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Research Article

SCREENING AND IDENTIFICATION OF HEAVY METAL-TOLERANT ENDOPHYTIC FUNGI *LASIODIPLODIA THEOBROMAE* FROM *BOSWELLIA OVALIFOLIOLATA* AN ENDEMIC PLANT OF TIRUMALA HILLS

SANI AISHWARYA[1], VENKATESWARULUNAGAM[2], VASUDEVA REDDY NETALA[1], VIJAYA TARTTE[2]

[1]Department of Biotechnology, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. [2]Department of Botany, Sri Venkateswara University, Tirupati, Andhra Pradesh, India. Email: aishwaryaaa14@gmail.com

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ABSTRACT

Aim: The aim of this study was to evaluate the heavy metal resistance potentiality of endophytic fungi isolated from the leaves of *Boswellia ovalifoliolata,* an endemic medicinal plant of Tirumala Hills.

Methods: Initially, isolation of fungal endophytes was carried out. Isolated fungi were screened for the heavy metal resistance against Co, Cd, Cu and Zn using growth and evaluated their maximum tolerant capacity. Molecular identification of endophytic fungi was carried out by 18S rRNA gene amplification and Sanger's nucleotide sequencing. Phylogenetic tree was constructed using NCBI Clustal W.

Results: Ten different endophytic fungi were isolated from the leaves of *B. ovalifoliolata*. Among the isolated endophytic fungi, five showed resistance to Co, Cd, Cu, and Zn. The most resistant fungus was identified as *Lasiodiplodia theobromae* based on 18S rRNA gene sequencing.

Conclusions: *L. theobromae* was isolated from *B. ovalifoliolata* and identified as one of the useful fungi involved in mycoremediation against heavy metal toxicity.

Keywords: Heavy metals, Endophytic fungi, Endemic plant, Bioremediation.

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INTRODUCTION

Soil pollution due to heavy metals has become one of the most severe worldwide environmental problems. Industrial activities have been leading to a continuous increase of heavy metal discharge into the environment including cadmium, lead, copper, chromium, and nickel [2] and eventually cause water and soil to be contaminated and become toxic. This problem has attracted considerable public attention because the continued increase of metal levels in soil and water poses a health risk to humans and animals through the food chain or contaminated drinking water [3]. Different conventional methods have been used for the removal and treatment of heavy metal pollution sites such as ion exchange, electrochemical treatment, reverse osmosis, precipitation, evaporation, and sorption [4,5]. However, these methods involve application of more reagents, high energy, high cost and result in ineffective and incomplete removal of metals, and also generate toxic sludge [2]. Bioremediation offers most economical and promising option to treat heavy metal in contaminated sites [6]. Different microorganisms are able to reduce the stress placed on plants by the presence of heavy metals, increase the availability of metal for plant uptake and promote plant growth [7,8]. Endophytic fungi are intriguing microbes live inside the healthy plant tissues and exhibits excellent metal-binding capacity [9] and provide more advantages over bacterial bioaugmentation [10]. Not only having the ability to protect against heavy metal toxicity, they also increase nutrient acquisition of host plants and enhance their metabolic activity to combat stress [11,12]. A wide range of fungi from all major taxonomic groups have been found in heavy metal polluted soil and some of them have evolved resistance to heavy metals [13]. The resistance and efficiency of endophytic fungi for removal of heavy metals vary greatly. Therefore, it is necessary to isolate and screen heavy metal-tolerant fungi. This study is aimed to isolate and screen heavy metal-tolerant fungi and to evaluate their efficiency to remove heavy metals from solid medium under laboratory conditions.

METHODS

Isolation of endophytic fungi

Endophytic fungi were isolated from the leaves of *Boswellia ovalifoliolata,* an endemic medicinal plant of Tirumala hills. The leaves of the *B. ovalifoliolata* were cut into smaller pieces and surface sterilized with 70% ethanol, 1% chlorox, and rinsed with sterilized water [1,14]. The plates were incubated at 25°C and observed for growth. The pure fungal cultures were transferred periodically onto fresh potato dextrose agar (PDA) plates. Pure cultures of endophytic fungi were stored in slant PDA and used for screening against heavy metals.

Screening of fungal isolates against heavy metals

Heavy metal-tolerant (50 ppm) fungal isolates were further screened for tolerance to Co, Cd, Cu and Zn at 100, 200, 400 and 600 ppm of heavy metals individually on PDA. All the fungal isolates were inoculated on PDA medium containing 100, 200, 400 and 600 ppm of each of the four heavy metals separately. The fungal isolate on PDA medium without adding any heavy metal is served as control for comparing the growth of fungal isolates on PDA medium containing different concentration of heavy metals. Observations on the growth of the fungi are recorded as normal growth or absent in comparison to control.

Identification of endophytic fungi

Morphological identification

After 3 days of an incubation period on PDA, a sterile wire loop was used to transfer the isolates from the agar onto a microscope slide. Crystal violet was used to stain the endophytic fungi for easier and better visualization. The slides were then viewed under an inverted microscope and identification was based on fungal morphological keys [15-18].

Molecular identification

DNA extraction

Fungal DNA was extracted for DNA amplification using the thermolysis method devised by Zhang *et al*. [19]. Pure colonies of fungal isolates were added into 100 µl of sterilized water in a 1.5 ml micro centrifuge tube and centrifuged at 10,000 rpm for 1 minute to homogenize it. After centrifugation, the supernatant was discarded and 100 µl of the lysis buffer (50 Mm potassium phosphate, 1 mM EDTA, and 1% glycerol) was pipetted into the micro centrifuge tubes. The tubes were placed in a water bath at 85°C for 30 minutes and used for DNA amplification.

DNA amplification

The DNA amplification method for fungal DNA was conducted according to Netala *et al*. [20]. The fungal DNA was amplified using 0.6 µl fungal primers internal transcribed spacer (ITS) 1 (5'-TCC GTA GGT GAA CCT GCG G-3') and 4 (5'-TCC TCC GCT TAT TGA TAT GC-3'), 12.8 µl deionized distilled water, 15 µl of 2X MyTaqRedMix and 1 µl of DNA template. The polymerase chain reaction (PCR) conditions were set up as 95°C, 3 minutes (initial denaturation); 95°C, 3 seconds (denaturation); 47°C, 30 seconds (annealing); 75°C, 2 minutes (elongation); 72°C, 5 minutes (final elongation) and 4°C (cooling). The PCR tubes containing the MASTER MIX and DNA template were amplified using bioer little genius thermocycler. The PCR products were run in 1% agarose gel electrophoresis using 1XTAE buffer at 90 V for 45 minutes and visualized under UV transilluminator.

DNA sequencing and phylogenetic tree analysis

The PCR products were sent to Beijing Genomic Institute, China, for nucleotide sequencing. The sequences obtained were analyzed against the NCBI (USA) database [21], and a phylogenetic tree was constructed from genetic distance and bootstrap values calculated using MEGA5 [22].

RESULTS AND DISCUSSION

Isolation of endophytic fungi

Endophytic fungal growth from the leaf tissues of *B. ovalifoliolata* was first observed after 48 hrs of inoculation. A total of 10 endophytic fungi were isolated from the leave tissues. The surface sterilization protocol was a critical prerequisite for isolating plant endophytic fungi. This study showed that the surface sterilization protocol was effective in removing epiphytic microorganism and that the fungal isolates can be considered to be true endophytic fungi. This made it possible to isolate and characterize endophytic fungi associated with healthy leaves.

Screening of fungal isolates against heavy metals

Ten fungal isolates were screened against Co, Cd, Cu and Zn. From the preliminary screening 10 fungi which showed different resistance pattern against individual heavy metal (Table 1). The fungi were further screened for their tolerance to Co, Cd, Cu and Zn at 50,100, 200, 400 and 600 ppm. All ten isolates showed resistance Co and Zn up to 600 ppm but only HEF3 isolate showed resistance to all four heavy metals Co, Cd, Cu and Zn at 600 ppm. The difference in metal tolerance may be due to the presence of various strategies of resistance mechanism exhibited by the fungi [23,24]. Most studies have been undertaken on filamentous fungal strains and mostly members from the genera *Aspergillus*, *Fusarium, Humicola,* and *Nannizzia* have been reported to possess resistance against heavy metals [13,23,25]. Recently, several studies have reported a similar trend among endophytic fungi being able to resist several heavy metals such as copper, zinc, and cadmium [26-28]. The preliminary screening of endophytic fungi against heavy metals showed the order of tolerance to heavy metals are Cd<Cu<Zn<Co. It is observed that as the concentration of heavy metal increased the growth of the fungi decreased due to toxicity of heavy metals. Fungal cell walls are typically composed of the polysaccharides chitin and cellulose and these constituents of the cell wall possess functional groups such as amino, carboxyl, hydroxyl and sulfate which have high metal binding capacities and are believed to have a significant potential for metal binding [29].

Identification of endophytic fungi

The isolate HEF3 resistant to Co, Cd, Cu and Zn at 600 ppm was identified as *Lasiodiplodia theobromae* and the morphological features include characteristic black pigmentation and mycelia was smooth branched, septate and subhylaline hyphae (Fig. 1). The fungus was characterized by PCR amplification of 18S rRNA gene using both forward and reverses ITS primers. The amplified PCR product was around the size of 500 bps. The Sanger's dideoxy nucleotide sequencing of amplified ITS region (ITS 1-5.8S-ITS 2) of 18 seconds rRNA gene resulted in 517 bps nucleotide sequence. The blast analyses, pairwise, and multiple sequence alignment revealed 98-100% identity with the sequences of *L. theobromae* strains and is designated as *L. theobromae* and has been deposited in NCBI Gen Bank (Accession Number KT804649.1). Multiple sequence alignment was carried out using Clustal W2 with default parameters. Phylogenetic tree was constructed by the neighbor-joining method with nucleotide pair wise genetic distance corrections (Fig. 2). *L. theobromae* is a cosmopolitan fungus with a worldwide distribution in the tropic and sub tropic regions, and there is no evidence of host specificity for the isolate [30,31]. *L. theobromae* can also be considered as a latent pathogen capable of endophytic infection [32].

CONCLUSION

Endophytic fungal isolates were isolated from *B. ovalifoliolata* an endemic plant of Tirumala hills. The 10 fungal isolates were screened for their tolerance for the four heavy metals (Co, Cd, Cu and Zn) in PDA medium containing heavy metals from 50 to 600 ppm. It was observed that there was decrease in number of fungal isolates tolerance to heavy metals with increasing concentration of heavy metal from 50 to 600 ppm. Majority of the fungal isolates were able to tolerate heavy metals up to 400 ppm. Among all the fungal cultures only one culture *L. theobromae* HEF3 showed resistance to all four heavy metals Co, Cd, Cu and Zn up to 600 ppm. The endophytic fungi *L. theobromae* remarkably differed in detoxification behavior from other isolated fungi in this study. The fungus showed a remarkable potential to actively grow in the presence of Co, Cd, Cu, Zn and reduce heavy metal concentration to less toxic levels. Further investigations

Table 1: Growth of the fungi observed at 600 ppm concentration of heavy metals (Co, Cd, Cu, and Zn)

Isolates	Co	Cd	Cu	Zn
HEF1	+	-	-	+
HEF2	+	-	-	-
HEF3	+	+	+	+
HEF4	+	-	+	+
HEF5	+	-	+	+
HEF6	+	+	-	+
HEF7	+	-	-	-
HEF8	+	-	-	+
HEF9	+	+	-	+
HEF10	+	-	+	+

+: Indicate the presence of growth, -: Indicates absence of growth, Co: Cobalt, Cd: Cadmium, Cu: Copper, Zn: Zinc

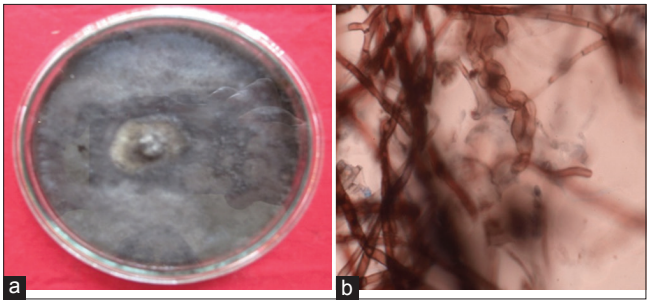

Fig. 1: (a and b) Morphological and microscopical features of *Lasiodiplodia theobromae*

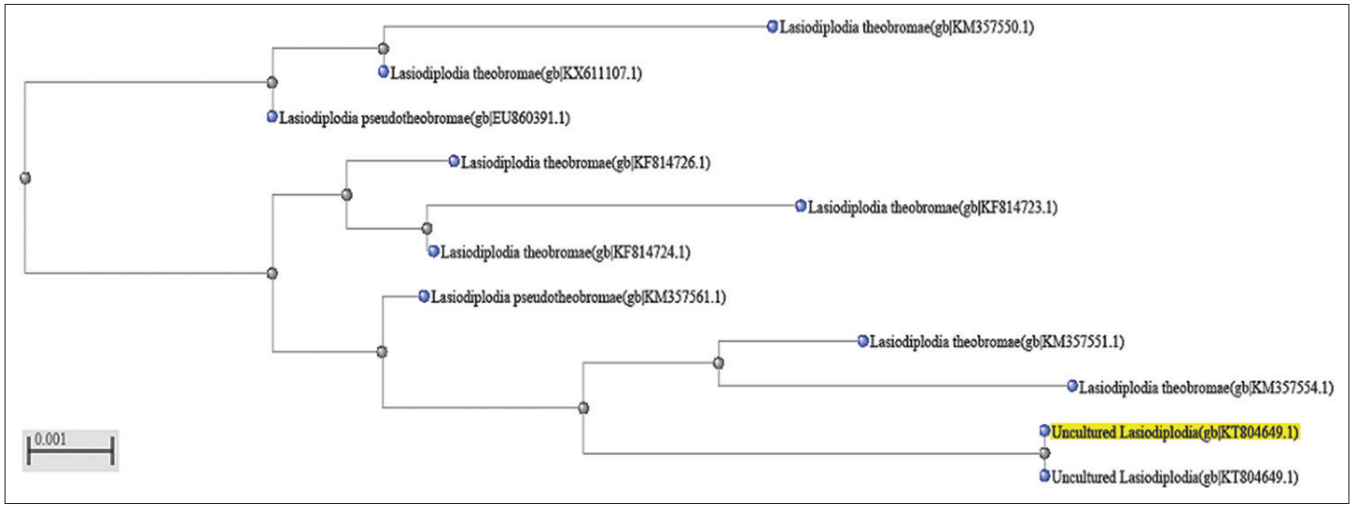

Fig. 2: Phylogenetic analysis of 18S rRNA gene of *Lasiodiplodia theobromae* with other fungal isolates

are needed to know the mechanism involved in fungi for showing resistance.

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