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Isolation And Identification Of Endophytic Fungi RhizopusDelemar From MemecylonUmbellatum In Gudiyum Forest, Tamil Nadu, India

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ABSTRACT

Fungal endophytes are micro fungi that live inplants without causing apparent symptoms of infection. Recently, more studies have focused on the endophytic fungi extracted from various medicinal plants. This study was conductedto identify endophytic fungi isolated from the medicinal plant Memecylonumbellatum which was collected from Gudiyum forest in East and South part of TiruvallureDist (TN). Among the four endophytic fungal isolates, the predominant isolate MU1 was identified as Rhizopus sp. by studying cultural and morphological studies. Further, the isolate was confirmed by molecular characterization using ITS sequence. The BLASTn analysis and phylogenetic analysis by NJ method of ITS sequence confirmed the fungal isolate as Rhizopusdelemar. This method is considered to be useful for identification purposes due to the rapid evolution of the ITS.

KEYWORDS: Endophytes, Memecylon umbellatum, Rhizopusdelemar, ITS sequence.

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INTRODUCTION

*Memecylonumbellatum*Burm. (Family: Melastomataceae) is small evergreen shrub or tree having young terete branches and bears numerous umbellate cymes. In English the plant is known as "Ironwood tree" while in Sanskrit as "Anjani". Geographically plants have been distributed mostly in coastal regions of the Deccan peninsula, the eastern and southern part of India all along Western Ghats and in the Andaman islands.[1]Variouspharmacological activities like antidiabetic, antiviral, antisporatic, anti-inflammatory, antimicrobial, antioxidant, antihelmenthic, hepatoprotective activity, anti-insect activity, antispasmodic, nephroprotective, anti-pyretic activity have been reported for *M. umbellatum*.[2]

Plants are broadly considered as sessile multicellular organisms, there is indeed an assortment of mutualistic networks connecting them with the environment. These comprise the multitrophic interaction of plants with other organisms, mainly microorganisms, which are diversely localized and have remarkable functional lifestyles.[3]Endophyte species are able to live inside plant tissues without inducing any apparent symptoms in their hosts; for this reason, endophytes have been receiving increasing attention from scientists since the latter part of the twentieth century. Fungi and bacteria are the most common microbes that exist as endophytes.[4]Manyendophytes, particularly from the kingdom fungi, have the potential to synthesize various bioactive natural products that may directly or indirectly be used as therapeutic agents against numerous diseases.[5]

The production of bioactive secondary metabolites by endophytes, including compounds mimetic to associated host plant metabolites, is important from both academic and industry perspectives.[6] The production of metabolites by microorganisms is known and explored, because the major of antibiotics are produced by fungi and bacteria, in this way,antimicrobial activities have been demonstrated in a variety of metabolites biosynthesized by the plant endophytes.[7]Endophytes are often studied at the morphological level, but, many of the endophytes either fail to sporulate or they are rare and difficult to identify. Therefore, molecular analysis based on DNA sequences is recognized as the most reliable method to reveal genetic relationship between the strains which could be unambiguously used to identify and evaluate the isolates at any taxonomic rank.[8]

MATERIALS AND METHODS

Study Site

Gudiyum forest are present in west part of Jaganathapuram village, Nagari District (AP), East part of Uttukottai, Tiruvallur District (TN), North part of Nagalapuram Village, Nagari District (AP) and South part of Poondi Village, Thiruvallure District (TN). It is located in lat: 13.28790 and Log: 79.80867 with an area covered with 148 acre of Tiruvallur District in Tamil Nadu. Climate of the

District is on the whole dry, except during North East monsoon season. Average annual rainfall in the Gudiyum forests is 1104 mm and in summer, maximum temperature is 21.5°C to 37.5°C.

Plant Sample

Memecylonumbellatum was collected from the study site and immediately transported to the laboratory for analysis. The nomenclature of collected sample was authenticated by Raw Material Herbarium and Museum (Reference number: NISCAIR/RHMD/Consult/2016/3003-30), New Delhi, India.

Isolation and Identification of Endophytic Fungi

Isolation of endophytic fungi was attempted with the plant samples using standard procedures.[9]The leaves of the plants were separated and were surface sterilized with 70% (v/v) ethanol for 30 s and then 5% (v/v) sodium hypochlorite for 3 min. They were subsequently washed thrice with sterilized water and blotted with sterilized filter papers. After sterilization each leaf was divided into four segments and placed on Potato Dextrose Agar (PDA) medium supplemented with streptomycin (100mg/L) to suppress bacterial growth. All the plates incubated at 27°C until fungal growth appeared. The plant segments were observed once a day for the growth of endophytic fungi. Hypal tips growing out the plated segments were immediately transferred into Potato Dextrose Agar slant and maintained at 4°C.Endophytic fungi were identified based on macro and micro morphological characteristics include colony diameter, texture, colour, dimensions and morphology of hyphae, and reproductive structures.[10]

Molecular Confirmation of the Fungal Isolate

For DNA extraction, the fungi were grown on potato dextrose broth (PDB) for 72 h at 30°C under shaking conditions (120rpm) and the resultant mycelium was harvested by vacuum filtration and stored at -70°C. The chilled mycelia (200mg) was ground in 500 µl of extraction buffer (50 mM^{-1} TrisHCl, pH 8.0; 700 mMNaCl, 10 mMEDTA, 1% (v/v) β-mercaptoethanol and 1% (w/v) SDS) and the 300 µl of equilibrated phenol and extraction buffer was added. The contents were homogenized and incubated for 15 min at 65°C. The DNA in the aqueous phase was purified with repeated extraction using equal volumes of saturated phenol, chloroform, isoamyl alcohol mixture (25:24:1). The DNA was precipitated with 9 parts of ice cold isopropyl alcohol and one part of Sodium acetate (3M, pH 8.0) and the precipitate was collected by centrifugation at 8000 rpm for 15 min. The DNA pellet was rinsed with 70% ethanol, air dried, suspended in 50µl of sterilized double distilled water and stored at 4 °C.[11]

PCR was carried out in Eppendorf PCR Master Cycler with ITS (internal transcribed spacer) universal primers ITS1(CTTGGTCATTTAGAGGAAGTAA) and ITS4 (TCCTCCGCTTATTGATATGC). The amplication was performed in a 50µl reaction mixture containing DNA- 10ng, Primer each - 0.5 µM, Taq Buffer - 1X, $MgCl_2$- 1.5mM, dNTP's - 500µM. Thermal cycling program was a followed 3 min for Initial denaturation at 94^oC, followed by 35 cycles of 30 sdenaturantion at 94^oC, 30 s primer annealing at 50$^oC$and 60sextension at 72^oC and 5 min at 72^oC for a final denaturation. The confirmations of the amplicons were done by running the samples in 1% agarose gel along with DNA ladder marker.

ITS were further sequenced using ABI 3730 automated sequencer at Eurofins Scientific India Pvt Ltd, Bengaluru, Karnataka, India. Two sequences received were assembled using CAP3sequence assembly program(Huang and Madan, 1999). The contig assembled is used for BLAST analyses using NCBI server and top 10 matching sequences were exported to FASTA format and used for the phylogenetic tree construction using MEGA 6 software.[12]

RESULTS

Isolation and Identification of Endophytic Fungi

A total of 4 fungal endophytes were isolated from the leaf fragments of the plant *M. umbellatum*evaluated in this study. Based on the dominance, one isolate (MU1) was selected for further study.The isolate MU1 was probably identified as *Rhizopus* sp. based on cultural and morphological characteristics such as colony shape, texture, color and morphology (Figure 1).

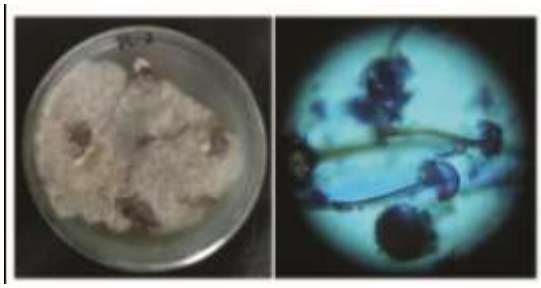

Figure 1:Cultural and Morphological Characters of the Isolate MU1

Molecular Confirmation of the Fungal Isolate

The fungal isolate was further confirmed by molecular study, the amplified ITS (5.8S and large subunit rDNA)products were sequenced using ABI 3730 automated sequencer. The obtained contingsequence has the length of 771 bp and was deposited in GenBank (Accession number: KY628942.1).

ITS of MU1 Fungal Isolate in FASTA Format

>KY628942.1 *Rhizopusdelemar* isolate MU1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

CACTGGCGTTTTCTATTGTGGTGTCGAGTGGTGCCTCGAGTACCAGAAGAACACGGGGCGCGCATACATA
CTTGGAAGTAAAAGTCGTAACAAGGTTTTTCCCTGGTGAACTTGCGGAAGGATCATTAATTATGTTAAAG
CGCCTTACCTTAGGGTTTCCTCTGGGGTAAGTGATTGCTTCTACACTGTGAAAATTTGGCTGAGAGACTC
AGACTGGTCATGGGTAGACCTATGTGGGGTTTGATCGATGCCCCTCCTGGTTTCAGGAGCACCCTTCATA
ATAAACCTAGAATTTCAGTATTATAAAGTTTAATAAAAAACAAGTTTTAACAGTGGATCTCTTGGTTTTC
GCATCGATGAAGAAGGTATCAAAGTGGGATAAGTAGTGTGAATTGCATATTCCGTGAATCATCGAGTCTT
TGAACGCAGCTTGCACTCTATGGTTTTTCTATGGAGTACGCCTGCTTCAGTATCATCACAAACCCACACA
TAACATTTGTTTATGTGGTAATGGGTCGCATCGCTGTTTTATTACAGTGAGCACCTAAAATGTGTGTGAT
TTTCTGTCTGGCTTGCTAGGCAGGAATATTACGCTGGTCTCAGGATCTTTTTCTTTGGTTCGCCCAGGAA
GTAAAGTACAAGAGTATAATCCAGCAACTTTCAAACTATGATCTGAAGTCAGGTGGGGATTACCCGCTGA
ACTTAAGCATATCATAAGCGGAGGGAGAAAAAAAAAAACCGTCCCGCCCCGGGGGCTGCCGGATCCCCGGT
G

The BLASTn analysis of assembled conting ITSsequnce showed 99 % homology with the fungi species*Rizopusdelemer* small subunit ribosomal RNAgene.The phylogenetic analysis (NJ method) of the sequence closely paired with the sequence of *Rizopusdelemer*(Figure 2). Hence, the isolate MU1 was confirmed as *Rizopusdelemer.*

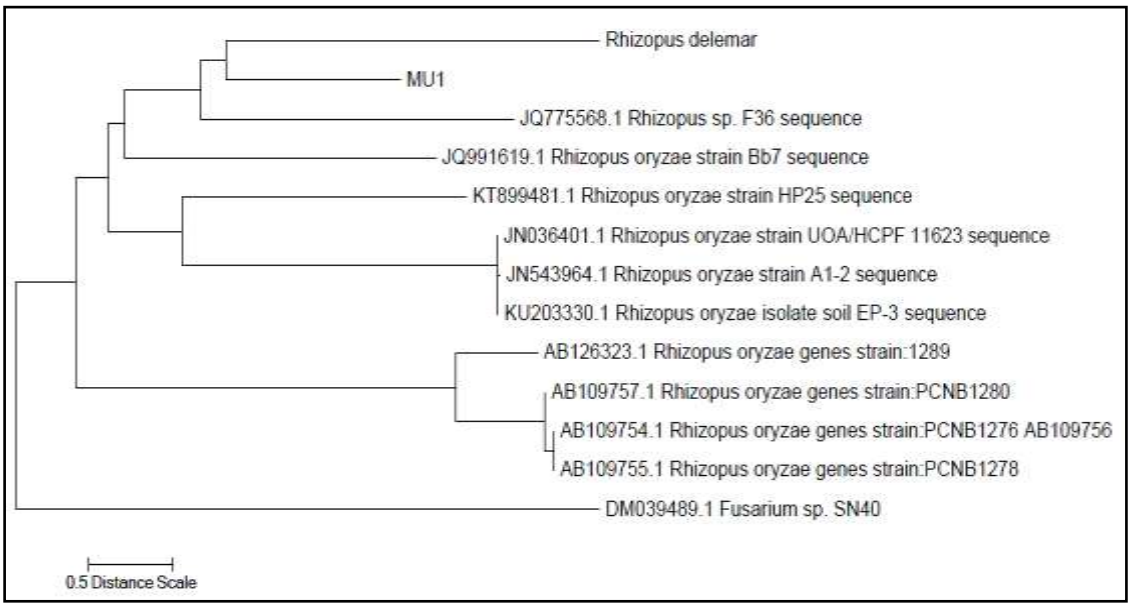

Figure 2: Phylogenetic Tree of ITS of MU1 Constructed by NJ Method

DISCUSSION

Endophytes are a rich source of functional metabolites which benefits host plants by preventing pathogenic organisms from colonizing them; they also stimulate the production of

secondary metabolites. Medicinal plants are good source for isolation of endophytic fungi that colonize the tissue without causing apparent symptoms.[13]Endophytic organisms have received considerable attention as they are found to protect their hosts against pests, pathogens and even domestic herbivores.[14]

Today, more and more studies have focused on the endophytic fungi extracted from various medicinal plants. Endophytic fungi are usually classified as clavicipitaceous (grass-inhabiting) and nonclavicipitaceous (generally non grass-inhabiting) based on phylogeny and life history traits. Moreover, Rodriguez *et al*.[15] distinguish three distinct functional groups in nonclavicipitaceous based on host colonization and transmission, in planta biodiversity, and fitness benefits conferred to host. Huang *et al*.[16]isolated 172 endophytic fungi from three medicinal plants. Gazis and Chaverri[17]indicated *Pestalotiopsis cf. hughesii* as the dominant species present in rubber leaves and sapwood.

M.umbellatum is a medicinal plant having a broad spectrum of medicinal value. All the parts of the plant is used as medicine, the fruits are edible, are eaten in time of famine and they are quite safe. The leaves are used in the dyeing industry to dye wool, silk and grass mats.[18]This study was carried to isolation and identification of endophytic fungi from *M.umbellatum* was collected from Gudiyum forest in East and south part of TiruvallureDist (TN). In the study, a total of 4 fungal colonies were isolated from the segments of the plant *M.umbellatum*. Among these, the predominant endophytic fungi colony MU1 was identified as *Rizopusdelemer*by cultural and morphological, and further confirmed by ITS sequence analysis.Many studies have traditionally used sequence data from the ITS region of fungi to identify.[19]ITS data are considered to be useful for identification purposes due to the rapid evolution of the ITS. However, most fungi are not represented in GenBank, and some GenBank records are misidentified or lack taxonomic information.[20] Therefore, BLAST and phylogenetic analyses of other genomic regions should be combined with those of the ITS region to improve the accuracy of identification.[21]

CONCLUSION

Medicinal plants are good source for isolation of endophytic fungi that colonize the tissue without causing apparent symptoms. Endophytic organisms have received considerable attention as they are found to protect their hosts against pests, pathogens and even domestic herbivores. In this study, an endophytic fungus of *Rizopusdelemer* was isolated from the medicinal plant *Memecylonumbellatum*and was confirmed by ITS sequence analysis. *M.umbellatum* having a broad

spectrum of medicinal value and this study support wide spectrum of endophyte with significant bioactive potential.

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