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Isolation, Characterization of Endophytic Fungi of *Mimusops elengi* (Bakul)

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ABSTRACT

Fresh bark pieces of *Mimusops elengi* Linn. were used for the isolation of fungal endophytes using standard methods. *Mimusops elengi* is a small to a large evergreen medicinal plant found all over the different parts of India. Seventy one endophytic fungi belonging to ascomycetes (11.27%), coelomycetes (39.44%) and hyphomycetes (49.29%) were isolated from 200 segments of *Mimusops elengi* collected from Dr. H. S. Gour University campus in Sagar District, Madhya Pradesh, India. The identification of these fungi was confirmed by the BLAST search of sequences of the ITS1-5.8S-ITS2 rDNA region against the NCBI/GenBank data and compared with deposited sequences for identification purpose. Sixteen different fungal species belonging to 16 genera viz. *Botryosphaeria mamane, Chaetomium globosum, Rhytidhysteron* sp., *Phoma putaminum, Pestaliopsis* sp., *Colletotricum gleosporoides, Phomopsis* sp., *Acremonium* sp., *Alternaria alternata, Beltraniella portoricensis, Cladosporium cladosporioides, Curvularia lunata, Fusarium chlamydosporum, Myrothecium verrucaria, Nigrospora oryzae* and *Torula herbarum* were isolated. The most frequently isolated endophytes were *Cladosporium cladosporioides* (22.53%), *Pestaliopsis* sp.,(19.71%), *Phomopsis* sp., (9.86%) and *Chaetomium globosum* (8.45%). This study shows that a great number of endophytic fungi exist in the samples obtained from bark samples of *M. elengi.*

Key words: Fungal endophytes, bark samples, *Mimusops elengi*, medicinal plants.

INTRODUCTION

Mimusops elengi Linn., commonly called Bakul, is a member of family *Sapotaceae*. It is a small to large evergreen tree found all over the different parts of India. All parts of the tree have medicinal properties. The earlier report reveals that the fruits are used in chronic dysentery, constipations; flowers are used as snuff to relieve a headache, lotion for wounds and ulcers. The bark is used to increase fertility in women and known to have anti-ulcer activity. The bark, flowers and fruits are acrid, astringent, cooling and anthelmintic (DMPA, 2000). Bark is used as a tonic (Chopra *et al.*, 2000; Joshi, 2000), febrifuge, as a gargle for odontopathy, inflammation, and bleeding of gums (DMPA, 2000). *Mimusops elengi* is a rich source of tannin, saponin, alkaloids, glycoside and ursolic acid (Anonymous, 1969). Bark established the presence of quercitol, lupeol, taraxerone, taraxerol, α- spinosterol, fatty acid ester of α-spinosterol, ursolic acid, betulinic acid, β-sitosterol, α and β amyrin (Misra and Mitra, 1967). The phytochemical investigation has revealed the presence of alkaloid isoretronecyl tiglate (Hart *et al*., 1968) and a mixture of triterpenoid saponins in the bark of *M. elengi* (Varsheny and Badhwar, 1972). The volatile organic matter from the bark of *M. elengi* had been analyzed. The major constituents were αcadinol, tau muurolol, hexadecanoic acid etc (Ruikar *et al.*, 2009). The whole plant contains lupeol like triterpenoid, β-amyrin, lupeol, α-taraxerol and ursolic acid (Jahan *et al*., 1995). Ganu and Jadhav (2010) also reported *in vitro* antioxidant and antihyperglycemic property of methanolic extract of *M. elengi*. In recent years, the quest for the isolation of new compounds from medicinal plants has become a fascinating area of research. Plants with ethnic pharmaceutical importance are being exploited because of their healing properties. However, large scale harvesting of medicinal plants has already become a major threat to biodiversity. As an alternative, the microbes which live inside such plants (endophytes) may offer tremendous potential sources of therapeutic compounds.

Fungal endophytes reside within the living tissues of higher plants without producing any apparent symptoms (Bills, 1996). Biologically and ecologically, they represent diverse nutritional requirements ranging from biotrophic parasites to facultative saprotrophs. They also represent a large reservoir of unexplored genetic diversity. They have been isolated from monocots (Alquati, 1999; Pamphile and Azevedo, 2002; Krishnamurthy and Hemalatha, 2003) and dicots (Bettucci *et al*,. 1999; Mahesh *et al.*, 2005) including mangroves (Maria and Sridhar, 2003) and sea grasses (Devrajan *et al.*, 2002). There are reports of endophytes isolated from algae, lichens, mosses, ferns and gymnosperms (Carroll and Carroll, 1978; Petrini, 1986; Petrini *et al.* 1990; Kralj *et al*,. 2006). The practical applications of these endophytes are manifold; as potential biocontrol agents, sources of novel metabolites for therapeutics, plant protection, other industrial applications, and as model systems for studying the host parasite interactions in natural ecosystems (Stone *et al.*, 2000; Schulz *et al*., 2002; Strobel *et al.,* 2004, Deshmukh and Verekar, 2009; Deshmukh *et al.*,2012; 2015).

Virtually very few reports are available on the association of endophytic fungi from tropical medicinal tree species (Deshmukh *et al.*, 2009; 2017; Periyasamy *et al.*, 2012; Verekar *et al.*, 2014; Rahier *et al.*, 2015; Mishra, *et al.* 2015; Bhatia *et al.,* 2016). Therefore, this study provides the first information on the isolation of fungal endophytes from *M. elengi*. We are currently pursuing fermentation of these microbes to obtain the secondary metabolites to facilitate screening against anticancer, anti-inflammation, anti-diabetic and antimicrobial activity.

MATERIALS AND METHODS

Bark samples of *M. elengi* were collected from Dr. H. S. Gour University campus in Sagar District, Madhya Pradesh (23° 88/ N latitude 78° 83' E longitude), India during the monsoon season of 2013 (July to September 2013). Bark pieces (5.0 × 5.0 cm) from the trunk were cut 1.5 m above the ground level with the help of sterile machete. The samples were placed in polyethylene bags, labeled, transported in the ice box to the laboratory and placed in a refrigerator at 4°C. All samples were processed within 24 h of collection.

Bark samples were halved, first immersed in 70% ethanol (v/v) for 1 min followed by second immersion in sodium hypochlorite (3.5%, v/v) for 3 minutes. The samples were rinsed 3 times with sterile distilled water and dried on sterile blotters under the laminar airflow to ensure complete drying. Bits of 1.0×0.1 cm size were excised with the help of a sterile blade. Two hundred bits from bark were plated on Potato Dextrose Agar (PDA)(Hi Media) supplemented with the antibiotic chloramphenicol (50 mg/l) to suppress the bacterial growth. Ten segments were plated per plate. The plates were incubated at 25°C ± 1°C with 12 h light and dark cycles for up to 6 weeks (Bills and Polishhook, 1991). Periodically the colonies were examined and each colony that emerged from segments were transferred to antibiotic-free PDA media to aid identification. All the fungal isolates have been cataloged as PM # series and maintained at the culture collection of the department by cryopreservation on PDA overlaid with 20 % glycerol (v/v) at -80°C in a deep freezer. The fungal identification was done based on colony morphology and conidial characters.

Molecular Characterization: Molecular characteristics of the cultures were studied by determination of their DNA sequences of ITS1-5.8S-ITS-2 region. Genomic DNA was extracted by the miniprep protocol of Lee and Taylor (1990). The ITS1-5.8S-ITS-2 rDNA was amplified using primers ITS1 and ITS4 as the forward and reverse primers as described by White *et al.* (1990). Amplification was performed in 100 µL reaction volumes containing 10X buffer 10µl, $MgCl_2$ (25mM) 2µl, dNTP (10mM) 2µl, ITS1 primer (20pm) 2µl, ITS4 primer (20pm) 2µl, Taq Polymerase (2.5U) 1µl, DNA Sample (5µg/ml) 3µl, and Milli Q Water 78µl. The PCR reaction was carried out using a Thermal Cycler (M.J. Research, PTC 200) with conditions as follows: denaturation for five minutes at 94°C, 34 cycles of (30 sec at 94°C, 30 sec at 55°C, 1 min at 72°C) extension for four minutes at 72°C and storage at 4°C. Negative controls were used in each set of reactions. The final products were analyzed by electrophoresis on 2.0% agarose gel (Sigma). The PCR product were purified using Qiagen Gel extraction kit (CAT No. 28704) and then sequenced using ITS1 and ITS4 primers at gene Ombio Technologies Pvt Ltd, Pune, India, using Applied Biosystems 3730 DNA analyzer.

Phylogenetic Analysis: Similarity analysis of the nucleotides was performed by BLAST searches against sequences available in GenBank (Altschul *et al.*, 1990). For phylogenetic tree construction, multiple sequences were obtained from GenBank and the alignments were performed using MEGA6 (Tamura *et al.*, 2013).

The colonization frequency (CF), expressed as a percentage was calculated according to Kumaresan and Suryanarayanan (2001) as follows:

$$\%CF = \frac{\text{Number of tissue segments colonized by a fungus}}{\text{Total number of tissue segments plated}} * 100$$

Dominant endophytes were calculated as percentage frequency divided by sum of the percentage of colony frequency of all endophytes X 100.

$$\text{Dominant endophytes} = \frac{\text{Percentage frequency}}{\text{Sum of \% of colony frequency of all endophytes}} * 100$$

RESULTS AND DISCUSSION

The results of isolation is presented in **Table 1**. They revealed that 71 isolates were obtained from 200 samples bark segments of *M. elengi*, they belongs to class ascomycetes (11.27%), coelomycetes (39.44 %), and hyphomycetes (49.29 %). The total colonizing frequency of bark sample was 35.5 %. These isolates belong to 16 different fungal species viz. *Botryosphaeria mamane, Chaetomium globosum, Rhytidhysteron* sp., *Phoma putaminum, Pestaliopsis* sp., *Colletotricum gleosporoides, Phomopsis*sp., *Acremonium* sp., *Alternaria alternata, Beltraniella portoricensis, Cladosporium cladosporioides, Curvularia lunata, Fusarium chlamydosporum, Myrothecium verrucaria, Nigrospora oryzae* and *Torula herbarum*.

Table 1. Endophytic fungi from Inner bark of *Mimosops elengi*

	Endophytic fungi	No. of endophytes	Colonization frequency*	Dominant fungi
	Ascomycetes			
1	*Botryosphaeria mamane*	1	0.5	1.428
2	*Chaetomium globosum*	6	3.0	8.450
3	*Rhytidhysteron* sp.	1	0.5	1.428
	Coelomycetes			
4	*Phoma putaminum*	2	1.0	2.816
5	*Colletotricum gleosporoides*	5	2.5	7.042
6	*Pestaliopsis* sp.	14	7.0	19.91
7	*Phomopsis* sp.	7	3.5	9.859
	Hyphomycetes			
8	*Acremonium* sp.	3	1.5	4.225
9	*Alternaria alternata*	3	1.5	4.225
10	*Beltraniella portoricensis*	2	1.0	2.816
11	*Cladosporium cladosporioides*	16	8.0	22.535
12	*Curvularia lunata*	4	2.0	5.633
13	*Fusarium chlamydosporum*	1	0.5	1.428
14	*Myrothecium verrucaria*	2	1.0	2.816
15	*Nigrospora oryzae*	3	1.5	4.225
16	*Torula herbarum*	1	0.5	1.428
	No. of isolates	**71**	**35.5**	**99.96**

* 200 segments were plated for frequency analysis

Fungal endophytes were isolated from fresh bark samples of *M. elengi* collected from Saugar University campus in Sagar , District, Madhya Pradesh, India on Potato Dextrose Agar. The colonization frequency of each fungus was calculated based on the number of segments colonized by a fungus over the total number of segments assessed and represented as percentage.

The DNA fragments were amplified using PCR by primers ITS1 and ITS4 which vary between 301 bp to 635 bp in length. The fragment contained the 3' end of 18S rDNA, ITS1, 5.8S rDNA, and ITS2 and the 5' end of 28S rDNA. The presence and similarity of the sequence in sixteen different isolates were ascertained using the CLUSTAL W multiple sequence alignment available at www.ebi.ac.uk/Tools/msa/clustalw2. The similarity based on the ITS region (ITS1, 5.8S rDNA, and ITS2 regions) between these isolates varied from 40 to 99% as per the alignment. **Figure 1** displays the Neighbour joining tree constructed by comparing the sequence identities of the ITS regions in different isolates.

The sixteen different isolates sequences were identified by BLAST analysis. Most significant BLAST hit obtained was considered as reference sequence for each sequence to be used in construction of phylogenetic tree. Using a very distant group like yeast as an outgroup for these fungal isolates would reduce the phylogenetic signal due to multiple ambiguously aligned residues; hence *Pichia* sp. and *Candida inconspicua* were used as outgroup sequences.

There are only a few reports on Indian medicinal plants. Mahesh *et al.* (2005) reported *Chaetomium crispatum, Chaetomium globosum, Pestalotiopsis* sp., *Phoma eupyrena, Phyllosticta* sp., *Acremonium acremonium, Aspergillus flavus, Aspergillus niger, Aspergillus oryzae, Cladosporium acaciicola, Cladosporium cladosporioides, Cochlonema verrucosum, Curvularia lunata, Fusarium clamydosporum, Fusarium moniliformae* var. *subglutinans, Fusarium oxysporum , Fusarium solani , Gliomastix* sp. *, Nigrospora oryzae, Penicillium* sp. , *Trichoderma* sp. and *Verticillium albo-atrum* from inner bark of *Azadirachta indica*. *Trichoderma, Penicillium* and *Pestaliopsis* sp. were the most dominant endophytic fungi. Similarly Gehlot *et al.* (2008) reported a large number of endophytic fungi from *Prosopis cineraria* and species of *Aspergillus*, *Fusarium* and *Alterneria* were found most dominant endophytes. Latter on Gond *et al.* (2012) isolated *Alternaria alternata, Aspergillus fumigatus*, *A. Niger, Cladosporium cladosporioides, Colletotrichum dematium, Chaetomium globosum, Curvularia lunata, C. oryzae, C. fallax, Drechslera ellisii, Fusarium oxysporum, Humicola grisea, Acremonium* sp., *Nigrospora oryzae, Penicillium* sp., *Phomopsis* sp., *Rhizoctonia* sp. from healthy leaf and stem tissues of *Nyctanthes arbortristis.* In the year 2013, Maheswari, and Rajagopal (2013) reported *Chaetomium globosum*, *Nodulisporium* sp., *Botryodiplodia theobromae*, *Colletotricum* sp., *Phoma chrysanthemicola , Pestalotiopsis* sp., *Phomopsis* sp. 1, *Phomopsis* sp.2, *Alternaria alternata*, *Alternaria tenuissima*, *Curvularia lunata*, *Curvularia geniculata*, *Drechslera hawaiiensis*, *Fusarium solani*, *Nigrospora oryzae*, *Nigrospora sphaerica, Trichoderma aeroviride*, *Mucor pusillus* and *Rhizopus oryzae*, along with some sterile mycelium from bark and leaf of *Kigelia pinnata*.

Many plant species representing grasses, palms, conifers, pines, ferns, mosses and lichens have been studied worldwide for the presence of endophytic fungi (Stone *et al.*, 2000). To date, very few medicinal tree species have been screened for their endophytic fungi. As the bark is attributed in the healing of various disorders, an attempt was made to isolate the endophytic fungi residing in the bark by employing stringent surface sterilization techniques. Our studies yielded mitosporic fungi as the major group of endophytic fungi. They out-numbered other groups of fungi such as zygomycetes and ascomycetes. Mitosporic fungal isolations as endophytes are common among plants inhabiting temperate, tropical and rainforest vegetations (Bacon and White, 1994).

So far, only a few publications had reported the isolation of fungal endophytes from the bark of tree species (Stierle *et al.* 1997; Brown *et al.*,1998) eg. Taxol producing fungus

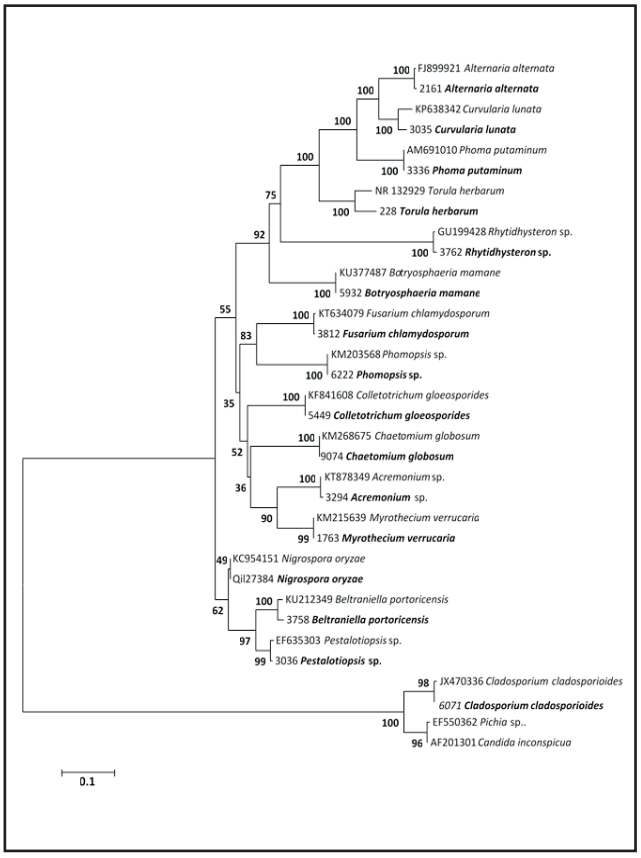

Fig. 1. Phylogenetic tree inferred using Neighbor-Joining method for query (organism name with four numeric and alphanumeric codes) and control ITS sequence (organism along with Genbank Accession numbers). The optimal tree with the sum of branch length = 3.28379180 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The analysis involved 34 nucleotide sequences including two outgroup sequences viz. *Pichia* sp. and *Candida inconspicua*.

Taxomyces andreanae was isolated from the bark of Pacific yew, *Taxus brevifolia* (Stierle *et al.*, 1997). Similarly Bills and Polishook (1991) isolated 69 fungal species from the bark of a single *Carpinus caroliniana* tree, which suggested the enormous extent of fungal diversity associated within a single plant. Deshmukh *et al.* (2009) reported an anti-inflammatory and anti-cancer compound the ergoflavin from the sterile mycelium isolated from *M. elengi* .

The exploration of woody perennials for organisms that might produce microbial metabolites for use as therapeutic agents needs much attention as it necessitates careful identification and selection of species unique to a particular host before the screening of metabolites for desired industrial applications.

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