

Molecular identification and Biosynthesis of silver nanoparticles using endophytic fungus *Alanphillipsia aloeigena*

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Abstract

The present study emphasizes on the isolation and molecular identification of endophytic fungi from the leaf, stem and flower of medicinal plant *Maytenus emarginata* (Willd.) Ding Hou. for the production of biologically important silver nanoparticles. Molecular characterization carried out by using 18S rRNA ITS universal primers and sequence data submitted to NCBI (Acc. No.MN794565). ITS sequencing of 18S rRNA region shows 100% homology with NCBI clade *Alanphillipsia aloeigena* isolate (NR137121), Biosynthesis of Silver nanoparticles was carried out using mycelium free filtrate incubated with 1mM aqueous solution of silver nitrate (AgNO₃). After observing the solution changes from colourless to brown colour indicates the bioreduction of silver ion to silver nanoparticles. The isolated endophyte is new report to *Maytenus emarginata* (Willd.) and it is good source of AgNPs.

Keywords: molecular, *Maytenus emarginata*, AgNPs and *Alanphillipsia aloeigena*

1. Introduction

Plant endophytic fungi are important components of plant micro-ecosystems. It is defined as the fungi which spend their whole life by colonizing intra or inter-cellularly within the healthy tissues of the host plants, without causing any symptoms of disease (Zhang *et al.* 2006) [29]. The fungal endophytic studies started during early 1900 when Freeman in 1904 described fungus from an annual grass in his paper (Padhi *et al.* 2013) [15].

Endophytes are found in a wide variety of plant tissue types such as roots, stems, leaves, tubers, buds, ovules, seeds, fruits, xylem and bark (Tan and Zou, 2001) [25]. Study revealed that approximately, there are near to 300,000 plant species on earth having host to one or more endophytes, and many of them may colonize different hosts. It has been estimated that thus endophytes may be hyperdiverse because there may be as many as one million different endophytic fungal taxa. (Strobel & Daisy 2003, Huang *et al.* 2007) [23].

Plant endophytic fungi are novel and important for production of natural bioactive compounds with their potential use in agriculture, medicine and food industry. Endophytes especially those secondary metabolites produced by fungal endophytes colonizing medicinal plants have been recognized as potential sources of novel natural products for, agricultural, pharmaceutical and industrial uses, (Strobel and Daisy, 2003) [23]

In recent years there is much attention is now being paid to endophytic biodiversity, molecular identification, biosynthesis of silver nanoparticles from endophytes and its biological activity and the relationship between endophytes and their host plants (Prabavathy *et al.*, 2015; Sherif moussa *et al.*, 2015; Magdalena and Dorota, 2018; Smitha Vijayan *et al.*, 2016; Vardhana *et al.*, 2018) [16, 20, 13, 22, 26].

Traditionally, endophytic fungi have been identified and classified on the basis of morphological and microscopic characters, later by ultra-structural and biochemical features, which however did not always distinguish between

analogies and homologies (Weber, 2009) [28]. To overcome lacuna of traditional identification species and socioeconomic relevance of fungi insist for alternate DNA based identification (Seifert, *et al.*, 2007) [18]. Taxonomy and phylogeny-based systematics now rely to a large extent on phylogenetic trees derived from molecular data used in phylogeny and taxonomy (Begerow, *et al.*, 2010, Nilsson, *et al.*, 2011) [2, 14]. DNA sequencing of the nuclear ribosomal internal transcribed spacer (ITS) region for the use in BLAST-based homology searches in the International Nucleotide Sequence Databases has been used as standard 'barcoding' marker by several mycologists for many years (Begerow, *et al.*, 2010) [2]. Because it is the most widely sequenced DNA region in fungi because of its higher degree of variation than other regions of rDNA (LSU and SSU) and for molecular systematic at the species level, and even within species (variety/strain/race) (Rashmi, *et al.*, 2013) [17]. Considering the importance of endophytes as potent producers of silver nanoparticles for biological applications, thus the aim of the present research is molecular identification of endophytic fungi from medicinal plant *Maytenus emarginata* (Willd.) Ding Hou by sequencing ITS region of the rDNA, and to carry out a phylogenetic analysis and comparison with other sequences deposited in NCBI database and biosynthesis of silver nanoparticles for biological activity.

2. Material and Methods

Collection of materials

Plant parts like stem, leaf and flower of *Maytenus emarginata* (Willd.) Ding Hou were collected from localities of Nanded and Hingoli district and brought to the laboratory used for the isolation of endophytic fungi.

Isolation of Endophytic Fungi

Endophytic fungi were isolated by Hallman *et al.* (2007) [9] and Selvakumar *et al.* (2014) [19] method. The collected plant

samples were washed in running tap water to remove the debris and epiphytic microorganisms and soaked in 0.1 % mercury chloride and surface sterilized by using ethanol. Followed by 2% Sodium hypochlorite solution for 2-4 minutes then dipped in sterile distilled water. The Sterile segments were then chopped into 3-4 mm x 0.1 cm lengths and inoculated in Petri dishes containing Czapek dox agar and potato dextrose agar (PDA) medium supplemented with streptomycin. The Petri dishes were sealed and incubated at room temperature (28±2°C) for 15 days. Fungal growths growing out of the pieces were sub cultured on separate Czapek dox agar medium plates and slants before use for the identification.

The colonization frequency (CF) percentage of endophytic fungi was calculated using the method (Kumar & Hyde 2004).

Colonization frequency

$$CF\% = \frac{\text{No of species isolated}}{\text{No of segments screened}} \times 100$$

Molecular Identification of Isolated fungal organisms-

The identification of endophytic fungi was done using 18s rRNA ITS universal primers at Sai biosystems Pvt. Ltd. Nagpur. The pure culture of isolated fungal strains was maintained in Czapadox agar slants at 28°C during the study.

Procedure

DNA Extraction

Fungal genomic DNA was isolated using the InstaGene™ Matrix Genomic DNA isolation kit

PCR Protocol

Using 18S rRNA ITS Region Universal primers gene fragment was amplified using MJ Research PTC-225 Peltier Thermal Cyclor.

Primer Details

Primer Name Sequence Details Number of Base

ITS1 TCCGTAGGTGAACCTGCGG 19

ITS4 TCCTCCGCTTATTGATATGC 20

Purification of PCR products

Removed unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). The PCR product was sequenced using the ITS1/ITS4 primers. Sequencing reactions were performed using a ABI PRISM® BigDye™ Terminator Cycle Sequencing Kits with AmpliTaq® DNA polymerase (FS enzyme) (Applied Biosystems).

Sequencing protocol

Single-pass sequencing was performed on each template using below 18s rRNA ITS universal primers. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were resuspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer (Applied Biosystems).

Primer Details

Primer Name Sequence Details Number of Base

ITS1 TCCGTAGGTGAACCTGCGG 19

ITS4 TCCTCCGCTTATTGATATGC 20

The program MUSCLE 3.7 was used for multiple alignments of sequences (Edgar 2004) [7]. The resulting aligned sequences were cured using the program Gblocks 0.91b. This Gblocks eliminates poorly aligned positions and divergent regions (removes alignment noise) (Talavera and Castresana 2007) [4]. Finally, the program PhyML 3.0 aLRT was used for phylogeny analysis and HKY85 as Substitution model. Tree Dyn 198.3 was used for tree rendering. (Dereeper *et al.*, 2008) [5].

Biosynthesis of Silver Nanoparticles

For screening of biosynthesis of extracellular silver nanoparticles (AgNPs). After isolation fungi were grown in the Czapadox broth supplemented with streptomycin at 28°C by putting on shaker at 120 rpm for incubation period of 72 hrs. After incubation biomass formed, harvested, filtered using Whatman filter paper number 1 and washed several times with sterile distilled water to remove any medium component. Harvested biomass was transferred into 100 ml of sterile distilled water and incubated for 48 hours. The cell-free filtrate was used in experiments. AgNPs was prepared by mixing AgNO₃ (1 mM of final concentration) with cell-free extract. The above flask was then incubated at room temperature under dark conditions, and observed for color development. Control was also prepared, containing only the cell filtrate without silver nitrate solution. The formation of silver nanoparticle is usually confirmed by visual observation of color change from pale white to reddish brown. This appearance of color change from colorless to brown is a clear indication of the formation of silver nanoparticles by reduction of silver in the filtrate extracellularly. AgNPs synthesis was carried out by the method of (Prabavathy *et al.*, 2015 and Kiran, 2017) [16, 11]

3. Results and Discussion

Isolation of endophytic fungi

The sterilized plant parts of *Maytenus emarginata* (Wild.) Ding Hou like stem, leaf and flower were grown on czapek dox agr medium for 5 days of incubation. After that different colonies were selected transferred on other plate for pure culture and slant was maintained. (fig.1 & 2).

Colonization frequency

The black colony of experimental fungus showed frequency of colonization high on flower (20%) followed by stem (15%) and leaf (6.66%).



Fig 1: Czapek dox Agar plate



Fig 2: Pure culture

Molecular identification of endophytic fungal isolate

The pure culture of endophytic fungus used for molecular identification through ITS sequencing of 18S rRNA region Universal primers gene with 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 includes

GTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGA
 TCATTACCGAGTTCTCGGGCTTCGGCTCGACTCTCC
 CACCCTTTGTGAACATACCTCTGTTGCTTTGGCGGC
 TTCTCGGGCTCCCCAGCCCGGGCCCGCCGCCAGA
 GGACCTTCAAACCTCAGTCAGTAAACGTCGACGTC
 TGATAAGCAAGTTAATAAACTAAAACCTTTCAACA
 ACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC
 AGCGAAATGCGATAAGTAATGTGAATTGCAGAATT
 CAGTGAATCATCGAATCTTTGAACGCACATTGCGC
 CCCTTGGTATTCCGAGGGCCATGCCTGTTCTGAGCG
 TCATTACAACCTCAAGCTCTGCTTGGTATTGGGCG
 CCGTCCCTACTGCGGACGCGCCTCAAAGACCTCGG
 CGGTGGCGTCTAGCCCTCAAGCGTAGTAGAAAACA
 CCTCGCTTTGGAGCGGTTGGCGTCGCCCGCCGGAC
 GAACCTTCTGTAGAACTACTTCTCAAGGTTGACCT
 CGGATCAGGTAGGGATACCCGCTGAACTTAA

After molecular identification authentication were carried out by comparing the result sequence to those available in National centre for biotechnology information (NCBI) data. NCBI blast data confirms endophytic isolate belong to *Alanphillipsia aloeigena* of class ascomycetes and received NCBI authentication number MN794565 and finally phylogenetic tree were constructed.

BLAST Homology of *Alanphillipsia aloeigena*

Endophytic fungal isolate 7 identified by ITS region sequencing were showed homology 100% with NCBI clade *Alanphillipsia aloes* (NR137122), *Alanphillipsia aloeigena* isolate (NR137121), *Alanphillipsia aloeigena* strain CPC 21286 (KF777137), *Alanphillipsia aloetica* (NR137123), and with *Alanphillipsia aloetica* strain CPC21109 (KF777139). So, by applying principle of parsimony isolate 7 confirmed as *Alanphillipsia aloeigena* (NCBI accession no. MN794565).

A phylogenetic tree (fig.3) finds relationships among different taxa ITS sequences and their hypothetical common ancestors (Hall, 2013). Thus, the phylogenetic relationship of two endophytic fungal isolates based on the ITS sequences were done and deposited in NCBI nucleotide database. Major taxa showing closest homology to our sequences were selected (Bosshard *et al.*, 2006) [3].

GenBank

Alanphillipsia aloeigena isolate 7 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

GenBank: MN794565.1
 FASTA: Graciosa

LOCUS: MN794565 560 bp DNA Linear PLN 06-Jan-2019

DEFINITION: Alanphillipsia aloeigena isolate 7 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.

ACCESSION: MN794565
 VERSION: MN794565.1

KEYWORDS: Alanphillipsia aloeigena
 Alanphillipsia aloeigena
 Eukaryota; Fungi; Dikarya; Ascomycota; Pezizomycotina; Dothideomycetes; Dothideomycetes incertae sedis; Botryosphaeriales; Botryosphaeriaceae; Alanphillipsia.

REFERENCE 1 (bases 1 to 560)
 AUTHORS: Sayad Shahin,K., Gachande,B.D. and Shaikh Najiroddin,F.
 TITLE: Biological activity of silver nanoparticles synthesized by endophytic fungi of Maytenus emarginata (Willd) Ding Hou
 JOURNAL: Unpublished

REFERENCE 2 (bases 1 to 560)
 AUTHORS: Sayad Shahin,K., Gachande,B.D. and Shaikh Najiroddin,F.
 TITLE: Direct Submission
 JOURNAL: Submitted (04-DEC-2019) P G Department of Botany, N.E.S. Science College Handed, Sneh Nagar, Handed, Maharashtra 431065, India
 COMMENT: Sequencing Technology :: Sanger dideoxy sequencing
 #Assembly-data-FASTQ

FEATURES
 Location/Qualifiers
 source 1..560
 /organism="Alanphillipsia aloeigena"
 /mol_type="genomic DNA"
 /isolate="7"
 /host="Gymnosporia emarginata"
 /db_xref="taxon:361624"
 /collection_date="18-Jan-2019"

Fig 3

Phylogram of *Alanphillipsia aloeigena* (fig.3)

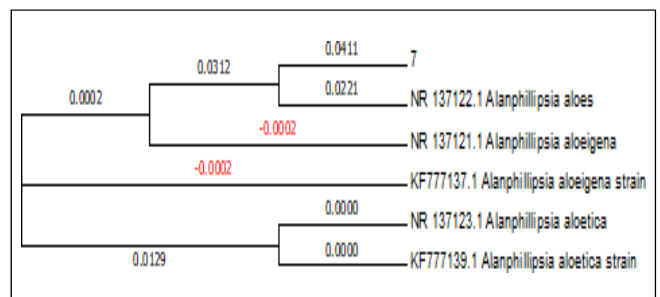


Fig 4

Taxonomy of *Alanphillipsia aloeigena* (fig.4)

NCBI Taxonomy Browser

Search for: *Alanphillipsia aloeigena*

Display: 3 levels using filter: none

current name: *Alanphillipsia aloeigena* Cross & M.J. Wing, 2013
 holotype of *Alanphillipsia aloeigena*: CBS:H:21419
 culture from holotype of *Alanphillipsia aloeigena*:
 CPC:21286, CBS:136408
 includes: *Botryosphaeriaceae* sp. JZG-2013a

NCBI BLAST name: ascomycetes
 Rank: species
 Genetic code: Translation table 1 (Standard)
 Mitochondrial genetic code: Translation table 4 (Mold Mitochondrial;
 Protozoan Mitochondrial; Coelenterate Mitochondrial; Mycoplasma;
 Spiroplasma)

Lineage (full):
 cellular organisms; Eukaryota; Opisthokonta; Fungi; Dikarya;
 Ascomycota; saccharomycetes; Pezizomycotina; Isonomiycetes;
 dothideomycetes; Dothideomycetes; Dothideomycetes incertae
 sedis; Botryosphaeriales; Botryosphaeriaceae; Alanphillipsia

Database name	Direct links	Links from type
Nucleotide	5	4
Protein	1	-
Popset	2	-
PubMed Central	3	-
Identical Protein Groups	1	-
Taxonomy	1	-

Fig 5

Preliminary Screening for Biosynthesis of Silver Nanoparticles production

The aqueous culture filtrate of *Alanphillipsia aloeigena* was analyzed for biosynthesis of AgNPs. After 24 hours of incubation of filtrate with silver nitrate, the reaction solution turned colorless to dark brown color which indicated the biosynthesis of AgNPs. (fig.5 & 6). The results of the present study show the color change from colorless to brown after addition of AgNO₃ to extracellular cell-free fungal filtrate which is the preliminary indication of the biosynthesis of AgNPs (Singh *et al.*, 2013) [21]. The biological reaction takes place between the extracellular fungal filtrate and the silver ion solution. The fungal enzyme nitrate reductase reduces silver ions leading to the formation of AgNPs (Arasu *et al.*, 2010) [1].

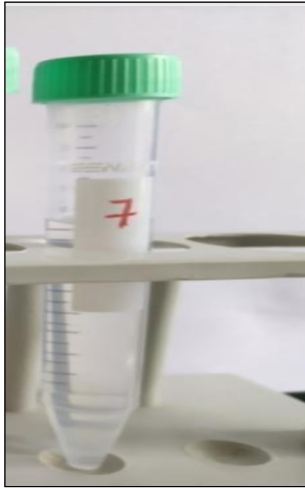


Fig 5: Fungal extracellular filtrate

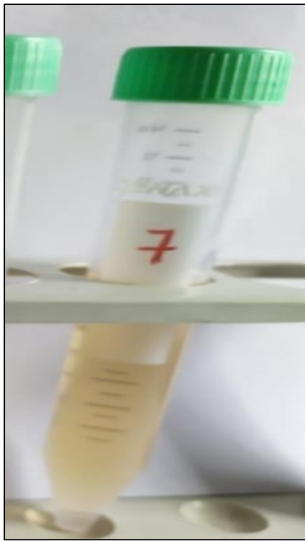


Fig 6: Filtrate with silver nitrate

4. Conclusion

Endophytic fungal isolate number 7 identified by ITS region sequencing were showed homology 100% with NCBI clade *Alanphillipsia aloeigena* isolate (NR137121) and *Alanphillipsia aloeigena* strain CPC 21286 (KF777137). So, by applying principle of parsimony isolate 7 confirmed as *Alanphillipsia aloeigena*. ((NCBI accession no. MN794565). *Alanphillipsia aloeigena* is first time reported as an endophyte to *Maytenus emarginata*. It is a good source of silver nanoparticles which can be used for biological activity in the near future.

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