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Endophytic fungi isolation and identification from Memecylon species of Western Ghats, India

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Abstract

Endophytic fungi have been documented as sources for novel secondary metabolites with useful medicinal properties. Interest in fungal endophytes is mainly due to their chemical diversity. These signify a virtually untapped source of chemical reservoir that finds applications in agriculture and therapeutics. Sampling and characterization of fungal endophyte diversity is an emerging challenge, which leads to the discovery of new species producing new compounds and a better understanding of their role in ecosystems. In the present study fungal endophytes were isolated from surface sterilized leaf and stem segments of five *Memecylon* species such as *M. umbellatum, M. edule, M. talbotianum, M. malabaricum* and *M. wightii*. A total of 156 isolates of endophytic fungi were obtained from 2000 tissue segments of five *Memecylon* species being investigated. Of the 156, endophytic isolates recovered, 86 sporulated and belonged to 10 genera, *Alternaria* (12.2%), *Pleosporales* (6.4%), *Stagonos poropsis* (3.8%), *Clados porium* (8.2%), *Fusarium* (7.3%), *Aspergillus* (12.2%), *Pestalopsis* (7.5%), *Collectotrichum* (21.6%), *Phoma* (4.4%) and fungal mycelia (19.1), which are identified based on morphological characteristics and the dominant endophytes such as *C. gleosporiades*, *Pleosporales* sp., *Stagonosporopsis cucurbitacearum* and other fungal endophytes are further confirmed based on PCR amplification and sequencing of 5.8srRNA gene region and their accessions are obtained KT375578, KT375576.1, KT375577.1 and KT375579.1. The study provides the first report on the isolation and identification of endophytic fungi from *Memecylon* species.

Keywords: Memecylon, endophytes, isolation, PCR, accessions.

1. Introduction

Medicinal plants have been traditionally used for many years for the treatment of several diseases. It is well known that plants harbour microbes, together called as endophytes [1]. Endophytic fungi have great importance in biotechnology as a source of novel bioactive compounds for example a well-known drug taxol identified from endophytic fungus *Taxomyces andreannae* from *Taxus brevifolia* and *Pestalotiopsis microspora* from *Taxus wallichiana* showed anticancer activity [2-3]. Since medicinal plants are recognized as depository of fungal endophytes, identification of endophytes from medicinal plants plays an important role in the production of novel secondary metabolites with useful biological activity, which also contributes to the diversity of microbes in the natural environment [4].

Memecylon is one of the genus of Melastomataceae family, which consists of about 300 species, mainly in the old world tropics. In India, the genus is represented by about 40 species, of which 21 are endemic to peninsular India[5-7]. Several phytoconstituents are reported from the aerial parts of the *Memecylon* species which include β-amyrin, α-tocophrol, sitosterol, oleanolic acid, ursolic acid and umbelactone and various pharmacological properties such as antioxidant, anti-inflammatory, antidiabetic properties are reported[8].

Some bacterial endophytes with antimicrobial property are reported from *M. edule* along with other

medicinal plants [9]. Fungal endophytes such as *Fusarium*, *phoma*, are reported from *M. malabaricum* along with several other tree species [10]. Similarly *Xylaria* a fungal endophyte with antimicrobial activity reported from 22 tree species including *M. malabaricum* [11]. So far very few reports are available on endophytes of *Memecylon* species. Hence the present study gives first information on the isolation, identification of endophytes from five *Memecylon* species.

2. Materials and Methods

2.1 Collection of plant material

Leaf and stem samples of *M. umbellatum*, *M. edule*, *M. talbotianum*, *M. malabaricum* and *M. wightii*, were collected from different regions of Western Ghats, Karnataka, India. Identification of the plant species based on their morphological characteristics was confirmed by plant taxonomist. The samples were placed in a refrigerator at 4°C until further use.

2.2 Isolation and Identification of Endophytes

Isolation of endophytes was done based on method described by Sunanya [12]. The endophytic identification was done based on the morphological characters based on identification manual [13]. The endophytic isolates were maintained in cryovials on PDA layered with 15% glycerol (v/v) at -80°C in an Ultra freezer (Cryoscientific Pvt. Ltd., Chennai, India) at the Department of Studies in Biotechnology, University of Mysore, Mysore, India.

2.3 DNA extraction and sequencing

The dominant endophytes were identified by extracting DNA using CTAB (Cetyl trimethyl ammonium bromide) method following the protocol of Doyle and Doyle (1990). Quantification of DNA was done by using NanoDrop® NDC-2000 Spectrophotometer. A ratio of 1.8 at absorbance $A_{260/280}$ confirmed high quality genomic DNA. DNA was amplified with the universal ITS primers ITS1 (5' TCC-GTA-GGT-GAA-CCT-GCG-G 3') and ITS4 (5' TCC-TCC-GCT-TAT-TGA-TAT-GC 3') in a Thermal Cycler (Eppendorff, Germany) programmed for a preliminary 3 min denaturation step at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and extension at 72°C for 1 min, and finally at 72°C for 10 min. Amplification products were separated alongside a medium range molecular weight marker on a 1.2% (w/v) agarose gel electrophoresis in TBE (Tris Borate-EDTA - Tris base 54 g for 500 mL, Boric acid 27.5 g; 0.5 M EDTA 20 mL and pH of 8) buffer stained with ethidum bromide and visualized under UV light, and amplified products were sent to sequencing (Chromos Biotech, Bangalore). The obtained sequences were checked for their homology using BLAST software analysis.

2.4 Data analysis

The percentage of colonization frequency (% CF) was calculated according to Fisher and Petrini[14] as follows:
% CF = (Number of tissue segments colonized by a fungus/ Total number of tissue segments plated) x 100**.**

The Jaccard's similarity indices *viz.* Simpson and Shannon diversity indices were calculated for the endophytes isolated from different *Memecylon* species sample (stem and leaf) using the Shannon calculator [15-16]. The endophyte species present in the particular sample type was taken as 1 and the same endophyte absent in the other sample type was taken as 0, calculated and represented in Table 1.

3. Results

3.1 Isolation of endophytes from *Memecylon* species

A total of 2000 (200 leaf and 200 stem) tissue segments of different *Memecylon* species were screened for fungal endophytes. A total of 156 isolates representing 10 fungal endophytic species were estimated. The total percentage of fungal endopytes recovered from leaf and stem samples of *Memecylon* species was found to be 150.2% the highest percentage of endophytes from both leaf and stem was observed in *M. malabaricum* followed by *M. umbellatum*, *M. edule*, *M. talbotianum*, and *M. wightii*. The *Colletotrichum gleosporioides* was the most dominant endophyte observed in all five *Memecylon* species followed by fungal mycelia, *Aspergillus flavus*, *Cladosporium*, *Pestalotiopsis* sp, *Fusarium oxysporoum*, *Pleosporales*, *Stagonosporopsis cucurbitacearum* and *Phoma* species (Table 1 and Figure 1).

Table 1: Frequency of endophytic fungi isolated from leaf and stems of five *Memecylon* species

Endophytic Fungi	*M. umbellatum*		*M. edule*		*M. talbotianum*		*M. malabaricum*		*M. wightii*		Total	Relative dominance
	leaf	stem	leaf	Stem	leaf	stem	leaf	stem	leaf	stem		
Colletotrichum gleosporioides	4.5	3.5	3.5	2.1	5.0	2.5	5.0	3.2	3.5	1.0	33.8	12.6
Cladosporium sp.	2.5	1.0	1.5	-	-	2.0	1.1	2.5	2.3	-	12.9	11.6
Fusarium oxysporoum	2.0	-	3.0	-	1.1	0.5	1.2	1.5	-	2.2	11.5	9.9
Fungal mycelia	1.0	1.0	1.5	2.2	2.2	1.0	4.2	3.5	1.0	1.5	19.1	7.8
Aspergillus flavus	-	4.5	-	-	1.4	1.5	3.5	2.2	2.5	3.5	19.1	9.9
Pestalotiopsis sp.	3.0	2.5	0.5	-	2.2	-	3.5	-	-	-	11.7	7.3
Alternaria alternata	4.5	2.5	1.0	3.5	1.1	1.0	2.5	0.5	1.0	1.5	19.1	16.6
Phoma sp.	-	-	2.0	1.5	-	-	2.0	-	0.5	-	6.0	9.5
Stagonosporopsis cucurbitacearum	1.0	-	1.0	1.0	1.0	1.5	1.0	-	-	0.5	7.0	7.1
Pleosporales	0.5	2.5	1.0	1.5	1.0	1.0	1.0	1.0	-	0.5	10.0	7.1
Total CF%	19.0	17.5	15	11.8	15	11	25	14.4	10.8	10.7	150.2	-
Total no of isolates recovered	20	18	17	12	14	12	25	15	12	11	156	-
Species richness	8	7	9	6	8	8	10	7	6	7	76	-
Shannon-Wierner diversity index	1.89	1.85	2.07	1.73	1.91	2.02	2.15	1.78	1.62	1.78	18.8	-
Evenness	0.91	0.95	0.94	0.97	0.92	0.97	0.93	0.91	0.9	0.91	9.31	-
Simpson diversity index	0.85	0.84	0.88	0.88	0.87	0.92	0.88	0.85	0.83	0.84	8.64	-

*Based on 200 bits

*Simpson's index of diversity (D).

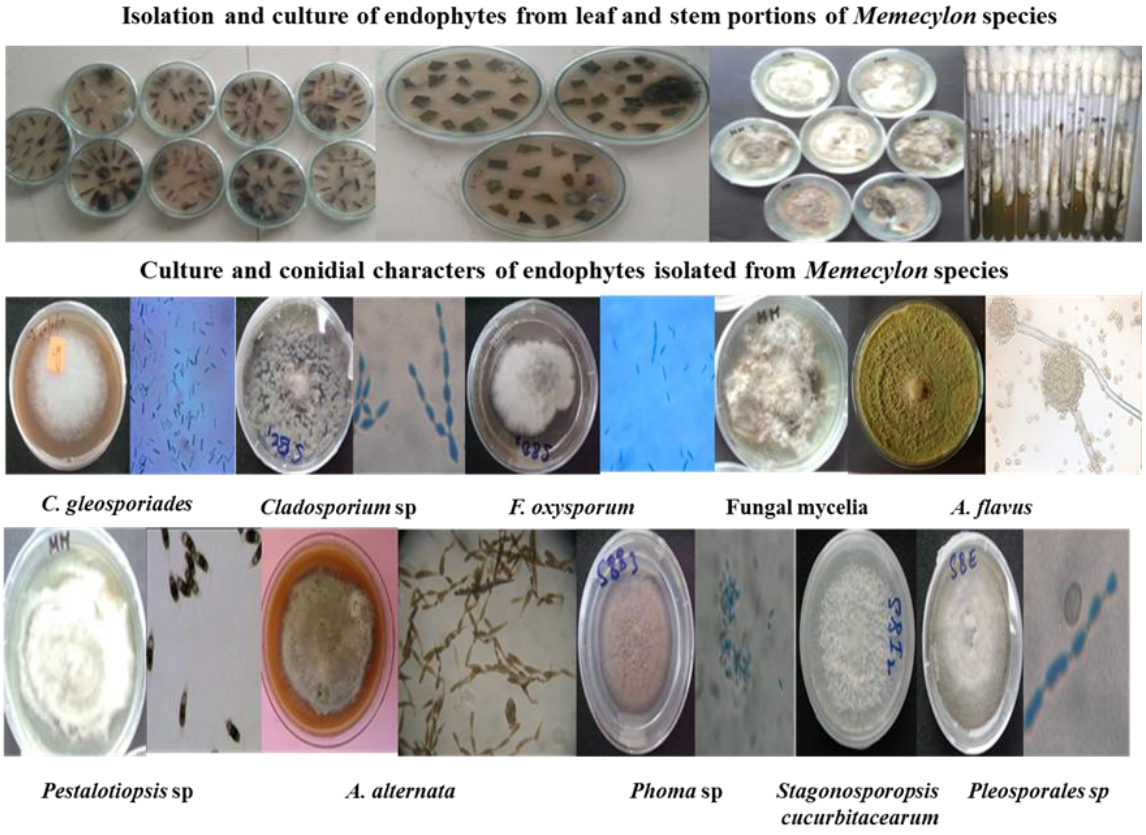

Figure 1: Isolation and morphological characters of endophytes isolated from *Memecylon* species

3.2 Diversity of fungal endophytes

The diversity of fungal endophytes was calculated based on total species richness a number of genera per sample (leaf and stem) were documented. The distribution of some endophytic taxa and their concentration in leaf segments was more compared to stem of some *Memecylon* species. Jaccard's similarity indices and Simpson and Shannon diversity were calculated for endophytes from leaf and stem samples using the Shannon calculator. Simpson and Shannon diversity indices differed between different samples endophytes from leaf and stem of *M. malabaricum* showed the highest diversity index compared to other *Memecylon* species. The diversity from all five *Memecylon* species is estimated as their Species richness (10), Simpson diversity (8.64) and Shannon diversity (18.8) (Table 1 and Figure 1 & 2).

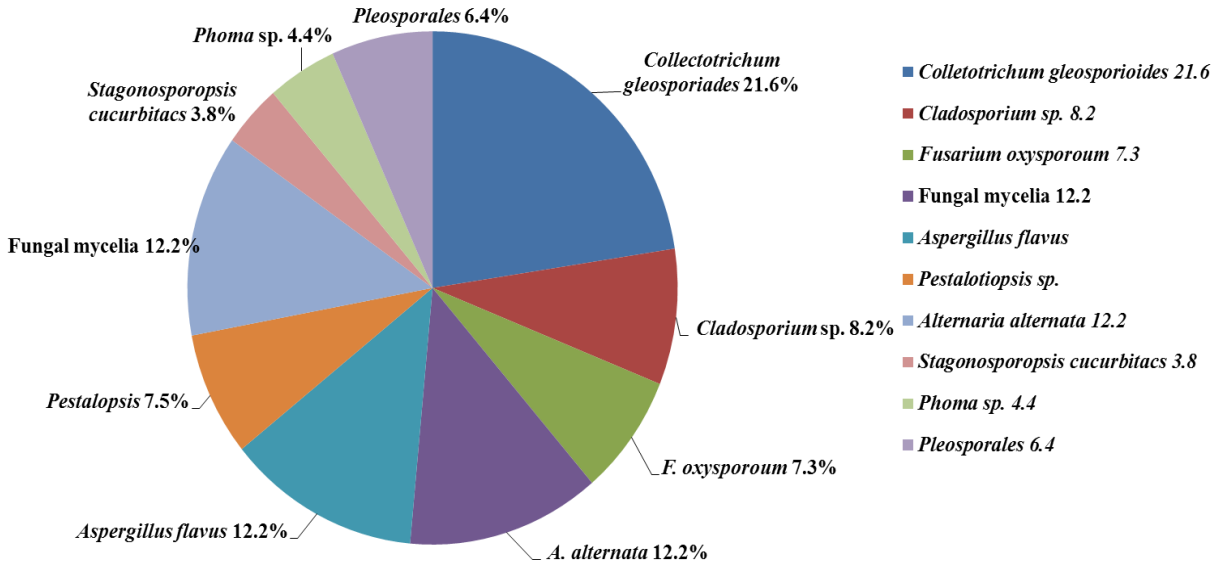

Figure 2: Distribution of Endophytic fungi in *Memecylon* species

3.3 Molecular identification of fungal endophytes based on ITS (5.8s rRNA) gene region

The DNA was extracted from the 1 dominant and 3 morphologically unidentified fungal endophytes and their absorbance at $A_{260/280}$ nm was 1.7, 1.82, 1.8 and 1.86 ng/µl which was acceptable. The amplified ITS DNA fragments of fungal endophytes showed a clear band when examined on agarose gel the size of the band was in the range 600-700bp, which was purified and sent for sequencing. The obtained sequences were identified using nucleotide blast and the accessions were identified as *C. gleosporiades*, *Pleosporales*, *Stagonosporopsis cucurbitacearum* and fungal mycelia. Further the sequences were submitted to genbank and their accession numbers are obtained KT375578, KT375576.1, KT375577.1 and KT375579.1 (Figure 3 and Table 2).

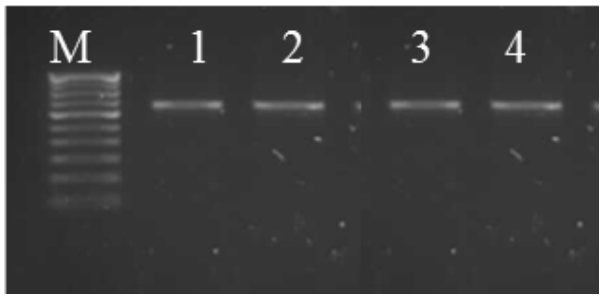

Figure 3: PCR amplification of ITS gene region of fungal endophytes Lane 1: Marker (100-1000bp), lane 1: *C. gleosporiades*, Lane 2: *Pleosporales*, Lane 3: *Stagonosporopsis cucurbitacearum* and Lane 4: Fungal mycelia

Table 2: Concentration of extracted DNA and their species identification result of fungal endophytes isolated from *Memecylon* species

Sl. No	Endophytic taxa	Concentration of DNA (ng/µl)	$A_{260/280}$	Genbank Accession No.	Similarity to nearest genus (%)
1	*C. gleosporiades*	250	1.7	KT375578	*Collectotrichum gleosporiades* (100%)
2	*Pleosporales,*	1080	1.82	KT375576.1	*Pleo sporales* (100%)
3	*Stagonosporopsis cucurbitacearum*	680	1.8	KT375577.1	*Stagonosporopsis cucurbitacearum* (98%)
4	Fungal mycelia	890	1.86	KT375579.1	Uncultured fungal mycelia (97%)

4. Discussion

The present study gives the first information on the fungal endophytes of *Memecylon* species. Since genus *Memecylon* is an ethno medicinal plant used for the treatment of skin diseases mainly herpes, stomach disorders, inflammation, diabetes etc, and several bioactivities are reported form this plant [7]. But there are only few reports available for *Memecylon* species regarding endophytes mainly few fungal endophytes are reported in *M. malabaricum*[10-11] and few bacterial endophytes are reported from *M. edule* [9]. Our results indicate that *C. gleosporiades*, *Cladosporium*, *Fusarium oxysporum* were the dominant fungal endophytes these findings are in accordance to the study reported by Govinda Rajulu and Murali [10-11] in different medicinal plants including *M. malabaricum*. The rate of isolation of endophytes occurred certain differences in the leaf and stem which show their attraction for diverse tissue categories and their capability for using within a definite substrate and a favourable habitat for their genetic adaptation and growth these results supports the findings of Ruma and Jin [16-17]. The common endophytic fungi observed in the present study were *A. flavus*, *C. gleosporiades*, *F. oxyspourm* these endophytes were found common on different host and also supported by several previous reports [12,17-18]. *Stagonosporopsis cucurbitacearum* and *Phoma* species are the endophytes isolated from *Memecylon* species, same as reported from *Glycine max* [19]. *Cladosporium* and *Pestalotiopsis* sp were isolated from *Memecylon* species as endophytes, and the same have been reported on the other hosts such as *Terminalia arjuna, Boswelia serrate, Artocarpus hirsutus* and *Vateria indica* [12, 16, 20].

The genomic DNA was extracted and amplified and sequenced using ITS primers for the dominant endophytic fungi. Sequences are identified with their respective accessions through BLAST analysis and submitted to Genbank and their accession numbers are obtained which is given in Table 2. Similar type of identification based on ITS gene sequence is reported for several endophytes from different medicinal plants [17,21]. In general Shannon-wiener diversity index and Simpson diversity index was high in stem portion of *M. talbotianum* and *M. wightii* and low in *M. umbellatum*, *M. edule* and *M. malabaricum* compared to leaf. Jin[17] have reported similar results from diversity studies on endophytes of *Dendrobium officinale*.

5. Conclusion

The present report gives information regarding fungal endophytes associated with different *Memecylon* species and their diversity which help in developing evidence on the accessible biodiversity and at the same time could add to the nationwide collections of microbes from these areas mainly Western Ghats. Further fermentation of these endophytes is carried out to get a wide array of secondary metabolites to enable screening against therapeutic targets.

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Conflict of interest: None

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