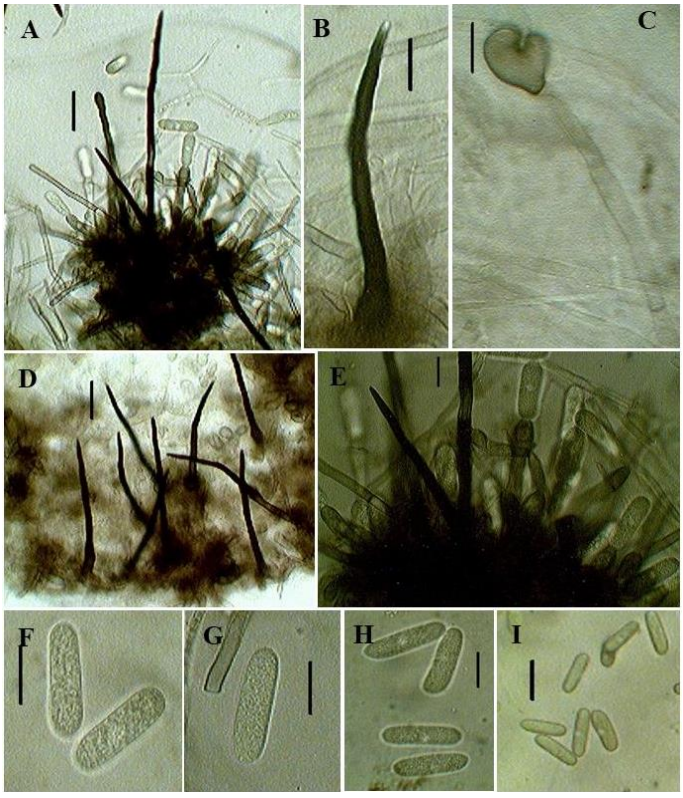

Fig. 9 – *Colletotrichum gigasporum*. A Setae and conidiophores bearing conidia. B Seta. C Appressorium. D Setae. E Conidiophore bearing conidia. F–H conidia. Scale bars: A, D, I = 20 µm, B–C, F–H = 10 µm.

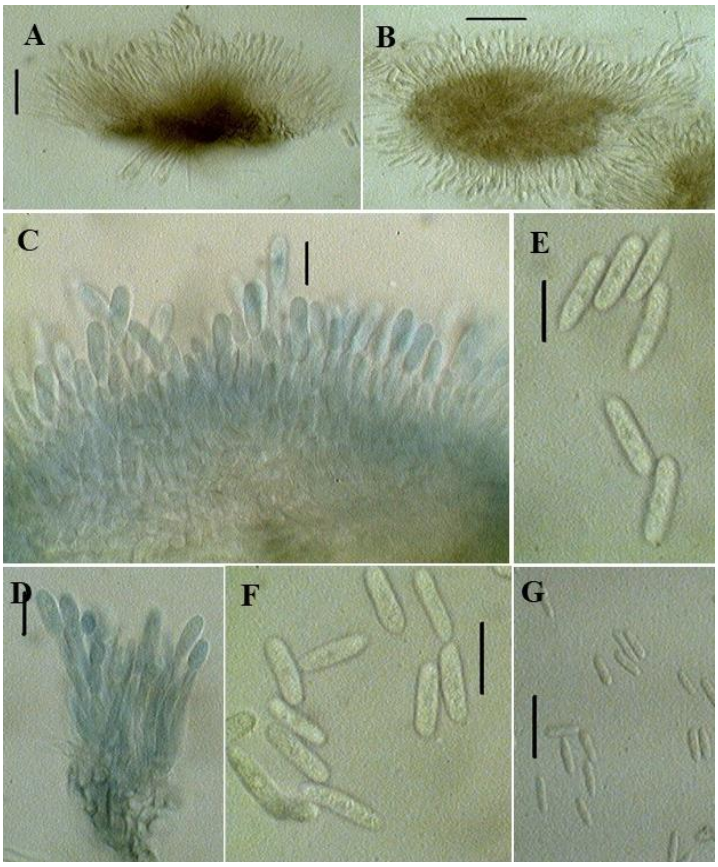

Fig. 10 – *Colletotrichum gloeosporioides*. A–D Conidiophores bearing conidia. E–G Conidia. Scale bars: A, B, G = 20 µm, C–F = 10 µm.

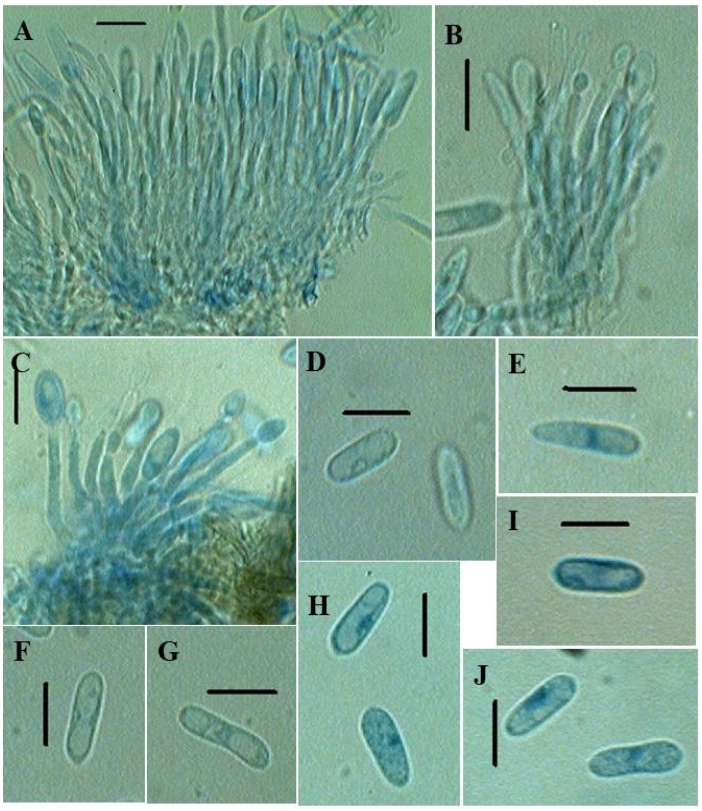

Fig. 11 – *Colletotrichum musae.* A–C Conidiogeneous cells bearing conidia. D–J Conidia. Scale bars = 10 µm.

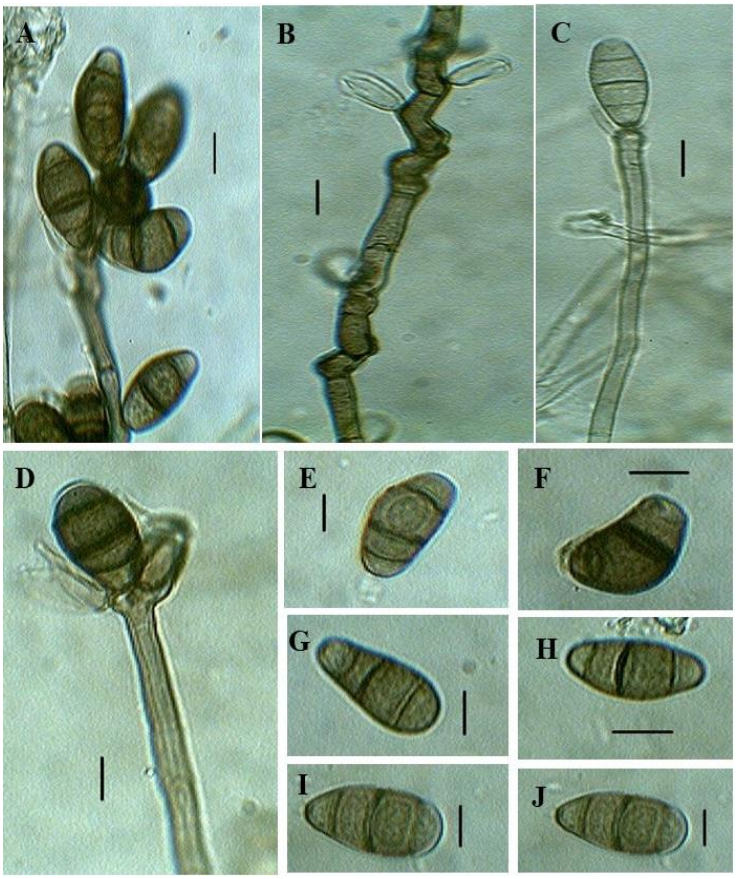

Fig. 12 – *Curvularia lunata.* A Conidiophore bearing group of conidia. B–D conidiophore bearing conidium E–J conidia. Scale bars = 10 μm.

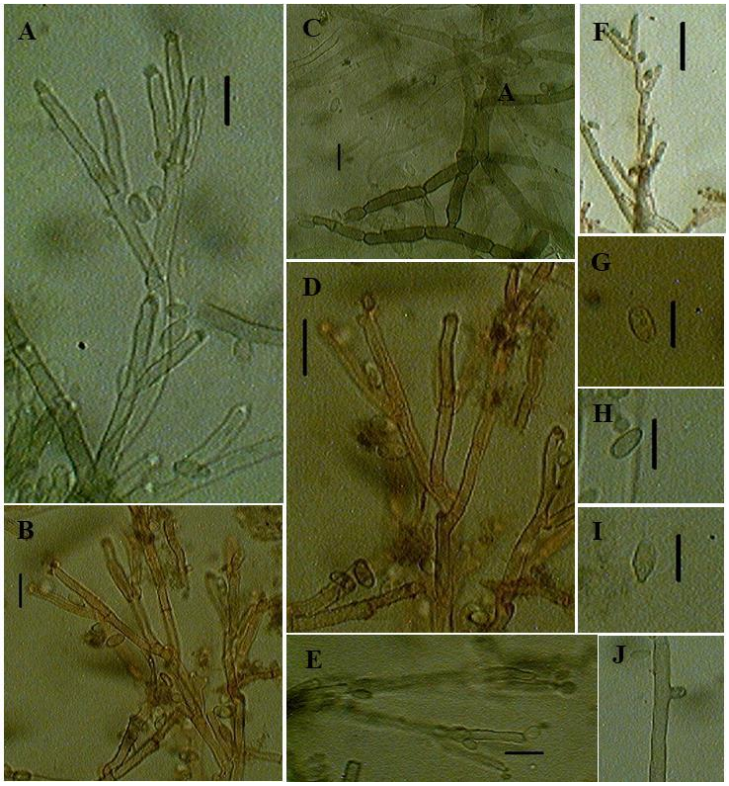

Fig. 13 – *Daldinia eschscholtzii*. A–B, D–F Conidiophore bearing conidia. C Branched hypha. G–I Conidia. J Hyphae having exudate on their surface. Scale bars: A–E, G–J = 10 μm, F = 20μm.

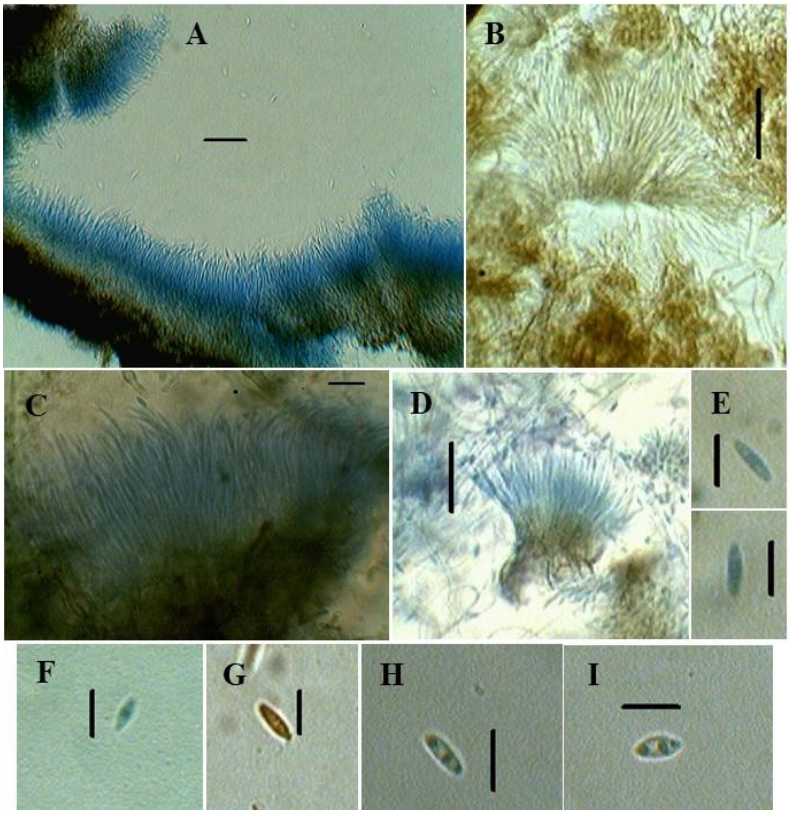

Fig. 14 – *Diaporthe phaseolorum*. A–D Conidiophore bearing conidia. E–I Conidia. Scale bars: A–D = 20 μm, E–I = 10 μm.

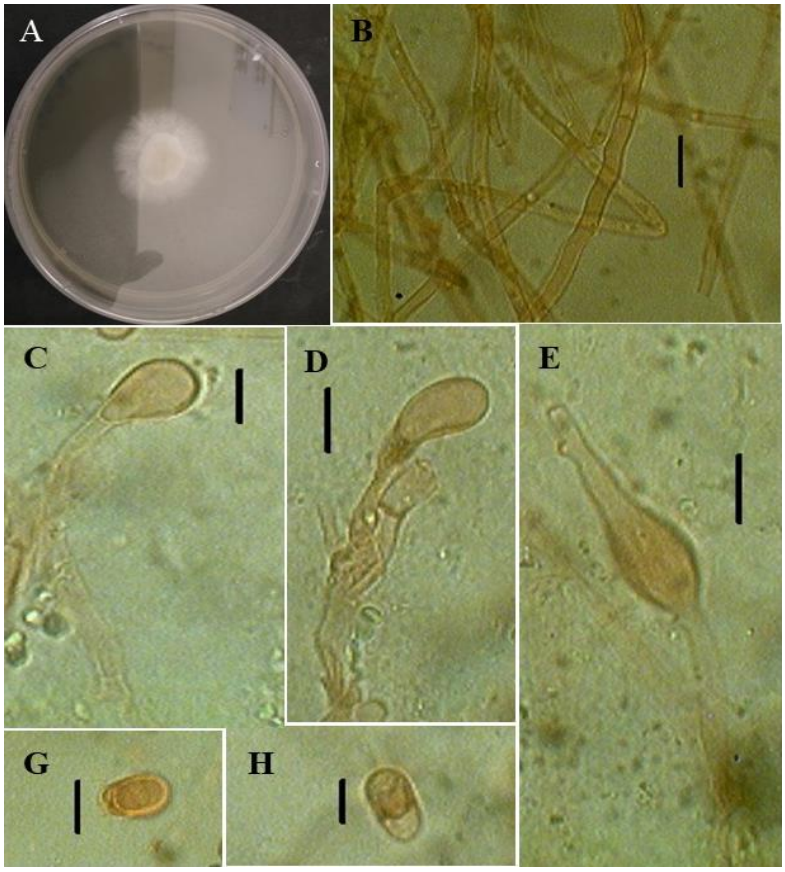

Fig. 15 – *Fomitopsis meliae*. A Colony on PDA. B Branched, septate granulated hyphae. C–E Cystidia. G–H Basidiospores. Scale bars = 10 μm.

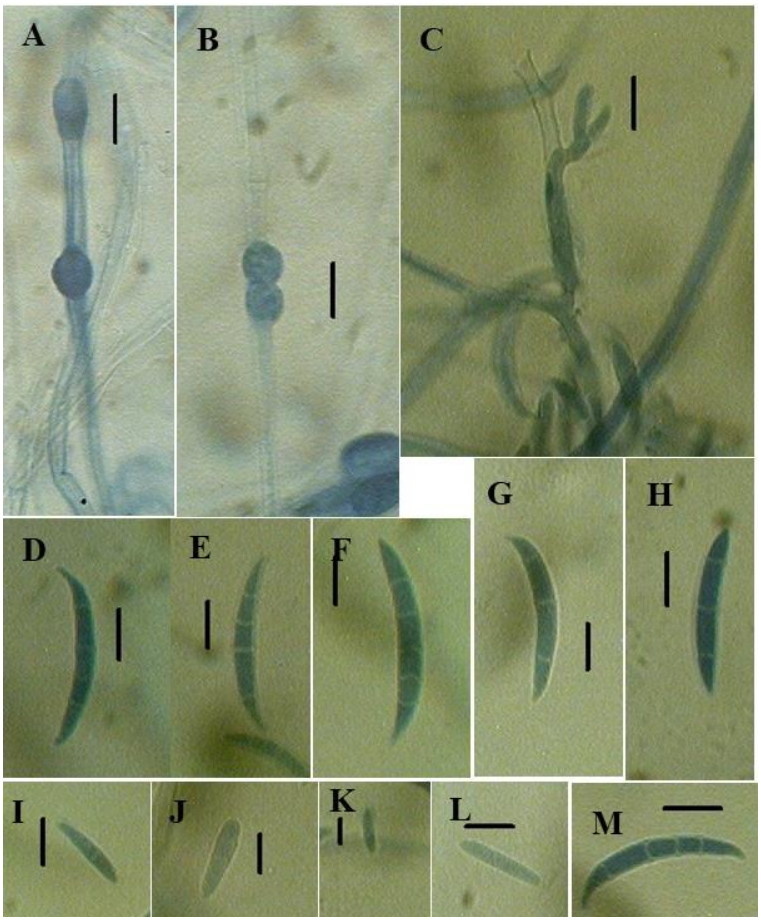

Fig. 16 – *Fusarium brachygibbosum*. A–B Chlamydospores. C Philaids. D–M Macro and microconidia. Scale bars = 10 μm.

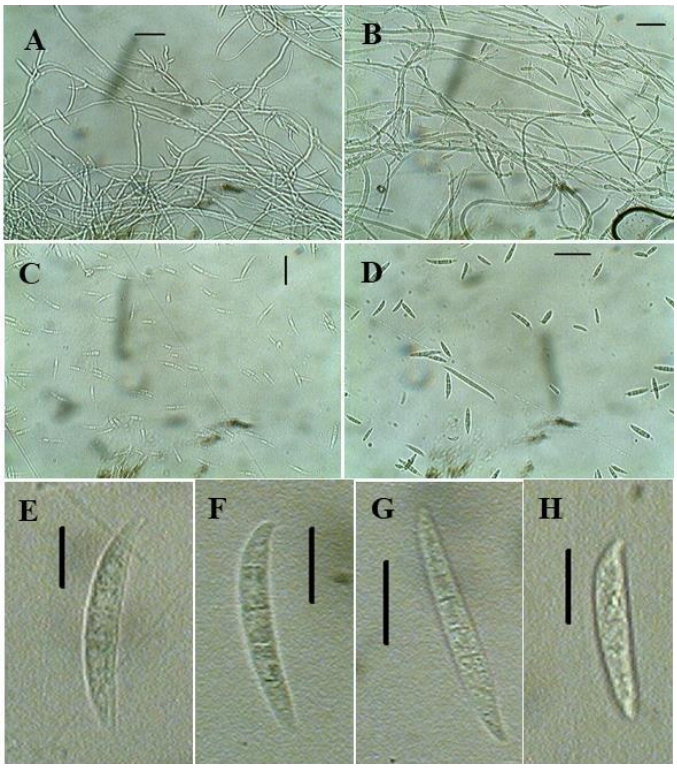

Fig. 17 – *Fusarium oxysporum*. A–B Hyphae and conidiophores bearing conidia. C–H conidia. Scale bars: A–D = 20 μm, E–H = 10 μm.

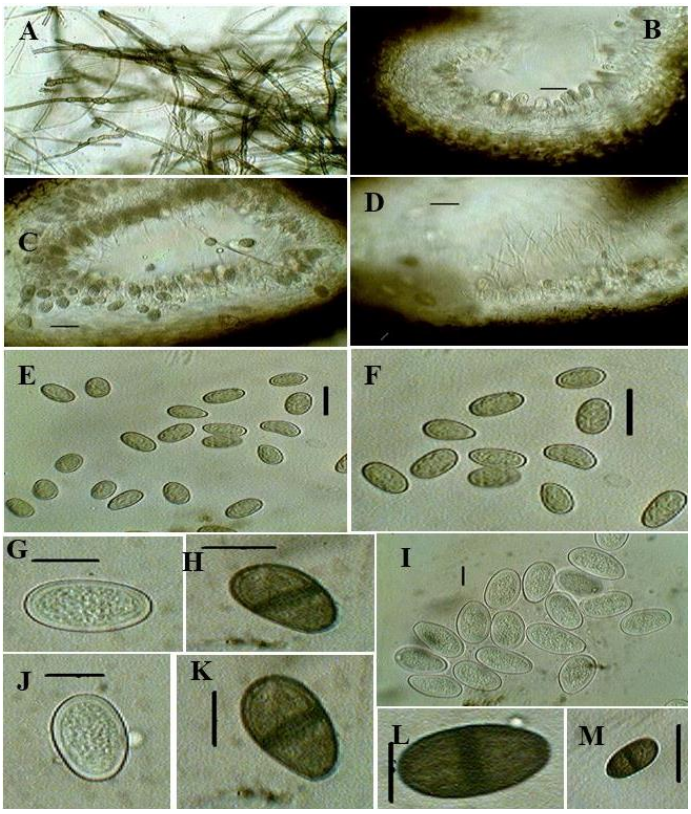

Fig. 18 – *Lasiodiplodia theobromae*. A Hyphae B–D Cross section of pycnidium having conidiogenous cells bearing conidia E–M Conidia. Scale bars: B–D = 40 µm, E–F = 20 µm, A, G–M = 10 µm.

Fig. 19 – *Nigrospora sphaerica*. A–G Conidiophores bearing conidia on conidiogenous cell. H–J Conidia. Scale Bars = 10 µm.

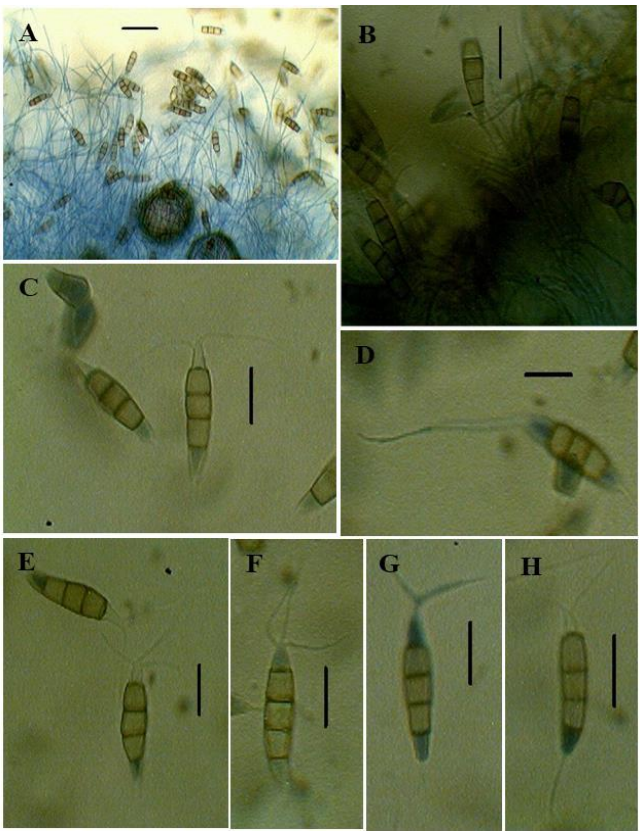

Fig. 20 – *Neopestalotiopsis clavispora.* A–B Conidia borne on conidiogenous cells. C–H Conidia having apical and basal appendages. Scale bars: A = 20 µm, B–G = 10 µm.

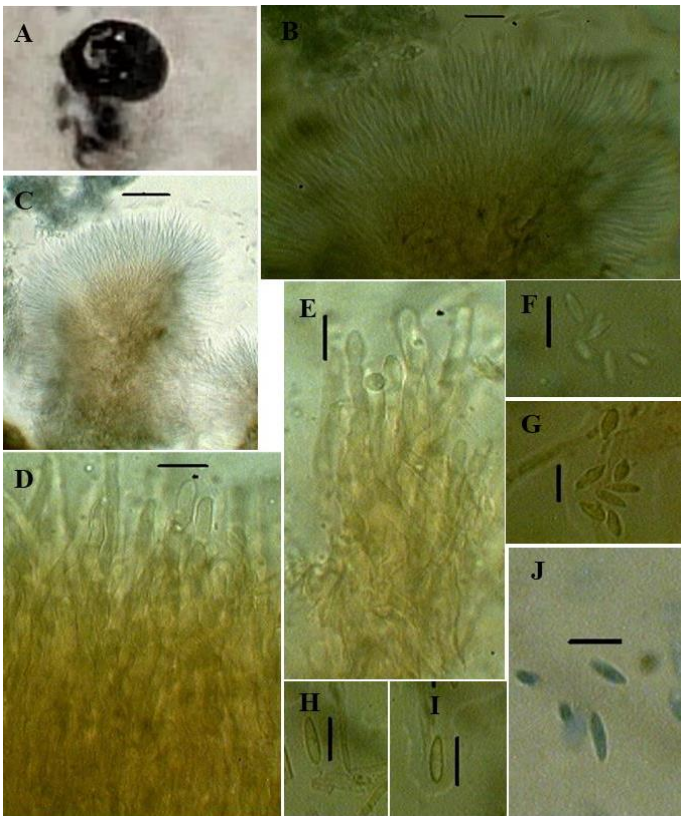

Fig. 21 – *Phomopsis* sp. A Pycnidium. B–E Conidiphores bearing conidia. F–J Conidia. Scale bars: B–C = 20 µm, D–J = 10 µm

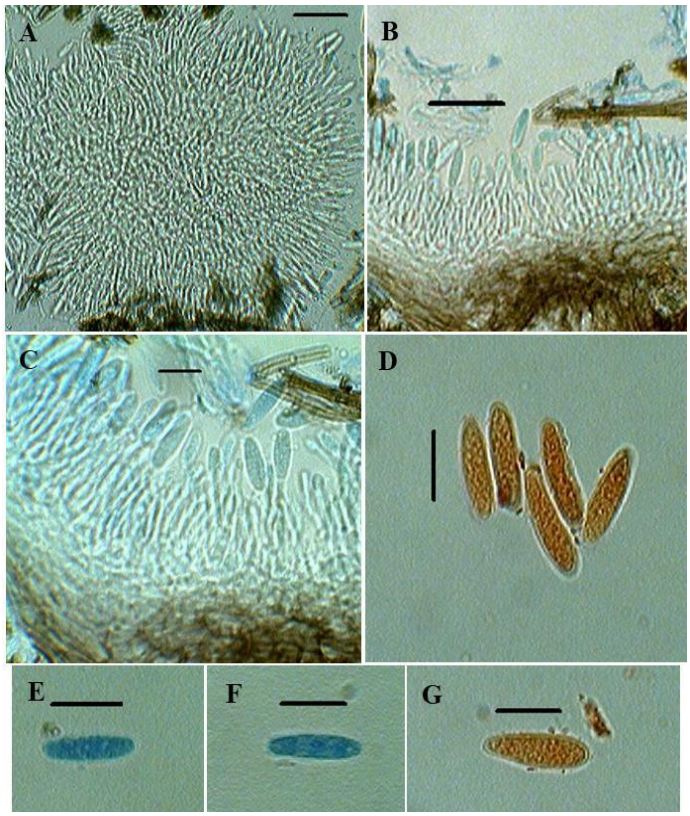

Fig. 22 – *Pseudofusicoccum adansoniae*. A–C Conidiogeneous cells bearing conidia. D–G Hyaline aseptate conidia. Scale bars: A = 20 µm, B–G = 10 µm.

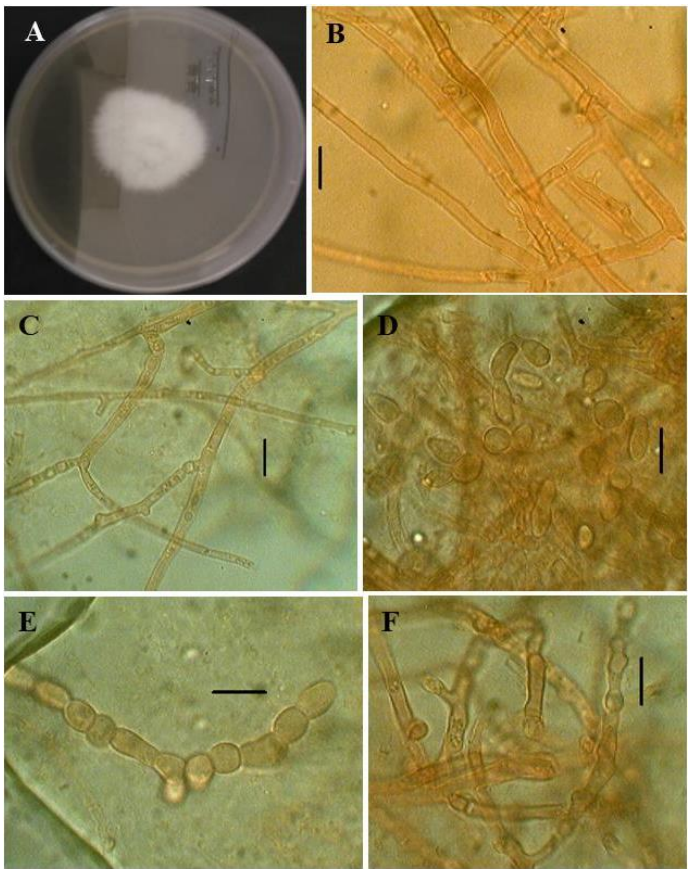

Fig. 23 – *Schizophyllum commune*. A Colony on PDA petri plate. B Branched septate hyphae. C Granulated hyphae. D–E Chlamydospores. F Developing Chlamydospores. Scale bars = 10 µm.

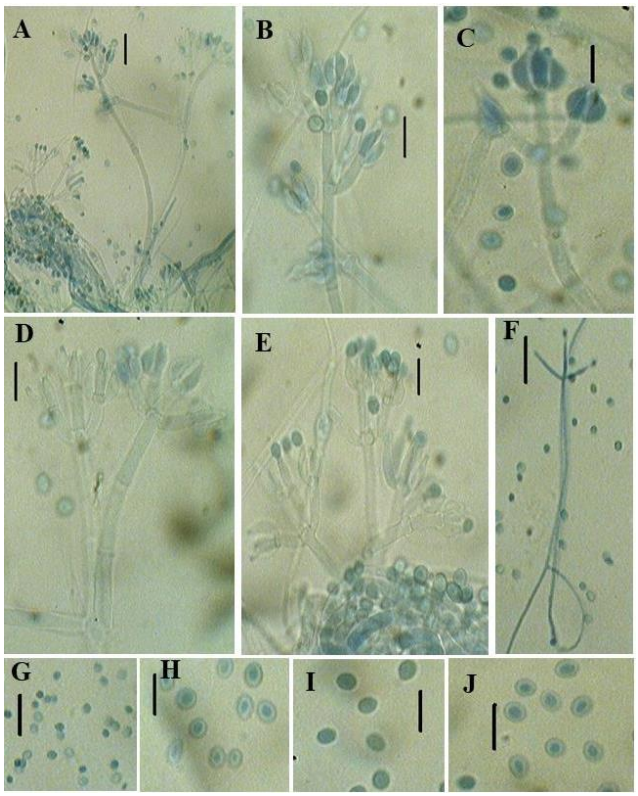

Fig. 24 – *Trichoderma viride*. A–F Conidiophore bearing phialides on which phialospores are borne. G–J Conidia. Scale bars: A, F, G = 20 µm, B–E, H–J = 10 µm.

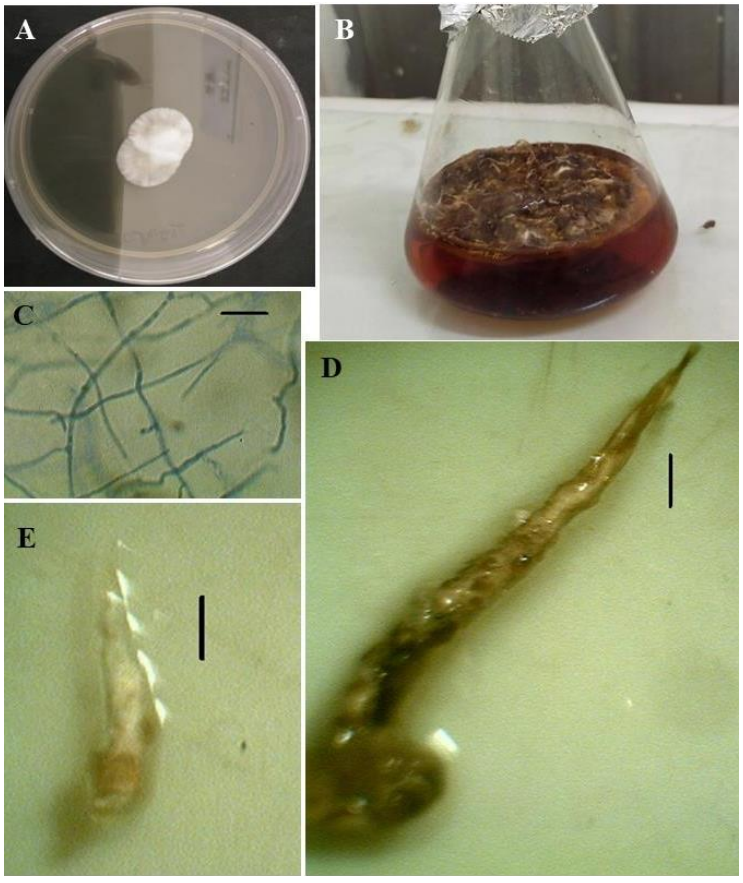

Fig. 25 – *Xylaria longipes*. A Colony on PDA plate. B Growth in Potato Dextrose Broth. C Branched septate hyphae. D–E Stromata. Scale bars = 20 µm.

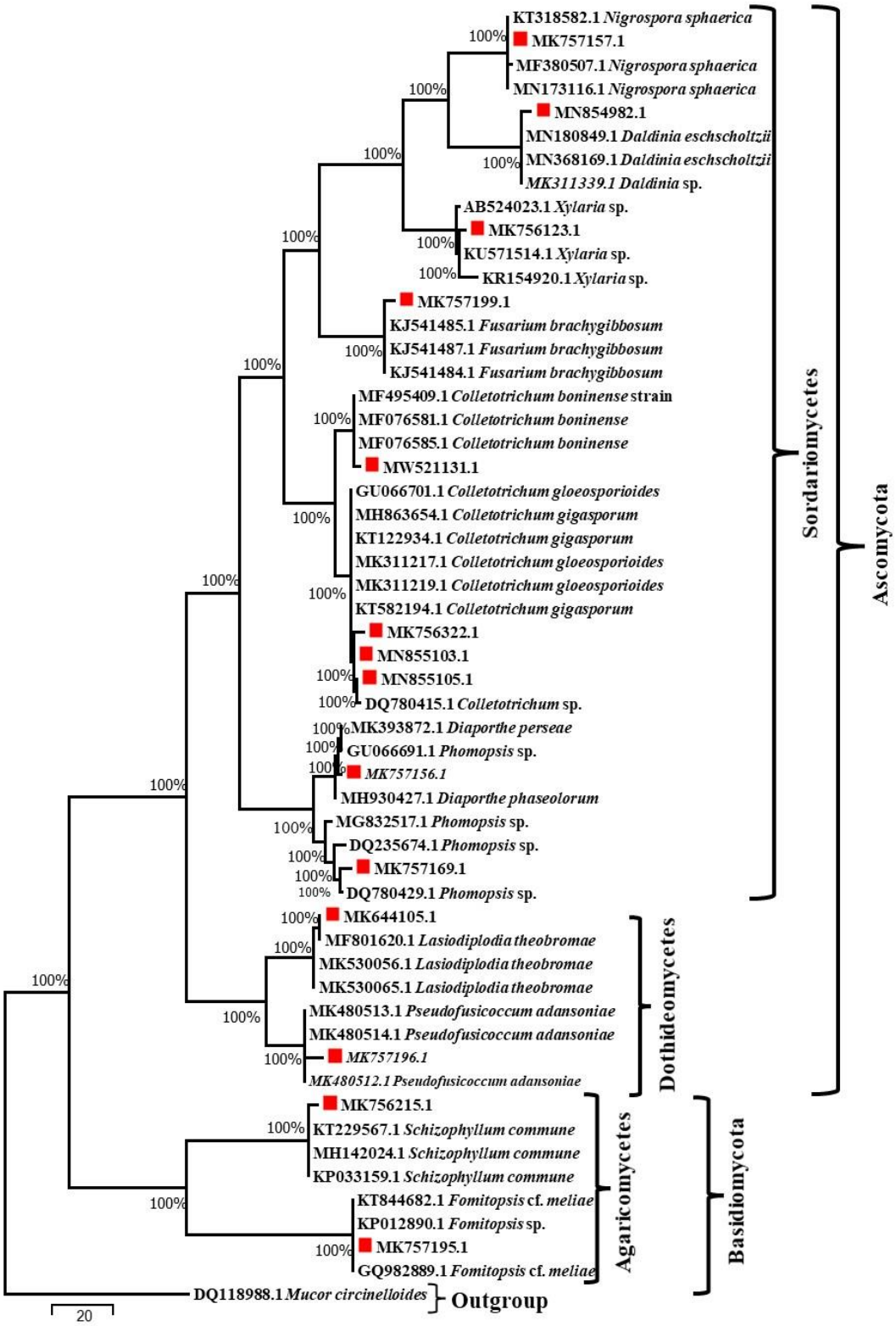

Fig. 26 – Phylogenetic tree of endophytic fungi isolated from *Dillenia indica* L. based on ITS region. The evolutionary history was inferred using the Maximum Parsimony method. 100% bootstrap value showed each genus was distinguished by monophyletic group in different subclades from the outgroup. The tree is drawn to scale, with branch lengths calculated using the average pathway method and are in the units of the number of changes over the whole sequence. The analysis involved 55 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 342 positions in the final dataset. Evolutionary analyses was conducted in MEGA7.

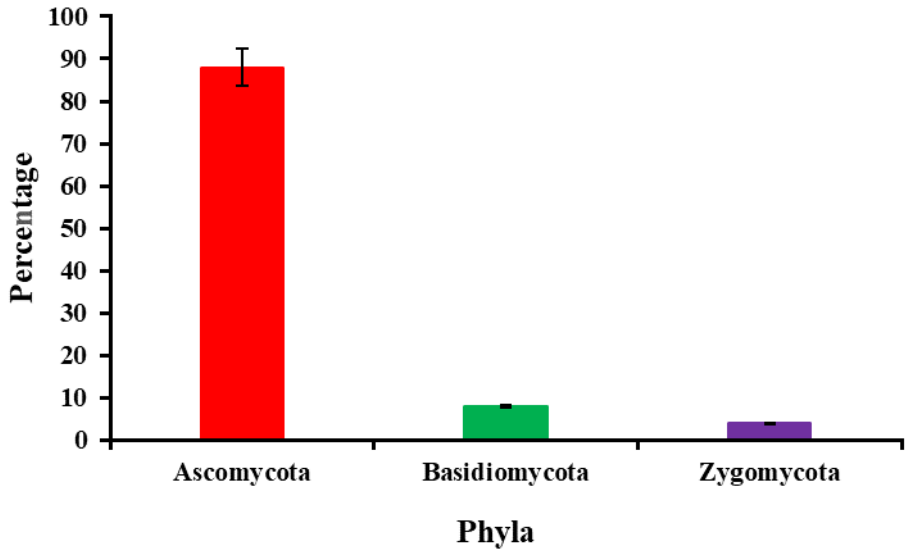

Fig. 27 – Percentage of occurrence of endophytic fungi belonging to different phyla isolated from leaves, fruits and stems of *Dillenia indica* L.

Dothideomycetes and Sordariomycetes were the dominant classes, followed by Agaricomycetes and Eurotiomycetes. The dominating genera were *Daldinia*, *Cladosporim*, *Colletotrichum*, *Fusarium*, *Nigrospora* and *Curvularia*. The genera-wise distribution of isolated fungi from different tissues (leaves, stems and fruits) of *Dillenia indica* L. in terms of percentage is presented in the pie chart (Fig. 28). Table 3 shows the colonization frequency of each isolated fungi. The percentage-wise distribution of isolated fungi of each species showed that the *Colletotrichum gloeosporioides*, *Daldinia eschscholtzii* and *Cladosporium cladosporioides* were found to be the most common isolates.

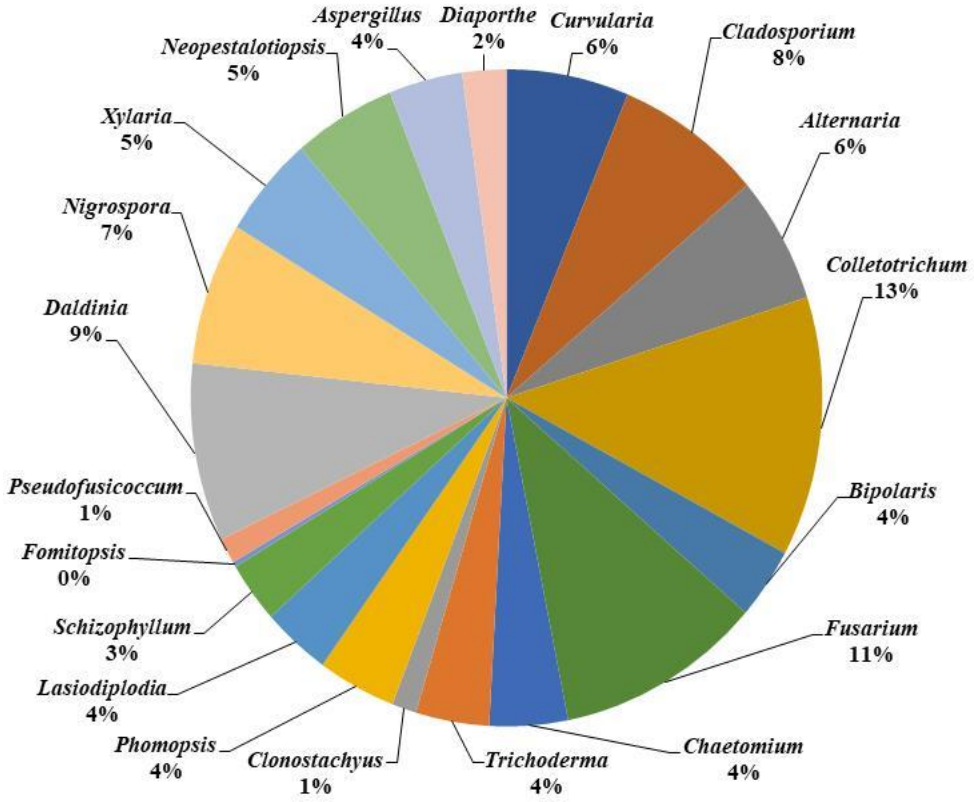

Fig. 28 – Genus-wise distribution of fungal isolates (in term of %) from *Dillenia indica* L.

Table 3 Colonization frequency of endophytic fungi isolated from different tissues (leaves, stems and fruits) of *Dillenia indica* L.

S. No.	Endophytic fungi	Colonization frequency (%)
1.	*Alternaria alternata*	4.87
2.	*Alternaria tenuissima*	1.21
3.	*Aspergillus fumigatus*	3.65
4.	*Bipolaris crotonis*	3.41
5.	*Chaetomium globosum*	3.9
6.	*Cladosporium cladosporioides*	7.31
7.	*Colletotrichum boninense*	2.19
8.	*Colletotrichum gloeosporioides*	6.82
9.	*Colletotrichum gigasporum*	0.48
10.	*Colletotrichum musae*	2.92
11.	*Clonostachys rosea*	1.21
12.	*Curvularia lunata*	6.09
13.	*Daldinia eschscholtzii*	8.5
14.	*Diaporthe phaseolorum*	2.19
15.	*Fomitopsis meliae*	0.24
16.	*Fusarium brachygibbosum*	5.85
17.	*Fusarium oxysporum*	4.39
18.	*Lasiodiplodia theobromae*	3.41
19.	*Neopestalotiopsis clavispora*	5.12
20.	*Nigrospora sphaerica*	6.82
21.	*Phomopsis* sp.	3.9
22.	*Pseudofusicoccum adansoniae*	1.21
23.	*Schizophyllum commune*	2.92
24.	*Trichoderma viride*	3.65
25.	*Xylaria longipes*	4.87

The diversity index values showed that the fungal communities inside the *Dillenia indica* L. were diverse in their relative abundance (Table 4). Simpson index (H) was highest in the leaf having value of 2.6, followed by the stems with the value of 1.59 and lowest in the fruits with the value of 1.07. Similarly, the value of Shannon Index was highest in the leaves i.e., 0.91 and lowest in the fruits i.e., 0.65. Evenness index was recorded highest in the fruits with a value of 0.98 and lowest in the stems (0.89). The frequency of isolated fungal species, along with their order, is displayed in the form of a bar graph (Fig. 29). The isolated taxa were also tissue-specific, as some species were recovered from one tissue and not from the other. The values of total isolation rate, colonization rate, percentage frequency and colonization frequency was 3.65%, 30.2%, 60% and 33.03% (Fig. 30).

Table 4 Diversity indices of endophytic fungi isolated from *Dillenia indica* L

S. No.	Diversity indices	Leaves	Stems	Fruits
1.	Simpson Index	2.6	1.59	1.07
2.	Shannon Index	0.91	0.77	0.65
3.	Evenness index	0.93	0.89	0.98

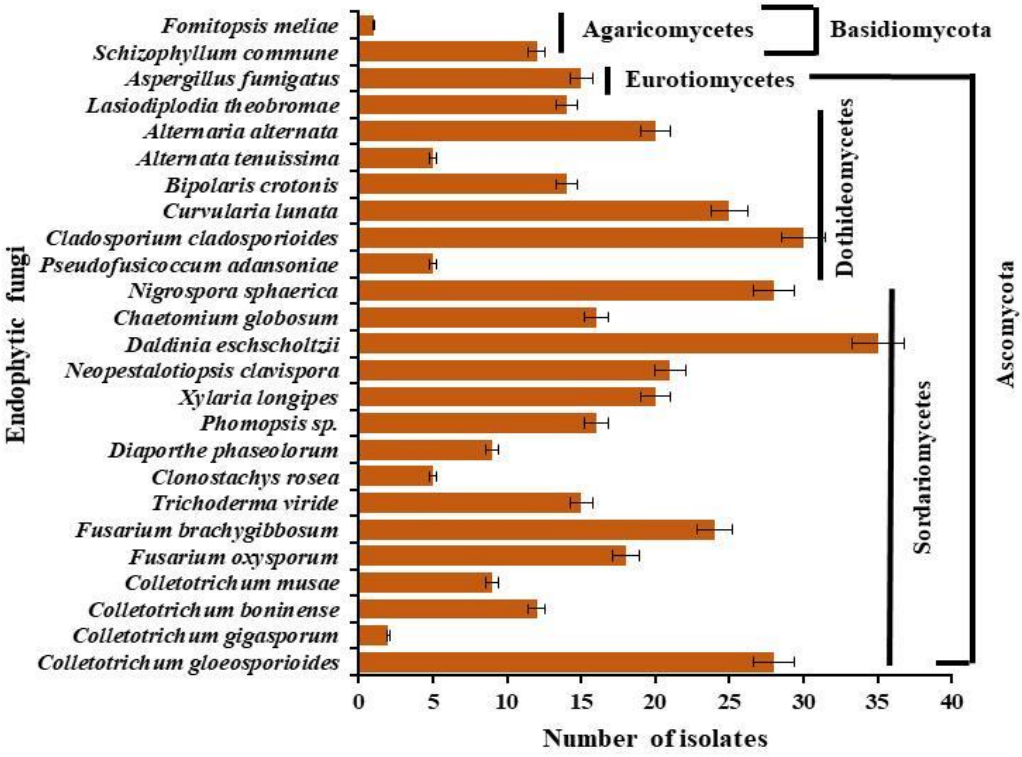

Fig. 29 – The frequency (relative abundance) of fungal endophytes isolated from different parts of *Dillenia indica* L.

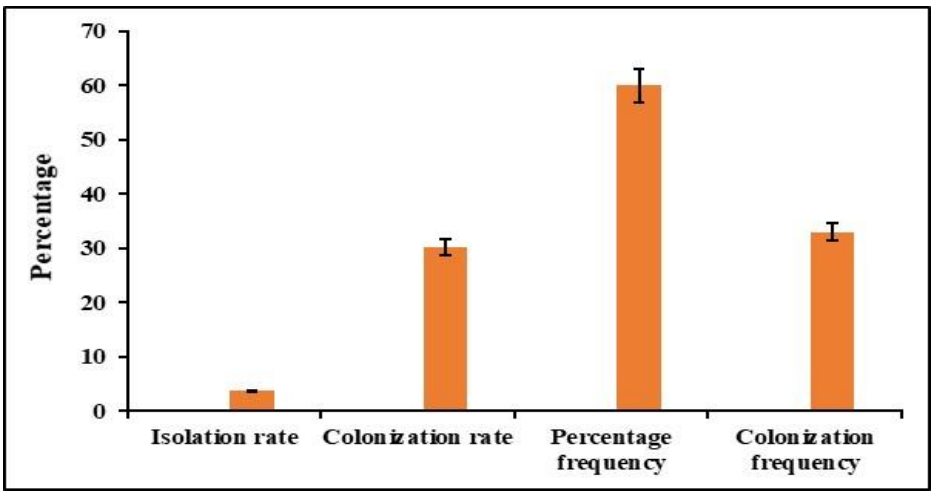

Fig. 30 – Isolation rate, colonization rate, percentage frequency and colonization frequency of fungal endophytes recovered from *Dillenia indica* L.

Discussion

Bioprospecting endophytic fungi from medicinal plants is an active area of research but there was no previous report on the diversity of fungal endophytes from *Dillenia indica* L. The present study focused on the isolation and identification of endophytic fungi from different parts (leaves, stems and fruits) of *Dillenia indica* L. In the present investigation, 798 isolates of endophytic fungi were isolated from leaves, fruits and stems of *Dillenia indica* L. The isolated fungal isolates comprised of 25 taxa belonging to 20 genera. The majority of the isolated fungal taxa belong to Ascomycota (22 species), followed by Basidiomycota (2 species) and Zygomycota (1 species). Ascomycota was the dominating phyla of endophytic fungi, as reported in the previous studies carried out by Park et al. (2017), Sarma et al. (2020). Sordariomycetes and Dothideomycetes were

the dominant classes which are in accordance with the findings of Li et al. (2016), Sarma et al. (2018). Leaves harbor more endophytes than stems and fruits. This may be due to the presence of stomata on the leaf surface and its larger surface area exposed to the environment. Similar results were obtained by Huang et al. (2008). Kumar & Hyde (2004) also found that the colonization rate in the leaves was higher than those in the roots, stems and petiole. The inflorescence of *A. cimicina* and *H. contortus* hosts a greater number of endophytic fungi than leaves and culm (Nischitha & Shivanna 2020). Endophytic fungi were reported by various scientists from different parts of the world. Manganyi et al. (2108) reported 60 endophytic fungi from *Sceletium tortuosum* and found that the *Fusarium, Aspergillus* and *Penicillium* were the most dominant genera. Arora et al. 2019 isolated 266 isolates comprising of 21 genera and 38 taxa and found that the *Phomopsis* and *Fusarium* were the commonly occurring genera. Toghueo et al. (2017) isolated 21 different taxa of endophytic fungi from medicinal plants *Cananga odorata*, *Terminalia catappa* and *Terminalia mantaly* and screened them for the production of industrially important enzymes i.e., amylase, cellulase, lipase and laccase. Sureshkumar et al. (2019) isolated endophytic fungi from the medicinal plant *Ficus racemosa* and characterized them on morphological and molecular basis.

Some isolated endophytic fungi showed tissue specificity, similar to studies of De Errasti et al. (2010), Wearn et al. (2012). The distribution of endophytic fungi is influenced by plants tissue type and environmental factors (Singh et al. 2017). The endophytic fungal communities of rice are mainly influenced by geographical conditions, different cultivars and tissue types (Su-Han et al. 2019). Some endophytes such as *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium oxysporum* isolated in this study were reported as pathogens and cause diseases to many plant species. *Trichoderma viride* reported in this study is an important biocontrol agent that protects plants from many pathogenic fungi (Padder & Sharma 2011, Sood et al. 2020). Endophytes are latent pathogens that may act as saprophytes when a plant dies or senescence its leaves to enhance its degradation (Promputtha et al. 2007).

Some endophytic fungi associated with *Dillenia indica* L. such as *Curvularia lunata, Alternaria alternata, Cladosporium cladosporioides, Fusarium oxysporum* and *Colletotrichum gloeosporioides* are cosmopolitan in distribution. These taxa were previously reported as endophytes in other medicinal plants (Mahmud et al. 2020, Gopane et al. 2021).

The Simpson index, Shannon index, and Evenness index varied among tissues of *Dillenia indica* L. High Simpson index and Shannon index were observed in leaves, followed by stems and fruits. In contrast, the value of the Evenness index was higher in fruits, followed by leaves and stems. Species richness of the endophytic fungal communities was higher in leaves than in stems which supports earlier findings (Sahani & Hemalatha 2018, Bhattacharya et al. 2020).

Endophytic fungi are a diverse group of microbes that play an essential role in the morphology and physiology of the host plants by different mechanisms (Zhou et al. 2016). The occurrence of endophytes inside the host plants depends on various environmental factors such as weather changes, stress conditions, etc. Many researchers have used the microscopic identification of fungal endophytes using standard mycological manuals as a traditional approach, but it is difficult to identify non-sporulating species up to species level (Kharwar et al. 2011, Gond et al. 2012). So, both morphological and molecular approaches have been applied to identify the isolated taxa.

Endophytic fungi form symbiotic relationships with their hosts, obtaining all essential nutrients from them while protecting them from stress, phytopathogens and herbivory (Khare et al. 2018). The current study provides information on endophytic fungi found in *Dillenia indica* L., allowing for further exploration of the hidden potential of these fungi.

In conclusion, the present study revealed 25 different fungal endophytes belonging to 20 genera associated with the ethnomedicinal plant *Dillenia indica* L. The dominant species were *Colletotrichum gloeosporioides, Daldinia eschscholtzii* and *Cladosporium cladosporioides.* The majority of the isolates belonged to the phylum Ascomycota and some isolates also belonged to Basidiomycota. Sordariomycetes was the dominant class of Ascomycota. *Dillenia indica* L is an ethnomedicinal plant with many medicinal values. So, the endophytic fungi isolated from this plant

may be a promising source of bioactive molecules. Further works on these fungi are going on to explore their bioactive potential.

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