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ISOLATION AND MOLECULAR IDENTFICATION OF ENDOPHYTIC FUNGI FROM *ACACIA NILOTICA*

Jawed Ahmed[*1], Dr. Ashfaque M. Khan[2] and Dr. M. M. Baig

[1](Department of Biotechnology, Maulana Azad College of Arts, Science and Commerce, Aurangabad, BAMU University, India).

[2](Department of Botany, Maulana Azad College of Arts, Science and Commerce, Aurangabad, BAMU University, India).

[3](Department of Biotechnology, Yashwantrao Chavhan Mahavidylaya, Nanded, SRTM University, India).

***Corresponding Author: Jawed Ahmed**

(Department of Biotechnology, Maulana Azad College of Arts, Science and Commerce, Aurangabad, BAMU University, India).

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ABSTRACT

In nature plant shows symbiotic relationship with some microorganisms act as undercover agents known as endophyte. Which produces potentially important bioactive compounds; these compounds have various functional roles in growth and protection of plants as per ecological niche. Medicinal plants have been recognized as a repository of fungal endophytes with novel metabolites. The present study was conducted to characterize and explore the endophytic fungi isolated from medicinal important plant *Acacia nilotica* of Marathwada region for the study of potential bioactive compounds. Endophytic fungi were isolated and characterized on the basis of sequence similarity using bioinformatics tool and molecular tools. Traditional methods of endophytes isolation and characterization have limitation and it is time consuming processes. Molecular identification of isolated endophytic fungi carried out using fungal specific 18S rRNA primer. Isolated endophytic fungi were show sequence similarity with *Aspergillus.*

KEYWORDS: Endophytic fungi, *Aspergillus sp.ZH14*, Molecular identification, 18S rRNA analysis, phylogenetic tree.

INTRODUCTION

Microorganisms, mainly bacteria and fungi, inhabit inside the plants known as endophyte. Bacon and White (2000) define endophytes: "microbes that colonize living, internal tissues of plants, without causing any immediate negative effect". The endophytic microorganisms penetrate plants tissue mainly by the root. Nowadays scientist have become more interested in bioprospecting of this undercover agents i.e endophytic fungi due to ability for production of potentially useful bioactive compounds which have been used in pharmaceutical and food industries. Endophytic fungi able to produce antibacterial, antiviral, anticancer, antioxidants, antidiabetic and immunosuppressive compounds (Strobel 2003, Dryfuss 2003) and biological control agents (Jalgaowala 2010 & Schardl 1991) which turn scientific communities for the study of this microorganisms.

Endophytic fungus communities consist of a broad range of fungal origins, such as Ascomycota, Basidiomycota and Zygomycota (Zheng and Jiang 1995; Sinclair and Cerkauskas 1996). Therefore, it is a tough task for mycologists to identify various endophytic fungi into genera or species based on morphological characteristics. Furthermore, it is verytime-consuming to make a complete identification. Therefore, DNA sequencing analyses coupled with morphology have been widely used in the investigation of endophyte diversity, particularly for ecology studies. Identification of microorganisms using genetic tool shows high sensitivity and specificity, also used for diverse hierarchical classification of microorganisms at different taxonomic levels. The 18S, 28S ribosomal genes, (ITS) internal transcribed spacer and (IGS) intergenic spacer regions mainly used in the study of fungi. Sequences generated by different tool have been extensively used in different level, it can be used for study of diversity and characterization of different community (Sterflinger and Prillinger 2001; Schabereiter-Gurtner et al. 2001), used in identification and detection (Ablitz et al. 2004; Abd-Elsalam et al. 2003; Wu et al. 200) used in study of different typing (Sutar et al. 2004) and used for the construction of phylogenetic relationships (LoBuglio and Taylor 1995; Peintne et al. 2003).

A.nilotica is well known multipurpose plant, used in treatment of different ailments (Singh et al., 2009). This medicinal plant is used in ayurvedic medicine practices to promote self healing, good health and durability and

provide the nutrients and therapeutic ingredients used to treat many diseases. It is well known source of polyphenols (Singh et al., 2009). *A. nilotica* is recognized by the following names: Acacia, *Acacia Arabica*, Babhul - Hindi and Napalese, Babla - Bengali, Babool - Unani, Babool Baum - German, Babhoola - Sanskrit, Babul, Babul Tree, Huanlong Kyain - Burmese, Kikar, Mughilan - Arabian Indogom - Japenese and Ummughiion – Persian (Steve, 2004). Leaf of *A. nilotica* shows various activity Chemoprventive, anitmutagenic, anti bacterial, anticancer, astringent, anti microbial, diarrhea, Aphrodisiac, dressing of ulcers, anti-inflammatory and Alzheimer's diseases (Kalaivani and Mathew, 2010; Shittu, 2010; Kalaivani et al., 2010).

MATERIALS AND METHODS STUDIED SPECIES

A. nilotica is a plant 5 to 20m high with a thick spherical crown, stems and branches usually sinister to black colored, grey-pinkish slash, fissured bark, exuding a reddish low quality gum. The plant has straight, light, thin, grey spines in axillary pairs, usually in 3 to 12 pairs, 5 to 7.5 cm long in young trees, mature trees commonly without thorns. The leaves are bipinnate, with 3 to 6 pairs of pinnulae and 10 to 30 pairs of leaflets each, rachis with a gland at the bottom of the last pair of pinnulae. Sample was collected from the Aurangabad region of marathwada.

Isolation of fungal endophytes

Plant leaf were collected and washed under running tap water to remove soil debris and then surface- sterilized with ethanol (70%) for 3 min, sodium hypochlorite (1%) for 1 min, followed by three rinses in sterile distilled water for 3 min each. Small sections of sterilized leaf (0.5–1.0 cm) were subsequently cultivated on PDA petri plates. Plates were then incubated at 28^0C for 3–4 weeks. After that time, emerging colonies were sub cultured to obtain pure isolates.

Molecular characterization of endophytes

Genomic DNA was extracted from the mycelia mat using the method described by Nicholson with slight modifications. Species identification of endophytic fungi was performed using the *18S rRNA* Specific Primer (1F and 4R). Amplification was conducted with 50 mL of PCR reaction mixtures, each containing 7 µL of total fungal genomic DNA, 1 µL of each primer (10 µM), and 13.5 µL of sterilized water. PCR was performed in a Veriti® 96 well Thermal Cycler (Model No. 9902) with the following program: 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 54 °C for 30 s and primer extension at 72 °C for 1 min, completed with a final extension at 72 °C for 7 min. The PCR amplicon was enzymatically purified and further subjected to Sanger Sequencing. Bi-directional DNA sequencing reaction of PCR amplicon was carried out with 1F and 4R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. Consensus sequence of 896 bp of *18S* gene in SSU region was generated from forward and reverse sequence data using aligner software.

Phylogenetic tree

Sequence alignments and phylogenetic tree were completed using MEGA software, version 7.0. Alignments were performed with ClustalW, DNA weight matrix ClustaW 1.6 and default parameters. Phylogenetic reconstruction was performed by using neighbor-joining method with p-distance substitution model and bootstrapping of 1000. Distance matrix was generated using RDP database. Additional 18S rRNA sequences for the different genera were retrieved from NCBI. The evolutionary distances were computed using the Kimura 2- parameter method and are in the units of the number of base substitutions per site. The analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 853 positions in the final dataset. Evolutionary analyses were also conducted using MEGA software version 7.0.

RESULTS

Data obtained from molecular analysis and phylogenetic tree were used to identify and characterize endophytic fungi isolated from *A. nilotica* medicinal plant. 18s rDNA sequence of the isolated strain were compared to sequences stored in Genebank database. Based on BLAST search of ribosomal DNA gene sequence, the endophytic fungi were found to be closest homolog of *Aspergillus sp*. Isolated endophytic fungi shows 99% similarity with *Aspergillus sp. ZH14* (Accession Number: JX303664.1).

Table description of 15 obtained sequences after BLAST.

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
JX303664.1	Aspergillus sp. ZH14	1561	1561	95%	0.0	99%
HQ393876.1	Aspergillus sp. PSFNRH-2	1561	1561	95%	0.0	99%
EU371048.1	Aspergillus sp. CPCC 480064	1561	1561	95%	0.0	99%
KJ869441.1	Aspergillus sp. S6 AD-2014	1544	1544	95%	0.0	99%
KX218387.1	Aspergillus neoflavipes strain AJR1	1500	1500	97%	0.0	98%
MG322181.1	Aspergillus sp. isolate DRC3	1496	1496	96%	0.0	98%
MF678562.1	Aspergillus terreus strain BTK-1	1491	1491	95%	0.0	98%
KX580630.1	Penicillium chrysogenum	1491	1491	95%	0.0	98%
KY010602.1	Penicillium chrysogenum strain A3	1491	1491	95%	0.0	98%

KT582272.1	Fungal sp. ZJ48	1491	1491	95%	0.0	98%
KT582268.1	Fungal sp. ZJ34	1491	1491	95%	0.0	98%
KT582267.1	Fungal sp. ZJ30	1491	1491	95%	0.0	98%
KT582266.1	Fungal sp. ZJ26	1491	1491	95%	0.0	98%
KT582252.1	Fungal sp. ZJ46	1491	1491	95%	0.0	98%
KT582251.1	Fungal sp. ZJ45	1491	1491	95%	0.0	98%

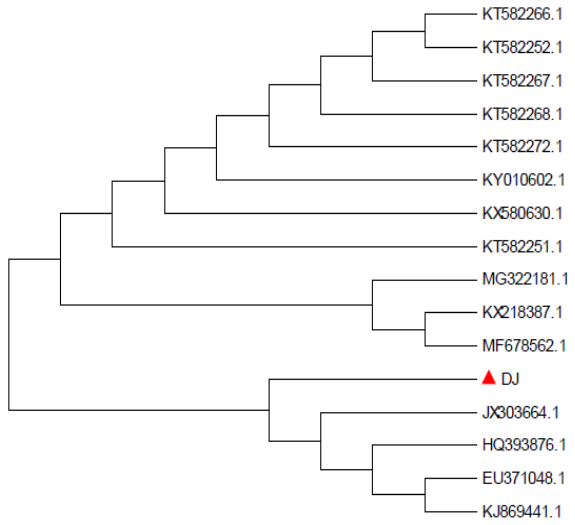

Fig. 1 Neighbor Joining (NJ) tree showed phylogenetic relationship between isolated sequences of endophytic fungi, based on the 18s rDNA sequences. Phylogenetic tree shows evolutionary history, which was inferred using the Neighbor-Joining method (fig 1). The optimal tree with the sum of branch length = 0.02252037 is shown.

DISCUSSION

Endophytic fungi are of highly diverse and intriguing group of microorganisms spend the whole or part of their lifecycle colonizing inter or intracellular in the healthy tissues of the plant typically causing no apparent symptoms of disease. These are considered as alternative of plant secondary metabolites as they are producing bioactive compounds of pharmaceutical and food industrial importance which also has applications such as antibiotics, antioxidants, anticancer and antiviral activities. And hence there is a need for isolation of endophytes and their exploitation at industrial level for producing pharmaceutically important bioactive compounds. The colonization and propagation of endophytes may in some ways offer significant benefits to their host plants by producing a plethora of substances that provide protection or increase the fitness of the hosts, such as enhancement of stress-, insect-, and disease-resistance, productivity improvement, and herbicide activities. Molecular techniques have been successfully used for identifying endophytic fungi in recent studies (Tedersoo et al., 2006; Morakotkarn et al., 2007). Culture-independent DNA methods, such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment length polymorphism (T-RFLP), and ITS sequencing, have been developed for the investigation of complex microbial communities. Bougoure et al. (2005) directly characterized the fungal community in hair roots using plant materials. Our study also shows that molecular identification based on ITS sequences can be used to complement or verify morphological identification of unknown endophytes. In the present study, endophytic fungi from a medicinal plant *A.nilotica* were isolated and identified. The isolated fungi was identified based on 18s rDNA sequencing. endophytes shows high diversity, for molecular characterization of endophytic fungi DNA sequences used as preferred marker. The preferred nucleotide marker has been ITS because of extensive database in NCBI Genebank. In addition, the ITS region is easy to amplify and, in many cases, provides enough variation to identify species.

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