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Traditionally, the plant, *Madhuca neriifolia* is used for various disease treatments. Very few research reports about this plant from the South West of Western Ghats of Karnataka, India, are available. Further, no research reports about the possible existence of endophytic fungi inhabiting this plant are found in the literature. Compounds from endophytic fungi are crucial elements in curing diseases. Therefore, it is necessary to explore the endophytic fungi that may be inhabiting this plant. Hence, this study is conducted to isolate and identify the possible existence of endophytic fungi in this plant. The test results are found to be positive. Four different endophytic fungi strains with different morphologies are isolated and identified from the leaves and twigs of this medicinal plant. The isolated fungi are identified based on the morphology of the fungal colony, PCR-amplified fungal internal transcribed spacer sequences (ITS) and by the phylogenetic analysis with the MEGA X software. All four different endophytic fungi are identified at the level of their species. They are *Alternaria alternata,Trichoderma asperellum, Aspergillus stellatus* and *Cladosporium cladosporioides*.

Key words – Endophytic fungi, *Madhuca neriifolia*, phylogenetic analysis, MEGAX

INTRODUCTION

Endophytes are microorganisms present in different parts of a plant body and establish a mutual relationship with the host plant without causing any diseases in it (Bacon *and White,* 2000). Usually, endophytes can be found in different forms like fungi, bacteria or actinomycetes in the host plant's body (Gouda *et. al.* 2016). Based on the critical nature of the endophytes, it can be predicted that endophytes may be beneficial, neutral or detrimental to their host plant (Sikora *et. al.* 2007).

In most of the cases, it was observed that the relationship between an endophyte is neither always pathogenic nor simply mutualistic (Wang *et. al.* 2007). Sometimes, the coexisting endophytes provide benefits to the host plant by protecting the plant from abiotic and biotic stresses (Gond *et. al.* 2010; Kharwar *et. al*. 2008). Fungal endophytes play a major role in the protection of the host plant from different pathogens through competition, antibiosis and mycoparasitism and it happens either directly or through proliferating within the host plant (Bailey *et. al.* 2006; Mejía *et. al.* 2008). Endophytic fungi can spend their life-cycle through intra or intercellular way to the whole or part of their life in the host plant's healthy tissues without causing any apparent symptoms of disease to the host plant (Li *et al.* 2008). It may be due to the enormous production of active novel compounds within it (Rodrigues *et al*. 2005). Different endophytic fungi show different biological activities, such as anticancer, antibacterial, antifungal, antitumor etc. (Rukachaisirikul *et al*. 2007). Therefore, it is necessary to study the endophytic fungi and the compounds existing in them and assess their effect in curing different diseases. Hence, a preliminary study has been conducted on the medicinal plant *Madhuca neriifolia* to isolate and identify the endophytic fungi existing in it.

Identifying endophytic fungi existing in a specific plant is a critical and difficult process. In earlier days, endophytic fungi were identified based on morphological characteristics of the genus and species. But it's quite difficult to identify all the

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species based only on morphology. Now, due to unprecedented advancement in molecular technology, it becomes comparatively easy and more reliable to identify the endophytic fungus from the fungal colony through a combined morphological study followed by molecular analysis. Some fungal species can not produce spores (sterile). In such types of fungi, the identification is solely based on molecular analysis using Internal Transcribed Spacer (ITS) region. ITS is normally used because of the presence of different variable regions in it (Guo *et al.,* 2000). In addition to the ITS region, the large subunit (LSU), Small subunit (SSU), B tubulin, RNA polymerase-I (RPB1), RNA polymerase-II (RPB2), EF1alpha, Chitin (ech42), Calmodulin etc. regions are also normally used in the identification of exact endophytic fungus species (Raja *et al.* 2017).

MATERIAL AND METHODS

Plant materials identification

The plant sample of *Madhuca neriifolia* was collected in the month of November from Seethanadi, the South West of Western Ghats of Karnataka, (13 0 28 / 30 // N 75 0 2 / 0 // E). Elevation range is above 80 meters from Mean Sea Level of India. *M. neriifolia* is a medicinal plant, which is found basically in river region of semi-ever green to evergreen forests of Western Ghats. This plant is found in Sri Lanka and India. In India, this is found in Bombay, Karnataka, and Kerala (Awasthi *et al*.1975). It is a moderate-sized tree, growing about 10 metres tall. Leaves grow up to 24 x 6 cm in length, crowded at the ends of the branches, oblong-elliptic to narrowly oblong-lanceolate, acute to obtuse at apex, glabrous, lateral nerves 14-22 pairs; pedicles up to 1.3 cm long. Young fruit oblong-lanceolate, glabrous; flowers yellowish white, 4 to 10 in clusters (Bhatt 2014; Awasthi *et al*. 1975). The morphological appearance of the plant is shown in Fig. 1. After morphological identification of the plant, it was processed for molecular identification. The molecular identification was done through DNA barcoding method and the barcode matK gene sequence. The result was submitted to NCBI data base and it was published in NCBI with the Accession No. – MN841760.

Isolation and sub-culturing of endophytic fungi

Healthy leaves and twigs of the selected medicinal plant were collected, packed with the transparent sterilized plastic bag and carried from the location of the plant species to the laboratory on the same day. Leaves and small twigs were processed following standard method with a minor modification at the level of the sterilising agent and its concentration. Ethanol (70%) was used for 5 sec followed by 0.01% mercuric chloride for 30 sec. to remove the epiphytic microorganism as well as other bacterial microorganisms.

Initially, the samples were washed with running tap water to remove the dust and debris which was attached to the outer part of the samples; it was done for 10 minutes. After cleaning the samples, it was soaked with 70% ethanol (v/v) for 5 sec. followed by 0.01% mercuric chloride ($HgCl_2$) for 30 sec. After 10 sec, finally, samples were rinsed with sterilized distilled water three times and dried with blotting paper and the samples were cut into 1 cm in length with the help of a sterilized blade for twigs and paper cutter for leaves. The samples were placed in 90 mm diameter Petri-dish containing Potato Dextrose Agar media (PDA). The PDA was made by using 200 g peeled potato cut into small pieces and boiled initially with 500 ml distilled water, followed by 200 ml distilled water. The potato liquid collected was by filtering the sample with the help of muslin cloth and made the final volume up to 1000ml by adding 200 ml of distilled water. Then 20 g of Dextrose was added followed by pouring and mixing agar (20 g) in the medium. The medium was autoclaved at 121^0C for 15 minutes. Antibiotic streptomycin (150 mg/l) was added to the medium to prevent bacterial contamination. After aseptic inoculation of the samples in the PDA medium, the Petri- dishes were sealed with parafilm (Size-M) and incubated for 12 h in daylight and 12 h at night at room temperature. After three to five days of inoculation, different fungal colonies were observed in the inoculated Petri plates. Each colony was transferred aseptically from the master plate to the newly prepared PDA plate; it took nearly two weeks. After the full maturity of each colony, it was again picked up from the edge of the individual colony with the fine-tipped needle and transferred into newly prepared different media like Malt Extract Agar (MEA), Czapek's Agar (CZA), Potato Dextrose Agar (PDA) containing Petri plates. Some endophytic fungal cultures were kept in the refrigerator for future reference while the remaining cultures were processed for morphological analysis and molecular analysis followed by genomic DNA extraction of those endophytic fungal cultures.

Microscopic observation of isolated endophytic fungi

The morphological identification was done through microscopic analysis. Newly grown endophytic fungal colony plates of MEA, CZA and PDA media culture plates were processed for microscopic analysis. Lactophenol cotton blue stain was used for the preparation of slides of the cultures. The endophytic fungi were picked up from the edge of the colony and transferred onto the lactophenol cotton blue stain containing sterilized glass slide and spread the endophytic fungi thoroughly and covered with slide and coverslip. After preparing the slides, the fungus morphology was observed under 10X, 40X and 100X magnification. Taxonomic identification was done based on the following characteristics as the morphology and growth pattern of the colony, hyphae, septa with spore morphology, colony colour on media, surface texture, margin characters, aerial mycelium, mechanism of spore production and conidial characteristics.

Extraction and PCR (ITS sequence) amplification of the endophytic fungal genomic DNA

According to the instruction of DNeasy Plant Mini Kit (QIAGEN), the endophytic fungal mycelial genomic DNA was extracted from the culture plates. The ITS-rDNA partial gene sequence was done for all endophytic fungal samples. Each endophytic fungal mycelial genomic DNA was extracted and amplified with the primer pair ITS4, ITS5 [(ITS4 R-5´TCCTCCGCTTATTGATATGC3´)and ITS5(5´GGAAGTAAAAGTCGTAACAAGG3´)]. The PCR amplification was carried out with total volume of 25 μl solution which contained following components; 10X buffer (2.5 μl), dNTPs (2 μl), ITS4 (1 μl), ITS5 (1 μl), Taq polymerase (0.25 μl), sterile dH_2O (19 μl). The DNA amplification was carried for 30 cycles which is as follows: 5 min initial denaturation for 94^0 C followed by1 min denaturation at 94^0 C, 30 sec primer annealing at 52^0 C, 1 min extension for 72^0 C and final 10 min extension at 72^0 C. With the help of gel documentation system, the PCR amplified product was visualized. After visualizing the PCR product, the quality and quantity of the PCR product was checked through purification.

The purified PCR product was processed for sequencing by ABI Prism® Big Dye®Terminator v3.1 cycle sequencing Kit (Applied Biosystem) 3100 Genetic Analyzer.The obtained sequences from the sequencer were edited by using Chromas Lite softwarefor inconsistencies.

Phylogenetic analysis

The obtained sequences were aligned and FASTA format was made and checked the identity of the sequences through BLAST in National Centre for Biotechnology Information (NCBI) website. The relevant target DNA sequences were retrieved from the BLAST search. The phylogenetic analysis was carried out based on the closely related sequences and the newly identified sequence comparison (Identity, query coverage) from the blast search in NCBI. BLAST search results were processed for construction of phylogenetic tree with the help of MEGA X software followed by multiple sequence alignment using MUSCLE, a maximum likelihood method by a bootstrap of 1000 replicates.

RESULTS AND DISCUSSION

Morphological identification of isolated endophytic fungi

After three to four days of culturing the endophytic fungi in PDA medium, potential different fungal colonies were observed on the medium. Each colony was transferred from the master plate to a new PDA containing plate. There was no contamination found in the pure culture plates after transferring the endophytic fungi from the master culture plate. Total four different endophyticfungi were isolated in pure form and their morphology was observed and analysed microscopically.

Characteristics of endophytic fungi isolate-1 from MEA medium

Endophytic fungus was transferred from PDA media to Malt Extract Agar medium (MEA). After seven days of inoculation, fungal colony appearedon MEA medium was mouse grey, floccose forming cushions on surface of colony, reverse slateblack. Hyphae variable in width, smooth to rough walled, subhyaline to olivaceous brown. Chlamydospores variable in shape and size, intercalary to lateral to terminal, simple to muriform, light olivaceous to sub

Table :1 Top five sequence from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT487794.1	*Alternaria alternata* KU20017.1	949	949	100%	0.0	100%
MT420650.1	*Alternaria* sp. isolate R9	949	949	100%	0.0	100%
MT640581.1	*Alternaria* sp. voucher HQU PS16	949	949	100%	0.0	100%
MT336603.1	*Alternaria alternata* strain Y. H. Yeh I0828	949	949	100%	0.0	100%
MT336602.1	*Alternaria alternata* strain Y. H. Yeh I0827	949	949	100%	0.0	100%

Fig: 1 *Madhuca neriifolia* plant.

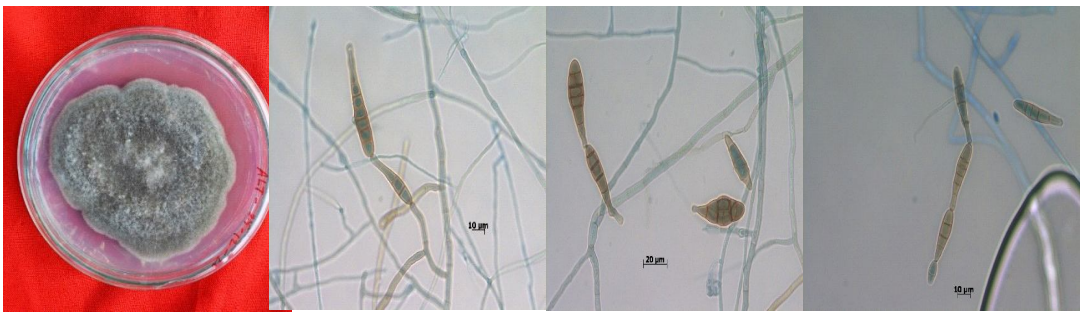
Table.2 : Top five sequence from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT529846.1	*Trichoderma asperellum* clone SF_570	1000	1000	100%	0.0	100%
MT529837.1	*Trichoderma asperellum*	1000	1000	100%	0.0	100%
MT529422.1	*Trichoderma asperellum*	1000	1000	100%	0.0	100%
MT529370.1	*Trichoderma asperellum*	1000	1000	100%	0.0	100%
MT367901.1	*Trichoderma asperellum* isolate UGM-LHAF	1000	1000	100%	0.0	100%

hyaline, globose. Conidiophore produced from superficial hyphae,simple to branched, up to 81.6 × 4.88 µm in dimension. Conidia variable in shape and size, dark brown, 2-3with transverse and longitudinal septa, up to 42.5 × 22.7 µm in dimension (Fig.2). These morphologicalfeatures of isolate-1are the key characteristics of fungal species *Alternaria,*thus, tentatively identified as *Alternaria alternata* (Woudenberg *et al.,* 2013).

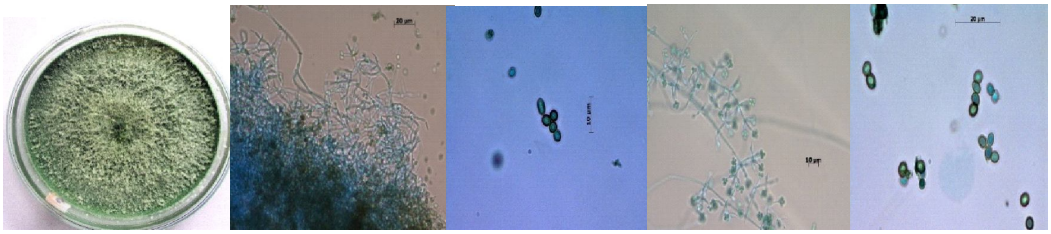
Endophytic fungal isolate-2 from PDA medium

Endophytic fungi colony was observed on Potato Dextrose Agar (PDA) medium after five days of



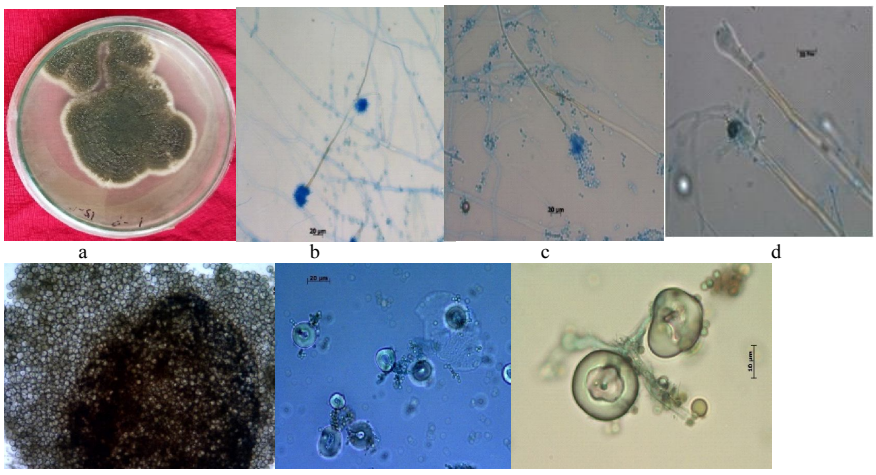
a b c d

Fig: 2 a) Pure culture of endophytic fungi *Alternaria alternata*, b) Hypha, conidiophore with conidia (10µm), c) Single, mature and branching conidia (20 µm). d) Branching chain of conidia with terminal conidia (10µm).



a b c d e

Fig: 3. a) Pure culture of *Trichoderma asperellum* b) Sparingly branched conidiophores. c) Conidia of *T. asperellum* (10µm). d) Phialides were elongated with long neck, two to three groups, variable in shape and size with conidia (10µm) e) Conidia of *T. asperellum* (20 µm**).**



a b c d

e f g

Fig:4. a) Pure culture of *Aspergillus stellatus*. b) Foot cell, conidiophore with compact conidia. c) Long conidiophore, biseriate sterigmata with conidia. d) Vesicles of *A. stellatus*. e) Compact mass of hulles cells. f) Single hulle cell (20 µm). g) Hulle cells (10 µm).

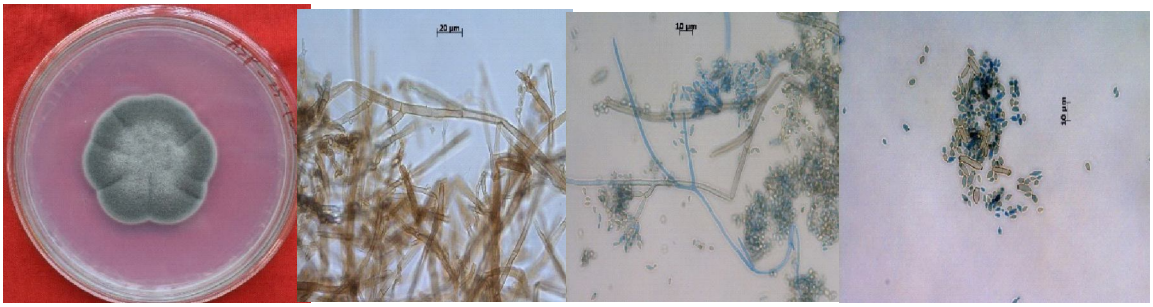


Fig: 5. a) Pure culture of *Cladosporium cladosporioides*. b) Olivaceous brown, branched, septate, smooth to rough walled hyphae. c) Branched, septate, smooth to rough conidiophore with conidia. d) Branched, catenate, cylindrical and variable shape conidia.

Table.3 : Top five sequences from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT122792.1	*Aspergillus stellatus* isolate white 3	937	937	100%	0.0	100%
MN796091.1	*Aspergillus stellatus* isolate FMF5	937	937	100%	0.0	100%
MN686298.1	*Aspergillus stellatus* strain CFE-76	937	937	100%	0.0	100%
MK376947.1	*Aspergillus* sp. strain 582PDA13	937	937	100%	0.0	100%
KU866665.1	*Aspergillus stellatus* strain DTO 325-A9	937	937	100%	0.0	100%

Table.4 : Top five sequences from BLASTn analysis

Gene Bank Accession No.	Description	Max score	Query cover	Query coverage	E value	Identity (%)
MT573533.1	*Cladosporium cladosporioides* strain BFMY-2	915	915	100%	0.0	100%
MT497437.1	*Cladosporium tenuissimum* isolate SS_69	915	915	100%	0.0	100%
MT497434.1	*Cladosporium tenuissimum* isolate SS_25	915	915	100%	0.0	100%
MT483945.1	*Cladosporium tenuissimum*	915	915	100%	0.0	100%
MT367253.1	*Cladosporium cladosporioides* strain HSX11#-10-1	915	915	100%	0.0	100%

Table.5: Gen Bank and NFCCI Accession Number

Strain name	Parts of isolation	Species name	Family name	Gen Bank Accession no	NFCCI Accession no
Isolate 1, MNMB1	Twigs	*Alternaria alternate* (Fr.) Keissl.	*Pleosporaceae*	OK326856	5054
Isolate 2, MB2	Twigs	*Trichoderma asperellum* Samuels, Lieckf. & Nirenberg	*Hyphocreaceae*	MN880489	5055
Isolate 3, MNMB3	Leaves	*Aspergillus stallatus* Curzi	*Trichocomaceae*	OK326861	5056
Isolate 4, MNMB5	Leaves, Twigs	*Cladopsorium cladosporides* (Fresen.) G.A. de Vries	*Cladosporiaceae*	OK326878	5057

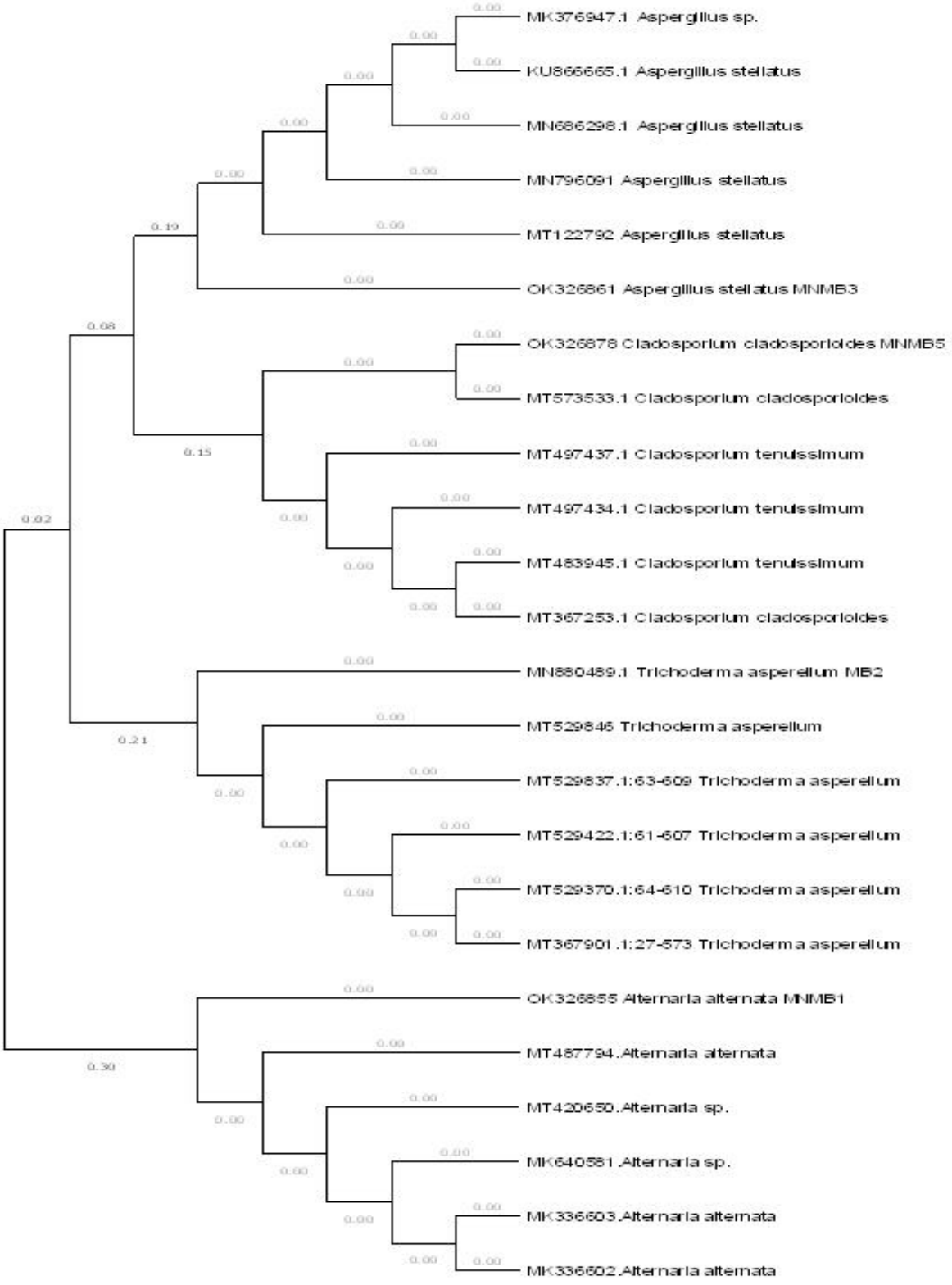


Fig: 5. Phylogenetic tree of isolated four endophytic fungi

inoculation. Morphology of colony on PDA medium looks dull yellowish green, postulate, fast growing, reverse pale yellow. Conidiophores are sparingly branched. Phialides are elongated with long neck, two to three in groups, variable in shape and size, 2-5 in number, measuring 11.6-9.1×3.1-2.5 µm. Conidia produced on phialides forming conidial heads, oval globose to sub-globose, wall thickened and darkened, smooth walled, measuring 3.7–5.1× 2.7–3.6 µm (Fig. 3). Above identified isolate-2 characteristics are the key features of the fungal species *Trichoderma*, thus, tentatively identified as *Trichoderma asperellum* (Nagamoni *et al.* 2002).

Endophytic fungi isolate-3 from CZA medium

Endophytic fungus was transferred aseptically from the PDA medium to Czapeks Agar (CZA) medium. After four days of inoculation on CZA medium, fungal colony was observed. The colony grown faster at 25^{0}C. Its appearance was olivaceous green, velvety, reverse light olivaceous. Hulle cells hyalinewere seen abundantly, produced in compact mass, smooth walled and measuring 21.8 ×21.2 µm in size. In ascomata, teleomorph is not seen, but hulle cells are present there abundantly. Conidiophores werelong, olivaceous, smooth walled, up to 510×6.2 µm in size. Vesicles were globose to sub-globose,olivaceous in form, smooth walled and up to 121.5×20.9 µm in size. Sterigmata of the fungus wasbiseriate, primaries smoothwalled, hyaline, upto 7.2×3.9µm in size. Secondary-ampulliform, hyaline, upto 4.5×2.5 µm in size. Conidia appearance was globose to sub globose, olivaceous, smooth walled and 2.5–2.9µm in size (Fig3). Above observed characteristics of the isolate-3 are the key feature of the fungal species *Aspergillus*, thus tentatively identified as *Aspergillus stellatus*.

Endophytic fungi isolate-4 from MEA medium

Endophytic fungus was transferred from PDA medium to Malt Extract Agar (MEA) medium. After five days of inoculation, fine colony was observed in the MEA medium. Appearance of the colony is olivaceous grey velvety, margin olivaceous green and reverse slate black. Hyphae are olivaceous brown, branched, septate, smooth walled to rough walled. Conidiophores are branched, septate, smooth to rough walled, fertile on terminal part of conidiophore, variable in size. Conidia are catenate, branched, base thickened and darkened, cylindrical, globose to pyriform to fusoid, olivaceous brown to sub hyaline, variable in shape and size-29.65 × 4.95 –15-7 × 4–3 µm (Fig 4). The morphological characteristicsof isolate-4 are the key features of the fungal species *Cladosporium*, thus, isolate-4 was tentatively identified as (*Cladosporium cladosporioides*. Crous *et al*. 2017).

ITS identification of the endophytic fungi

The ITS region of the extracted isolates was amplified with the help of ITS4 and ITS5 primers and Blast aligned to identify the corresponding fungal ITS sequences in GenBank. Blast alignment was done in FASTA format. The fungi are identified at the level of species by taking the percentage identity. For all the four isolates, 100% query coverage and 100% identity at the species level are observed. The isolate-1 is identified as *Alternaria alternata*, isolate-2 as *Trichoderma asperellum*, isolate-3 as *Aspergillus stellatus* and isolate-4 as *Cladosporium cladosporioides* (Tables 1, 2, 3, 4).

The phylogenetic tree was constructed based on the isolated endophytic fungi ITSsequences. These newly identified sequences were submitted to the GenBank and the obtained accession numbers are given in Table 5.

The present study has been conducted to isolate and identify the taxonomy and phylogeny of endophytic fungi from the medicinal plant *M. neriifolia* and to observe their seasonal variation in three different seasons- ie., summer, rainy and winter. In this study, only the leaves and twigs of this plant have been selected. However, different endophytic fungi can be present in various parts of the plant body, such as flowers, fruits, leaves, twigs, stems, inner bark, roots, and shoots.

M. neriifolia belongs to the family Sapotaceae; plants of this family are known for high medicinal value, andhence, there is a high possibility of the presence of valuable compounds with medicinal properties. Kuralarasi and Lingakumar (2018) have reported the availability of endophytic fungi in *Madhuca longifolia* from India. However, there are no reports found about the presence of endophytic fungi in the plant *M. neriifolia.* Hence, to check the availability of endophytic fungi, the plant *M. neriifolia* has been collected from Seethanadi, SouthWest of Western Ghats, Karnataka. SinceWestern Ghats region is known

for its richness in medicinal plants, a study from this specific region may help us in future to understand the regional differences of endophytic fungi ecology that this plant can host in different regions of the world. Further, extraction of endophytic compounds and their applications in curing different diseases may confirm the medicinal value of this plant as well as the endophytic fungi. PDA, MEA and CZA media have been used for the culturing of the fungal samples extracted from leaves and twigs of the plant *M. neriifolia*. After pure culturing of the extracted samples, it was sub-cultured for further analysis. For isolation and separation of endophytes, preventive measures have been under taken for unwanted epiphytic fungi contamination. Sterilized media was kept as the control to observe the possible contamination in the media containing plate every time. No contamination was observed in control and inoculated culture plates. Therefore, it is confirmed that the four endophytic fungi identified in the present study are pure in nature without any contamination.

Morphologically, the extracted fungi showed different fungal growth rates and spore production rates for various media. The production of spores is dependent on the types of media and the nature of the fungi-whethersporulatingor non-sporulating fungi. The production of spores were more prominent for isolate-1 and isolate-4 in the MEA medium than PDA medium. Contrary to it, for isolate-2, the sporulation was found to be better in PDA. In the case of isolate-3, the sporulation was good in CZA) medium. The identification of the extracted fungus was carried out based on the morphological and molecular analyse data. This combined approach gives accurate results in the identifying process of a species. The morphological identification is based on the morphology of the colony, mycelia structure, conidiophores, and size, shape, colour and texture of the conidia. Molecular analysis was carried out with the ITS4, ITS5 sequence analysis. ITS sequencing plays a major role in identifying an endophytic fungal species. Due to the advances of having different variable regions, the ITS4 and ITS5 primers have been selected for molecular identification (Peay *et al,* 2008).The phylogenetic tree has been constructed based on the similarity of the sequences. MEGA X software has been used to construct the phylogenetic tree for all the four endophytic fungi. The combined process confirms identification of four endophytic fungi such as *Alternaria alternata, Trichoderma asperellum*, *Aspergillus stallatus* and *Cladosporium cladosporides*. In this study, based on the morphological and preliminary molecular analysis, it is also confirmed that the isolates are 100% pure at species level and hence no further sequencing with reference to LSU, SSU, ?-tubulin, RPBI, RPBII, EF1 alpha, ech42, and Calmodulin (Raja *et.al.* 2017) was carried out.

The extracted fungal samples were sent to National Fungal Culture Collection of India (NFCCI) for further authentication. The NFCCI results are in agreement with our results. The molecular sequence analysis data were deposited in NCBI and the fungal cultures were deposited in NFCCI for further references. The accession numbers of NCBI and NFCCI are listed below in Table 5.

There are several reports on the isolation and identification of endophytic fungi from various plants. *A. alternata* and *C. cladosporoides* were isolated from the inner bark of the medicinal plant - *Mimus opselengi* which belongs to the family Sapotaceae. Further, the anticancer compound ergoflavin ($C_{30}H_{26}O_{14}$) was extracted from the leaf of thisplant. The plant *Madhuca indica,* belonging to the family *Sapotaceae,* also showed the endophytic fungi such as *A. alternata, Alternaria* sp*., Aspergillus* sp*., Trichoderma* sp*., Cladosporium cladosporoides* and many other hyphomycetes, coelomycetes and ascomycetes from the leaves, stems and bark of the plant from different regions of India. *Madhuca longifolia* plant bark showed the existence of endophytes such as:*Alternaria sp., Colletotrichum sp., Diaporthea sp., Phomopsis sp., Mycosperellacea sp., Fusarium sp.,* and *Pestolopsis sp.* (Kuralarasi and Lingakumar, 2018).These results clearly indicate that irrespective of the regional differences, different plant species of family Sapotaceae found across India hosts similar type of endophytic fungi. Seasonal variation in terms of inhabiting endophytic fungal diversity was observed in different medicinal plant species. In this study, it is found that all the four fungi are not seen in all the seasons (summer, rainy and winter). *Alternaria* and *Trichoderma* were found in the twigs of the plant only in the rainy season, *Aspergillus* sp. were found only in leaves during winter season. But *Cladosporium* were found in both the leaves and twigs of the plant throughout summer, rainy and winter seasons . Colonization of different endophytic fungi is

dependent on the presence of bioactive compounds of the selected materials, their rates of growth (Arnold *et al.* 2000), leaf-age and the seasonal variation (Osono and Masuya, 2012).

In this study also, seasonal variation in terms of colonization of different endophytic fungi in the selected materials were observed. The variation might be influenced by the factors like geography of the selected plant species (mean sea level) and environmental conditions (temperature, humidity percentage) (Strobel and Daisy, 2003), growth pattern of the leaves (Arnold *et al.* 2000), age of the selected leaves, dominant behaviour of the microbes present inside the plant body, samples collection time, host and microbes interaction and their seasonal priority. Above factors might also influence the colonization of different endophytic fungi in this plant species. There is evidence that the presence of endophytes during seasonal variation in *Madhuca longifolia, M. elengi,* and *M. indica* which belongs to the same family Sapotaceae (Kuralarasi and Lingakumar, 2018). Similarly, different plant species like *Heisteria concinna* and *Ouratea lucens* (Arnold *et al.,* 2000), *Theobroma cacao* (Arnold and Herre, 2003) and *Camellia japonica* (Osono, 2008) also showed seasonal variation in endophytic association.

CONCLUSION

Endophytic fungi have the potential to help not only the plant body (host) in different ways but also can provide effective secondary metabolites or active novel compounds for treating diseases. Numerous medicines have been isolated from different endophytic compounds. Therefore, identification of different endophytic fungi living in different medicinal plants which have a history of usages as medicine is required. It can confirm the medicinal value of such plants. Study of the endophytic fungi available in a traditionally used medicinal plant and the influences of the active compounds in curing different diseases found in such endophytic fungi may lead to the discovery of novel drugs. Study of the effectiveness of endophytes in curing diseases may give a better understanding of the working pattern of a traditional medicinal plant in disease treatment.The present study is able to confirm the presence of four endophytic fungi viz., *Alternaria alternata, Trichoderma asperellum, Cladosporium cladosporoides,* and *Aspergillus stellatus*. These fungi are known for their medicinal values. Therefore, further pharmacological studies are required to know the potential and effectiveness of these fungi in treating different diseases which may give a better an insight in understanding how this plant helps in treating diseases.

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REFERENCES

Arnold, A. E., Maynard, Z., Gilbert, G. S., Coley, P. D., Kursar, T. A. 2000. Are tropical fungal endophytes hyperdiverse? *Ecology Letters* **3**: 267-274.

Arnold, A.E., Herre, E.A. 2003**.** Canopy cover and leaf age affect colonization by tropical fungal endophytes: ecological pattern and process in *Theobroma cacao* (Malvaceae). *Mycologia* **95**: 388–398.

Awasthi,Y.C., Bhatnagar, S. C., Mitra, C. R. 1975. Chemurgy of Sapotaceous plants: *Madhuca* species of India. *Springer: The New York Botanical Garden.* **29**: 380-389.

Bacon, C. W., White, J. F. 2000. Microbial endophytes. Marcel Dekker,New York, Pp 341-388.

Bailey, B.A., Bae, H., Strem, M.D., Roberts, D.P., Thomas, S.E., Crozier, J., Samuels, G.J. , Choi, Ik-Y., Holmes, K.A. 2006. Fungal and plant gene expression during the colonization of cacao seedlings by endophytic isolates of four *Trichoderma* species. *Planta* **224**: 1149-1164.

Bhat, G. K. 2014. *Flora of South Kanara*. Published by Taxonomy Research Centre, Department of Botany, Poomaprajna College, Udupi, India. pp 928.

Crous, P. W., Braun, U., Schubert, K., Groenewald, J. Z. 2007. Delimiting *Cladosporium* from morphologically similar genera. *Stud. Mycol.* **58 :** 33-56.

Gond, S.K., Verma, V.C., Mishra, A., Kumar, A., Kharwar RN. 2010. Role of fungal endophytes in plant protection. *Mycoscience* **53**:113-121.

Gouda, S., Das, G., Sen, S.K., Shin, H. S., Patra, J.K.. 2016. Endophytes: A Treasure House of Bioactive Compounds of Medicinal Importance. *Front. Microbiol* .**7:** Article 1538.doi: 10.3389/fmicb.2016.01538

Guo, L.D., Hyde, K.D., Liew, E.C.Y. 2000. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA. *New Phytol.* **147**: 617–630.

Kharwar, R.N., Verma, V.C., Strobel, G., Ezra, D. 2008. The endophytic fungal complex of *Catharanthus roseus* (L. G. Don). *Curr. Sci.* **95** : 228-233.

Kuralarasi, R., Lingakumar, K. 2018. Isolation and antibacterial activity of endophytic fungi from *Madhuca longifolia* Bark. *J. Plants Stud.* **6**: 36-39.

Li, J., Zhao, J.L., Xu, L.J., Zhou, L.G., Li, X. 2008. Endophytic fungi from rhizomes of *Paris polyphylla* var. *yunnanensis*. *World J. Microbiol.Biotechnol.* **24**: 733–737.

Mejía, L.C., Rojas, E.I., Maynard, Z., Bael, S.V., Arnold, A.E., Hebbar, P., Samuels, G.J., Robbins, N., Herre, E. A. 2008. Endophytic

fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biol. Cont.* **46**: 4-14.

Nagamoni, A.,Manoharachary, C., Agarwal, D.K.,Chowdhry, P.N. 2002. Monographic contribution of *Trichoderma*. Associated Publishing Company. ISBN 10: 8185211558.

Osono, T., Masuya, H. 2012. Endophytic fungi associated with leaves of Betulaceae in Japan. *Can. J.Microbiol.* **58**:507–515.

Peay K.G., Kennedy P.G., Bruns T.D. 2008. Fungal community ecology: a hybrid beast with a molecular master. *Bioscience* **58**: 799-810.

Raja, H.A. Miller, A.N., Pearce, C. J., Oberlies, N. H. 2017. Fungal Identification Using Molecular Tools: A Primer for the Natural Products Research Community. *J. Nat. Prod.* **80**: 756–770.

Rodrigues, K.F., Costa, G.L., Carvalho, M.P., Epifanio, R.D.A. 2005. Evaluation of extracts produced by some tropical fungi as potential cholin esterase inhibitors. *World J.Microbiol.Biotechnol.* **21** :1617–1621.

Rukachaisirikul, V., Sommart, U., Phongpaichit, S., Sakayaroj, J., Kirtikara, K. 2007. Metabolites from the endophytic fungus *Phomopsis* sp. PSU-D15. *Phytochem.* **9** :783–783.

Sikora, R.A., Schäfer, K., Dababat, A.A. 2007. Modes of action associated with microbially induced in planta suppression of plant-parasitic nematodes. *Australas. Plant Pathol*. **36**: 124-134.

Strobel, G., Daisy, B. 2003. Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* **67**:491–502.

Wang, B., Priest, M.J., Davidson, A., Brubaker, C.L., Woods, M.J., Burdon, J.J. 2007. Fungal endophytes of native *Gossypium* species in Australia. *Mycol. Res.***111** :347-354.

Woudenberg, J.H.C., Groenewald, J.Z., Binder, M.,Crous, P.W. 2013. *Alternaria* redefined. *Stud. Mycol.* **75**:171-212