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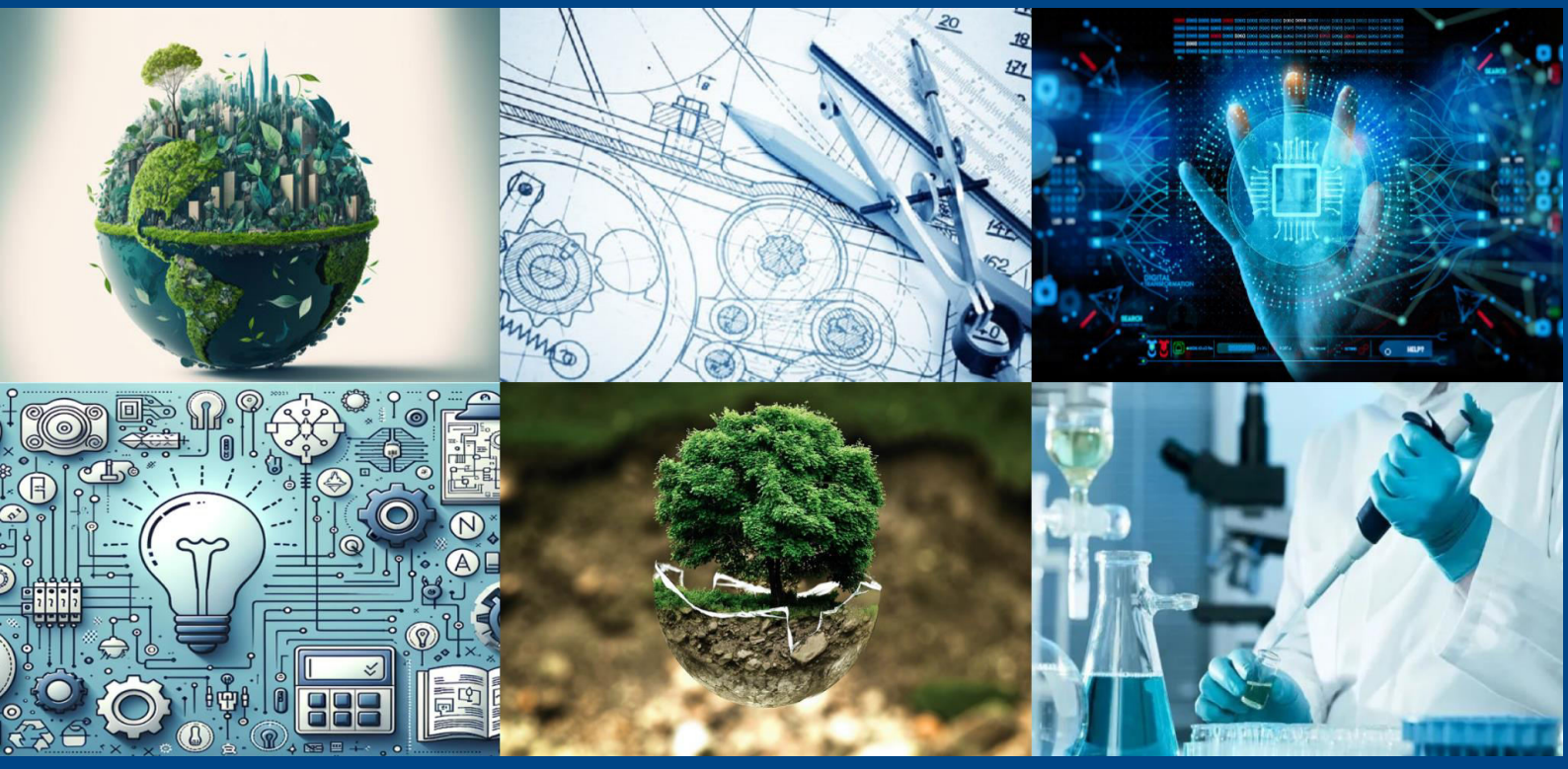

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Molecular identification of Endophytic fungi isolated from *Microstachys chamaelea (L.) Müll.Arg.*

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ABSTRACT: Endophytes are mutualists microorganisms that colonize in healthy tissues of live host plants showing no apparent symptoms. These endophytes can produce a variety of secondary metabolites having a wide range of essential properties they can be used for various medicinal, environmental and agronomic purposes. This study was carried out to isolate endophytic fungus living in the shoot of *Microstachys chamaelea* plant and to identify and characterize these endophytes. Total 12 endophytic fungi divided into 11 genera *Aspergillus, Fusarium*, *Colletotrichum*, *Cladosporium*, *Trichoderma*, *Alternaria*, *Nigrospora*, *Penicillium*, *Mucor*, *Rhizopus*, *Curvularia* fungal species were isolated and identified. This study shows that *Microstachys chamaelea* leaves and stem inhabitant by diverse group of endophytic fungi rather than flower. Endophytes were identified by 18SrDNA sequencing using ITS1 and ITS4 primers. Genomic DNA was isolated using Cetyl trimethyl ammonium bromide method of the endophytes. Further investigations are in needed to antimicrobial screening and isolation of bio active compounds and for a new drug discovery.

KEYWORDS: Endophytic fungi, *Microstachys chamaelea*, Molecular identification

I. INTRODUCTION

Endophytic fungi are considered as the hidden, unutilized resource have been reported from various plant species, which contribute to the diversity of microorganisms in innate environment and produce various bioactive compounds and novel metabolites [19, 20]. The extracts of endophytic fungi have been reported to show antimalarial, antimicrobial and cytotoxic activities on human cell lines [15]. Diverse variety of natural products have recently been identified from endophytic fungi that include substances that have shown promising anti-cancer, antioxidant, anti-viral, immune-suppressing and other bio-activities [12, 18] . A total of 20 types of fungal species were isolated from *Argemona mexicana*. More than 200 endophytic fungi were isolated from leaves and stems of *L. sidoides* which represented species belonging to Ascomycota, Coelomycetes and Hyphomycetes [7]. 27 fungal genera belonging to Ascomycota, Zygomycota and Basidiomycota from roots of *Pseudotsuga menziesii* and *Pinus ponderosa* using a combination of morphology and ITS sequence data[6]. In endophyte studies the most widely used DNA barcode in molecular identification is the ITS region [5, 11, 14] by using that 24 endophytic fungi belonging to *Garcinia mangostana* and *G. parvifolia* and studied the antibacterial activity by using filtered broth suspension[16].

Microstachys chamaelea belonging to the family Euphorbiaceae was selected to investigate the diversity of endophytic fungi associated. Leaf of *Microstachys chamaelea* shows pharmacological potential such as leaf decoction taken with butter is considered a tonic, and is applied to the head as a treatment for vertigo and decoction of the leafy stems is used as a bath to relieve teething pain in babies.

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II. MATERIALS AND METHODS

Plant samples Collection

The healthy plant of *Microstachys chamaelea* were collected from Karigatta hill (12°25′05″N 76°43′17″E) located on the Srirangapatna, Mandya district, Karnataka, India. The plant authentication and taxonomic identification of the plant was carried out by the personnel of the Herbarium of the Botanical survey of India, Coimbatore. The fresh plant samples were collected and placed in sterile zipped polythene bags. All samples were processed within 24hr of collection.

Isolation of Fungal endophytes:

The collected plant were aseptically transferred to the laboratory immediately and thoroughly cleaned under running tap water to remove soil debris/dust from the surface. The fresh plant leaf, stem and flowers were cut into 1.0 cm× 1.0 cm with the help of a sterile blade under aseptic conditions. The sequential immersion of the in ethanol(70%) for surface sterilization for 3 minutes, Sodium hypochlorite(5%) for two minutes and ethanol (70%) for 30s was followed by a final rinse in sterile distilled water to remove the chemical components present on it. The samples were air-dried [5,10]. Fungal isolation was done using the "Tissue Planting Method" on water agar (WA) medium (Fig. 1). 10-15 segments were plated per plate. The plates were wrapped in parafilm and incubated at 27±2º C in a light chamber with 12 hours of light followed by 12 hours of dark cycles for 3-4 weeks and were observed for emergence of colonies.The fungi from these colonies were sub-cultured to obtain pure isolates [2]. Furthermore, the hyphal tips of morphologically distinct endophytic fungi were carefully collected and transferred to newly prepared potato dextrose agar.

Morphological identification:

Morphological identification of the isolated endophytic fungi was done based on colony characters comprising of shape and size of colony, color of colony and hyphal characters by using the identification relevant literatures [1,3,10, 17].

Molecular characterization of endophytes:

The nuclear DNA was extracted from the freeze-dried mycelial mat using the cetyl-trim ethyl ammonium bromide (CTAB) method with trivial modifications. The concentration of DNA was measured using nanodrop spectrophotometer at 260 and 280 nm. The DNA was amplified with the PCR technique using a PCR kit. Molecular identification of the endophytic fungus was carried out by the ITS region amplification using universal ITS primers-ITS1 (5'-TCCGTAGGTGAACCTG CG-3') and ITS4 (5'- TCCTCCGCTTATTGATA TGC-3') using an initial denaturation temperature of 95°C for 5 min, 35 cycles of denaturation was performed at 94 °C for 1 min, annealing at 55 °C for 30 s, extension at 72°C for 2min, and the final extension of 72°C for 10 min. Amplified products were analyzed with horizontal agarose gel electrophoresis through 1% agarose gel supplemented with ethidium bromide along with the 100bp DNA marker. The purified products of PCR were sequenced in genetic analyzer (Dextrose technologies pvt.ltd. Bangalore). The resulting sequences were then analyzed using the Basic Local Alignment Search Tool (BLAST), and the most related sequences were retrieved from GeneBank NCBI [9,13].

III. RESULTS AND DISCUSSION

Total 12 endophytic fungi divided into 11 genera *Aspergillus, Fusarium*, *Colletotrichum*, *Cladosporium*, *Trichoderma*, *Alternaria*, *Nigrospora*, *Penicillium*, *Mucor*, *Rhizopus*, *Curvularia* fungal species were isolated and identified.

The morphological characterization of endophytic fungi isolated from *Microstachys chamaelea* was carried out through microscopic examination of stained fungal mounts. Figure 2 illustrates the microscopic structures of twelve distinct fungal isolates, designated Mc1 to Mc12, identified based on their conidial morphology, hyphal arrangement, spore shape, septation, and pigmentation.

The isolate Mc1 (Fig A) was identified as *Aspergillus luchuensis*, displaying dense hyphal networks and characteristic conidial heads. Mc2 (Fig B), *Fusarium graminearum*, was recognized by its septate hyphae and canoe-shaped macroconidia. Mc3 (Fig C), corresponding to *Colletotrichum garzense*, exhibited curved conidia and sparse mycelial growth, typical of the genus. The isolate Mc4 (Fig D), identified as *Cladosporium herbaroides*, showed darkly pigmented, branched conidiophores with blastoconidia.

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Mc5 (Fig E), representing *Trichoderma longibrachiatum*, revealed fine, branched hyphae and clustered conidia under lactophenol cotton blue staining. Mc6 (Fig F), *Alternaria alstroemeriae*, was marked by chain-like conidia with transverse and longitudinal septa. Mc7 (Fig G), identified as *Nigrospora* species, had spherical black spores, commonly detached from short conidiophores. The isolate Mc8 (Fig H), *Penicillium digitatum*, was recognized by its brush-like conidiophore arrangement and globose conidia. Mc9 (Fig I), corresponding to *Mucor jansseni*, showed broad, aseptate hyphae and large sporangia, indicative of zygomycetous fungi. Mc10 (Fig J), *Rhizopus oryzae*, exhibited stout, non-septate hyphae and prominent sporangiophores.

Isolate Mc11 (Fig K), identified as *Curvularia* species, demonstrated curved, multicellular conidia with dark pigmentation. Finally, Mc12 (Fig L), *Alternaria alternata*, was characterized by muriform conidia and a branching conidiophore system. The results clearly establish that *Microstachys chamaelea* hosts a taxonomically diverse range of endophytic fungi. These isolates represent multiple genera, including ascomycetous and zygomycetous members, many of which are known for their ecological significance and biotechnological potential. The wide diversity observed could be linked to the plant's unique phytochemical environment, which supports colonization by a broad array of fungal taxa (Table 1 and Fig. 2). The biodiversity analysis showed that colonization of endophytic fungi was more significant and more diverse in leaf and stem than flower.

Similarly from Euphorbiaceae family plants 27 fungal species belonging to 15 fungal genera in addition to one variety were isolated and identified for the first time from *Euphorbia geniculata* plants[7] and two endophytic fungi LA(1) and LC(2) were isolated from the leaves of a Nigerian plant *Euphorbia hirta*. The fungi were subjected to solid state fermentation on rice medium and metabolites were extracted using ethyl acetate. The fungal crude extracts were screened for antimicrobial and antioxidant activities[4].
The biodiversity analysis showed that colonization of endophytic fungi was more significant and more diverse in leaf and stem than flower. Molecular characterization of total 12 isolated endophytic fungi taxa with their GenBank accession numbers is depicted in Table 1.

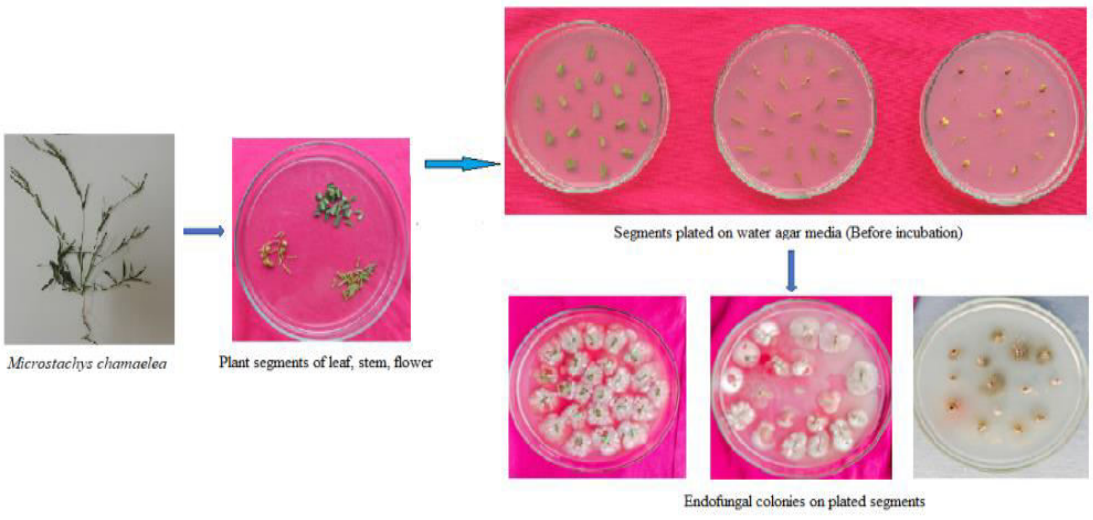

Fig.1. Tissue planting and Colonies of endophytic fungi on PDA medium

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Table1. Morphologically identified endophytic fungi associated with leaf, stem and flower of *M. chamaelea* by comparing ITS sequences with GeneBank data using a BLAST search.

Isolates No.	Sequence ID	Fungal species	Leaf	Stem	Flower
Mc_1	XR_005976915.1	*Aspergillus luchuensis*	+	+	+
Mc_2	LG262884.1	*Fusarium graminearum*	+	+	+
Mc_3	NR_176192.1	*Colletotrichum garzense*	+	+	-
Mc_4	NR_119655.1	*Cladosporium herbaroides*	+	+	-
Mc_5	NR_137309.1	*Trichoderma longifialidicum*	+	+	-
Mc_6	MH863036.1	*Alternaria alstroemeriae*	+	+	-
Mc_7	OP572420.1	*Nigrospora sp.*	+	+	+
Mc_8	NW_014574583.1	*Penicillium digitatum*	+	+	-
Mc_9	MH870818.1	*Mucor janssenii*	+	+	+
Mc_{10}	EE001248.1	*Rhizopus oryzae*	+	+	+
Mc_{11}	MZ695824.1	*Curvularia sp.*	+	+	-
Mc_{12}	OQ248210.1	*Alternaria alternata*	+	+	-

'+' and '-' represents the presence and absence of fungi, respectively.

Molecular confirmation and metabolite profiling of isolated endophytic fungi will further strengthen their potential applications in agriculture, medicine, and industry.

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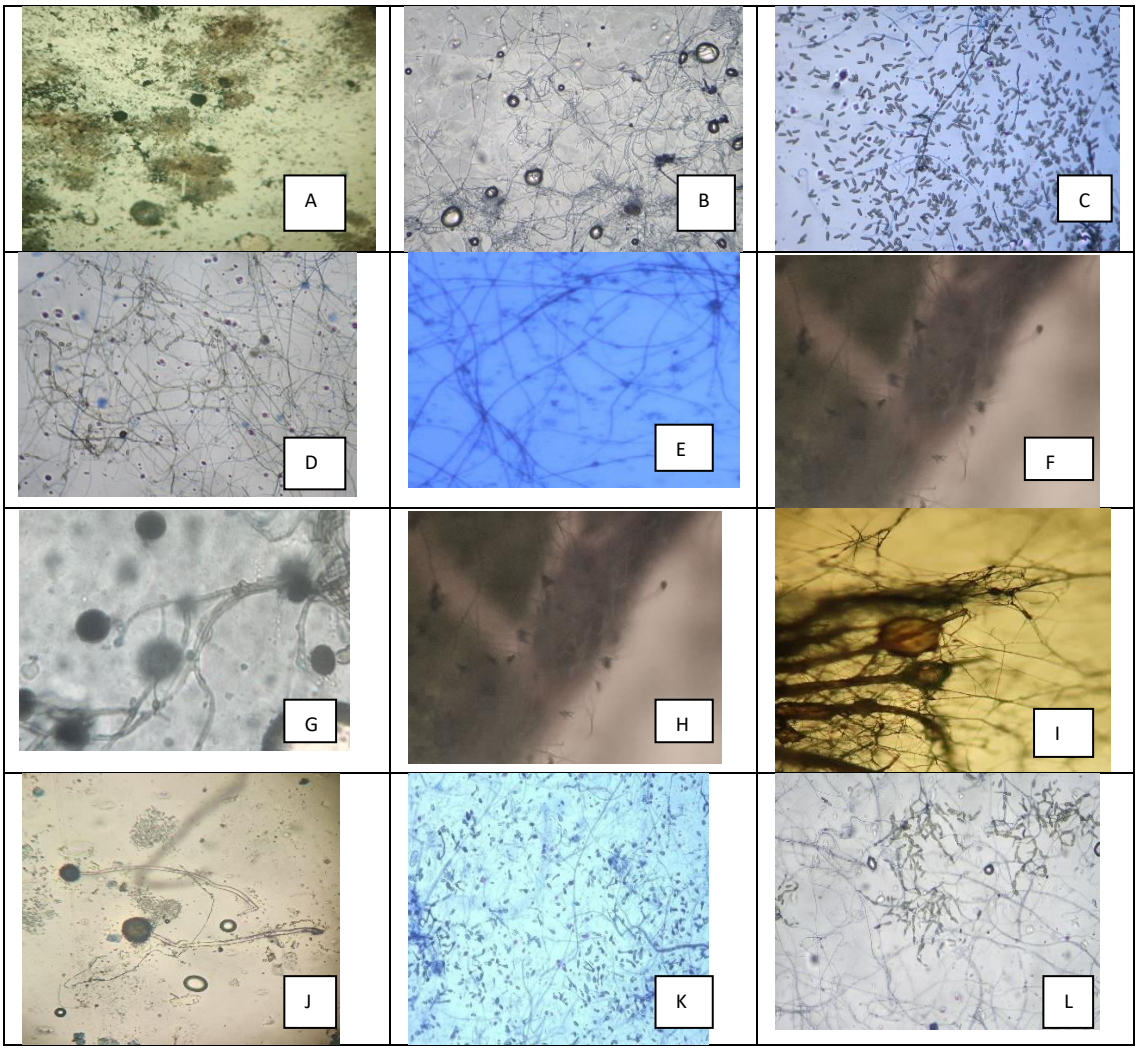

Figure2. Isolated endophytic fungi from *Microstachys chamaelea* are **A.** *Aspergillus luchuensis* , **B.** *Fusarium* graminearum, **C.** Colletotrichum garzense, **D.** *Cladosporium herbaroides*, **E**. Trichoderma *longifialidicum*, **F.** *Alternaria alstroemeriae*, **G.** *Nigrospora sp.*, **H.** *Penicillium digitatum*, **I.** *Mucor* jansseni, **J.** *Rhizopus oryzae*, **K.** *Curvularia sp.*, **L.** *Alternaria alternata*.

IV. CONCLUSION

Endophytic fungi are source for extraction of medicinally important metabolites has been gaining increasing interest. Metabolites produced by endophytes are being recognized as a versatile arsenal of antimicrobial agents.

Euphorbiacea is least explored family for endophytic characterisation and their active molecule isolation . Hence current study focused on isolation of endophytic fungi from different parts of *Microstachys chamaelea* plant. From the study it is concluded that endophytic fungi abundantly found on leaves and stem rather than flower. Which were further investigated for their chemical screening and antimicrobial property.

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