Isolation and Characterization of Endophytic Fungi from
*Boswellia Ovalifoliolata*. An endemic medicinal plants of Tirumala hills.

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ABSTRACT

The aim of the work is to isolate endophytic fungi from *Boswellia ovalifoliolata* an endemic medicinal plant from Eastern Ghats of Tirumala hills, India. Six endophytic fungi were isolated from leaf and stem samples. Based on morphological identification they were identified as *Xylaria sp, Fusarium equiseti*, *Cladosporium sp, Lasiodiplodia theobromae*, *Aspergillus niger*, *Cheatomium sp*. The overall colonization frequency of both stem and leaf was 17.5%. Among the endophytic fungi *Fusarium equiseti* was found to be the core group of fungus with colonization frequency of 30%.

**Keywords:** Endophytic fungi, endemic plant, Colonization frequency.
INTRODUCTION

Fungal endophytes are symbiotic organisms, and colonize the inner tissues of healthy plants to establish a harmonious relationship with their host without causing any visible disease symptoms [1-2]. Fungal endophytes have been isolated from various plant species, ranging from herbaceous crop plants to higher giant woody forest trees, suggesting their universal presence in nearly all higher plants [3-5]. They may contribute substantially to global fungal diversity but the proportion of this ecological group to total species richness in kingdom fungi remains unknown [6-7]. Previous studies have indicated that the association of fungal endophytes can significantly enhance plant growth, including biomass as well as production yield [8-9]. The beneficial effects of associated fungi (both endophytic fungi and ecto-mycorrhizae) on host plants mitigate biotic and abiotic stresses by increasing nutrient availability, enhancing tolerance to contaminants, and competing with and essentially inhibiting pathogenic organisms [10-11].

MATERIAL AND METHODS

Collection of plant material

*Boswellia ovalifoliolata* an endemic medicinal plant from Eastern Ghats of Tirumala hills, India. Plant parts such as leaves and stems were used for isolation of endophytic fungi.

Isolation of endophytic fungi

Endophytic fungi isolation was followed by Hallman [12]. Leaves were washed thoroughly under running tap water and then with liquid detergent teepol for 1-2 min to remove the adhered dust particles. Then the leaves were rinsed with Milli-Q water for two minutes and then transferred to laminar airflow (LAF). Under LAF, surface sterilization of leaves was carried out using 5% H₂O₂ for 45 sec and then the leaves were rinsed using 80% alcohol for 1 min. Finally, the leaves were rinsed thoroughly with Milli-Q water for 5 min. Moisture on the leaves was removed by placing them on sterile blotting paper and then cut into small segments of 5X5 mm. Leaf segments were placed on potato dextrose agar (PDA) plates and the plates were incubated at 28 ± 2°C for 8-10 days.

Colonization Frequency

The Colonization Frequency (CF) was calculated using the method Suryanarayanan [13]

\[
\text{Colonisation frequency} = \frac{\text{No of species isolated}}{\text{No of segments screened}} \times 100
\]

Morphological identification

All cultures were incubated at 28⁰ c for 7 days. Colonies were identified based on color of conidia, mycelia, reverse colors, texture, color , zonation and sporulation. All the isolates were subjected to microscopic analysis for characterization and identification.

RESULTS

Isolation of endophytic fungi

A total of 160 leaf and stem samples of *Boswellia ovalifoliolata* were inoculated on PDA medium(Table:1). Among them 28 isolates of six different fungal species were isolated . Out of 28 isolates, six were found to be *Xylaria* sp, six were found to be *Fusarium equiseti*, four were found to be *Cladosporium* sp. Two were found to be *Lasiodiplodia theobromae*. Eight were found to *Aspergillus* sp and two were found to be *Cheatomium* sp. *Fusarium* sp showed highest colonisation frequency (C.F) of 30% followed by *Aspergillus* sp and *Xylaria* sp (C.F=20%), *Cladosporium* (C.F=16%), *Lasiodiplodia theobromae* (C.F=13%) and the lowest colonisation frequency of 6% was observed by *Cheatomium* sp. The total isolation frequency was found to be 17.5%.
Table 1: colonization frequency of endophytic fungi isolated from Boswellia ovalifoliolata

<table>
<thead>
<tr>
<th>s.no</th>
<th>species</th>
<th>Site of isolation</th>
<th>N.O of samples</th>
<th>Fungi isolates</th>
<th>Colonization frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Xylaria sp</td>
<td>leaf</td>
<td>30</td>
<td>6</td>
<td>20</td>
</tr>
<tr>
<td>2.</td>
<td>Fusarium equiseti</td>
<td>leaf</td>
<td>20</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>3.</td>
<td>Cladosporium sp</td>
<td>stem</td>
<td>25</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td>4.</td>
<td>Lasiodiplodia theobromae</td>
<td>leaf</td>
<td>15</td>
<td>2</td>
<td>13</td>
</tr>
<tr>
<td>5.</td>
<td>Aspergillus sp</td>
<td>stem</td>
<td>40</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td>6.</td>
<td>Chaetomium sp</td>
<td>leaf</td>
<td>30</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td><strong>Total</strong></td>
<td>-</td>
<td>160</td>
<td>28</td>
<td>17.5</td>
</tr>
</tbody>
</table>

Morphological identification

**Xylaria sp**

Fruiting bodies were black in colour grown up to 2-3 cm in length (Fig.1.a) often growing in groups of three clustered into finger-like subcylindric at first, becoming flattened upper branches appear powdered white, finally tipped black when mature, stalk black and hairy. The mycelia was initially white and turned in to red in colour with irregular margins. Hyphae are thin-walled and branched (Fig.2.a).

**Fusarium equiseti**

Colonies are usually fast growing white, cottony, flat (Fig.1.b). Conidiophores are short, single, lateral monophialides in the aerial mycelium, later arranged in densely branched clusters. Chlamydospores are terminal, hyaline, smooth and short hyphal branches (fig.2.b).

**Cladosporium sp**

The colony was velvety to suede like in texture, slightly heaped and greyish green in colour (fig.1.c), edges of the colony are feathery. The colonies are diffuse and mycelia form mats and grown upward and seem velvety. Hyphae are erect and septated, conidia are small, oval shaped, single celled and smooth walled (fig.2.c).

**Lasiodiplodia theobromae**

Colonies with abundant aerial mycelium reaching to the lid of Petri plate, aerial mycelium is black in colour (fig.1.d). Isolates had irregularly shaped colonies with dense, fluffy and a moderate growth rate. The fungus produced stromata and pycnidia. From fruiting bodies liquid exudates. solitary, globose, thick-walled, non-papillate with a central ostiole. Paraphyses hyaline, cylindrical, thin-walled, aseptate and branched (fig.2.d).

**Aspergillus niger**

The colour of the colony was green in colour (fig.1.e) consisting of a dense felt of erect conidiophores. Conidiophores terminate in a vesicle covered with layer of subtending cells which bear small whorls of phialides (fig.2.e). The vesicle, phialides, metulae and conidia form the conidial head. Conidia are one-celled, smooth walled, hyaline pigmented are produced in long dry chains which may be divergent.

**Chaetomium sp**

Chaetomium sp colonies are cottony and white in colour, the colony reverse is brown (fig.1.f) Hyphae are septated Perithecia are large, dark brown to black, fragile and globose to flask shaped and have filamentous, hair-like, brown to black appendages (setae) on their surface. Ascocarps covered with pale, thin-
walled, flexuous hairs (fig.2.f). Terminal hairs sparse, olivaceous brown and fading towards the tips, punctuate and erect.

Fig 1: Colony morphology of endophytic fungi isolated from Boswellia ovalifoliolata
a. Xylaria sp b. Fusarium equiseti c. Cladosporium sp d. Lasiodiplodia theobromae e. Aspergillus niger
f. Cheatomium sp.

Fig 2: Microscopic identification of endophytic fungi
a. Xylaria sp b. Fusarium equiseti c. Cladosporium sp d. Lasiodiplodia theobromae
e. Aspergillus niger f. Cheatomium sp.

DISCUSSION

Endophytes are residing asymptptomatically in internal tissues of all higher plants are of growing interest as promising sources of biologically active agents [14]. The protection mechanism of the endophytes is exerted directly by releasing metabolites to attack any antagonists, or indirectly by inducing host defence mechanisms [15]. Endophytes can also promote plant growth through different mechanisms like production of phytohormones, synthesis of siderophores [16], nitrogen fixation, solubilisation of minerals [17], ethylene suppression [18] or via assisting phytoremediation [19]. However only a few plants have been studied for their
endophytic diversity and their potential to produce bioactive compounds because they occupy unique biological niches as they grow in so many environments [20]. In the present study altogether of six endophytic fungi were isolated from leaves and stems of *Boswellia ovalifoliolata* an endemic medicinal plant of Tirumala hills. Some hyphomycetous forms viz., *Cladosporium* sp, *Lasiodiplodia theobromae*, *Aspergillus* sp, [21-22]. were isolated as endophytes in the present study. A significant variation was observed in the colonization frequency. In this investigation low rate of colonization of endophytic fungi may be attributed due to the secretion of the certain antifungal and antibacterial components [23]. Previous studies reported that Eighteen different endophytic fungi were isolated from different tissues of bark, stem and leaf segments of five medicinal plants found within in Kudremukh range of Western Ghats of India, the dominant species isolated were *Curvulana clavata*, *C.lunata*, *C.pallescens* and *F.oxysporum*. The highest species richness as well as colonization frequency was found in the leaf segments of the host plant species [24].

**CONCLUSION**

In this study a total of 28 fungal endophytes were isolated from 160 leaf and stem explants (95 leaf explants and 65 stem explants) of *Boswellia ovalifoliolata* an endemic medicinal plant from Eastern Ghats of Tirumala hills, India. The isolated 28 fungal endophytes belongs to six different genera which includes. The leaf sample shows colonization frequency of 16.85 where as stem samples shows colonization frequency of 18.46%. *Xylaria* sp, *Fusarium equiseti*, *Cladosporium* sp, *Lasiodiplodia theobromae*, *Aspergillus niger*, *Cheatomium* sp. Colonization frequency (%) of fungal endophytes isolated from leaf and stem samples of *Boswellia ovalifoliolata* was ranged from 6 to 20%. Total colonization frequency of 17.5% was determined from *Boswellia ovalifoliolata*.

**ACKNOWLEDGMENT**

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

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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.

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 exhibit 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 [1,11,12]. 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 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 GCT GGG 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 MEGAS [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 leaf 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 Cu 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].

Identifying 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 subhyaline hyphae (Fig. 1). The fungus was characterized by PCR amplification of 18S rRNA gene using both forward and reverse ITS primers. The amplified PCR product was around isolate 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 and character endophytic fungi associated with healthy leaves. These results suggested that fungal isolates have potential to actively grow in the presence of Co, Cd, Cu, and Zn and reduce heavy metal concentration to less toxic levels. Further investigations are required to understand the potential of these fungal isolates in heavy metal remediation.

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

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Co</th>
<th>Cd</th>
<th>Cu</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>HEF1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>HEF2</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HEF3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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<td>HEF4</td>
<td>+</td>
<td>-</td>
<td>+</td>
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<td>HEF5</td>
<td>+</td>
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<td>+</td>
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<td>+</td>
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<td>HEF8</td>
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<tr>
<td>HEF9</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>HEF10</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

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

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Introduction
Review of Literature
Materials and Methods
Results
Discussion
Summary and Conclusion
References
ROLE OF ENDOPHYTIC FUNGI IN RESTORATION OF HEAVY METAL CONTAMINATED SOILS


ABSTRACT

Heavy metal contaminated soils may pose risk and hazards to humans and the ecosystem. Therefore, in order to maintain good quality of soil and keep them free from contamination, continuous efforts have been made to develop technologies that are easy to use, sustainable and economically feasible. Physiochemical approaches have been widely used for remedying polluted soil. However they experience more difficulties for a large scale of remediation because of high costs and side effects. Phytoremediation has been proposed as a low cost, environmental friendly and effective method to remove toxicants from contaminated soils. However phytoremediation of heavy metal still has to deal with some important shortcomings such as phytotoxicity, slower than mechanical method and a limited mechanical uptake. Nevertheless plant-associated endophytes can overcome these constraints, which can assist plants to accumulate higher amount of metals without increasing phytotoxicity. Many endophytic fungi have been found to be resistant to heavy metals and / or capable of degrading organic contaminates and endophyte-assisted phytoremediation has been documented as a promising technology for in-situ remediation of contaminated soils. Fungi posses the biochemical and ecological capacity to decrease the risk associated with metals, metalloids and radionuclides either by chemical modification or by influencing chemical bioavailability. Furthermore the ability of fungi from extended mycelial networks makes them well suitable for bioremediation processes. The application of filamentous fungi can be a promising method or a valuable complement in situation of bacterial malfunction, in which bacterial cells fail to form the mycelia network to react with contaminants. This paper reviews the heavy metal resistant endophytic fungi and their role in phytoremediation and discuss some issues that have been raised surrounding this area of research.

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INTRODUCTION

Heavy metal contamination refers to the excessive deposition of toxic heavy metals in the soil caused by human activities [1]. Heavy metals in soil include lead (Pb), cadmium (Cd), zinc (Zn), mercury (Hg), arsenic (As), silver (Ag), chromium (Cr), copper (Cu), iron (Fe), and the platinum group elements.

In recent years, with the development of the global economy, both type and content of heavy metals in the soil caused by human activities have gradually increased, resulting in the deterioration of the environment [2-4]. Heavy metals are highly hazardous to the environment and organisms. It can be enriched through the food chain. Once the soil suffers from heavy metal contamination, it is difficult to be remediated.

In the past, soil contamination was not considered as important as air and water pollution, because soil contamination was often with wide range and was more difficult to be controlled and governed than air and water pollution. However, in recent years the soil contamination in developed countries paid more and more attention and became a hot topic of environmental protection worldwide. In the world's top ten environmental events, two events have related to heavy metal contamination [5]. Heavy metal contamination not only explicitly damage the environment in a short period. Nevertheless, when it exceeds the environmental tolerance, or when environmental conditions have changed, heavy metals in the soil may be activated and cause serious ecological damage. So heavy metal contamination is usually chemical Time Bombs (CTBs) [6].

If the air and water are polluted, the pollution problem can be reversed certainly by dilution and self-purification after switching off the sources of pollution. However, it is difficult to use dilution or self-purification techniques to eliminate heavy metal contamination and to get soils improved. Some soils contaminated by heavy metals are likely to take one or two hundred years to be remediated [6]. Therefore, heavy metal contamination needs relatively high cost of remediation and the remediation cycle is relatively long.

Rapid and unorganized urban and industrial developments have contributed to the elevated levels of heavy metals in the urban environment of developing countries such as China and India [7-9]. Heavy metals are non-biodegradable and persistent environmental contaminants, which may be deposited on the surfaces and then absorbed into the tissues of vegetables. Plants take up heavy metals by absorbing them from deposits on the parts of the plants exposed to the air polluted environments as well as from contaminated soils [10-12]. A number of studies have shown heavy metals as important contaminants of the vegetables [13-15]. Heavy metal contamination of vegetables may also occur due to irrigation with contaminated water [15-17]. Heavy metal contamination of vegetables cannot be underestimated as these foodstuffs are important components of human diets. Vegetables are rich sources of vitamins, minerals, and fibers, and also have beneficial antioxidative effects. However, intake of heavy metal-contaminated vegetables may pose a risk to the human health. Heavy metal contamination of the food items is one of the most important aspects of food quality assurance [18-20]. International and national regulations on food quality have lowered the maximum permissible levels of toxic metals in food items due to an increased awareness of the risk these metals pose to food chain contamination [18].

Environmental contamination by heavy metals poses significant risks to public health and the ecosystem. Current research interests are moving towards the application of in situ strategies to reduce costs and to resolve pollution dispersal problems. Among in situ strategies, phytoremediation is considered to be a cost-effective and sustainable remediation method. However, phytoremediation is with some critical shortcomings, such as phytotoxicity [55]. Nevertheless, some plant-associated bacteria can overcome these constraints, which can assist plants to accumulate higher amounts of metals without increasing phytotoxicity [55]. The first and best-established approach for in situ detoxification of metal-contaminated soil is based on the partnership between the microorganisms and higher-plant. Endophytes are organisms that inhabit plant organs and their presence is generally inconspicuous [59]. Bioaugmentation with endophytes has several benefits over traditional bioaugmentation, such as enriching the soil with a consortium of bioremediation strains. Therefore, the objectives of this study were to characterize the features of a metal resistant endophytic fungus to investigate the potential to remove metals from contaminated soils.

Sources of heavy metals

Excess heavy metals in the soil originate from many sources, which include atmospheric deposition, sewage irrigation, improper stacking of the industrial solid waste, mining activities, the use of pesticides and fertilizers [21]. Heavy metals in the atmosphere are mainly from gas and dust produced by energy, transport, metallurgy and production of construction materials. Excepting mercury, heavy metals basically go into the atmosphere in the form of aerosol and deposit to the soil through natural sedimentation and precipitation, etc.

Transport, especially the automotive transport, causes serious heavy metal contamination (Pb, Zn, Cd, Cr, Cu, etc.) of the atmosphere and soils [22]. Heavy metals come from burning leaded gasoline and the dust produced by automobile tire wear. The amount of heavy metals which went into the soil through natural deposition and raining sedimentation are related to the level of development of heavy metal pollution. Wastewater can be divided into several categories, sanitary sewage, chemical wastewater, industrial mining wastewater and urban mining mixed sewage, etc. Heavy metals are brought to the soil by irrigative sewage and are fixed in the soil in different ways. It causes heavy metals (Hg, Cd, Pb, Cr, etc.) to continually accumulate in the soil year by year. Sewage irrigation is a feasible way to solve the problem of crop irrigation in the arid area. However, heavy metal contamination caused by sewage irrigation must be paid enough attention. Quality of irrigative sewage must be strictly controlled within the national quality standard for irrigation water.

There are a variety of solid wastes which have complex composition. Of which mining and industrial solid waste contamination is the most serious. When these wastes are in the process of being piled or governed, heavy metals move easily due to
the facilitation of sunlight, raining and washing. And they spread to the surrounding water and soils at the shape of funnel and radiation.

![Diagram of metal dispersion in the environment](image)

**Dispersion of metals in the environment [23].**

Fertilizers, pesticides and much are important agricultural inputs for agricultural production [21, 24]. Nevertheless, the long-term excessive application has resulted in the heavy metal contamination of soils. The vast majority of pesticides are organic compounds, and a few are organic - inorganic compound or pure mineral, and some pesticides contain Hg, As, Cu, Zn and other heavy metals [25]. Heavy metals are the most reported pollutants in fertilizers. Heavy metal content is relatively low in nitrogen and potash fertilizers, while phosphoric fertilizers usually contain considerable toxic heavy metals. Heavy metals in the compound fertilizers are mainly from master materials and manufacturing processes. The content of heavy metals in fertilizers is generally as follows: phosphoric fertilizer > compound fertilizer > potash fertilizer > nitrogen fertilizer [26]. Cd is an important heavy metal contaminant in the soil. Cd is brought to soils with the application of phosphoric fertilizers. Many studies showed that, with the application of a large amount of phosphate fertilizers and compound fertilizers, the available content of Cd in soils increases constantly, and Cd taken by plants increases accordingly. In recent years, the mulch has been promoted and used in large areas, which results in white pollution of soils, because the heat stabilizers, which contain Cd and Pb, are always added in the production process of mulch. This increases heavy metal contamination of soils [27].

**Table 1: Sources of heavy metals [28].**

<table>
<thead>
<tr>
<th>METALS</th>
<th>INDUSTRIES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chromium (Cr)</td>
<td>Mining, industrial coolants, chromium salts manufacturing, leather tanning</td>
</tr>
<tr>
<td>Mercury (Hg)</td>
<td>Chlor-alkali plants, thermal power plants, fluorescent lamps, hospital waste (damaged thermometers, barometers, sphygmomanometers), electrical appliances etc.</td>
</tr>
<tr>
<td>Arsenic (As)</td>
<td>Geogenic/natural processes, smelting operations, thermal power plants, fuel.</td>
</tr>
<tr>
<td>Copper (Cu)</td>
<td>Mining, electroplating, smelting operations</td>
</tr>
<tr>
<td>Vanadium (Va)</td>
<td>Spent catalyst, sulphuric acid plant</td>
</tr>
<tr>
<td>Molybdenum (Mo)</td>
<td>Spent catalyst</td>
</tr>
<tr>
<td>Zinc (Zn)</td>
<td>Smelting, electroplating</td>
</tr>
<tr>
<td>Cadmium (Cd)</td>
<td>waste batteries, e-waste, paint sludge, incinerations &amp; fuel combustion</td>
</tr>
<tr>
<td>Nickel (Ni)</td>
<td>Smelting operations, thermal power plants, battery industry</td>
</tr>
<tr>
<td>Lead (Pb)</td>
<td>lead acid batteries, paints, E-waste, Smelting operations, coal-based thermal power plants, ceramics, bangle industry</td>
</tr>
</tbody>
</table>

The heavy metal Chromium (Cr) is released from the Mining, industrial coolants, chromium salt manufacturing, leather tanning industries. Mercury (Hg) is released from the Chlor-alkali plants, thermal power plants, fluorescent lamps, hospital waste (damaged thermometers, barometers, and sphygmomanometers), electrical appliances etc. Arsenic (As) is released from the Geogenic/natural processes, smelting operations, thermal power plants, fuel. Copper (Cu) is released from the Mining, electroplating, smelting operations. Vanadium (Va) is released from the Spent catalyst, sulphuric acid plant. Molybdenum (Mo) is released from the spent catalyst. Zinc (Zn) is released from the Smelting, electroplating. Cadmium (Cd) is released from the waste batteries, e-waste, paint sludge, incinerations & fuel combustion. Nickel (Ni) is released from the Smelting operations, thermal power plants, battery
industry. Lead (Pb) is released from the lead acid batteries, paints, E-waste, Smelting operations, coal- based thermal power plants, ceramics, bangle industry.

IMPACT OF HEAVY METALS ON PLANTS

Low concentration of soil heavy metals, regardless of necessary or unnecessary to plants, will not affect the growth of plants in a certain range. But if the concentration is too high, the content of heavy metals enriched by the plant exceeds its tolerance threshold, and thus the plant will be poisoned and it even leads to death of the plant [1]. Plants exposed to high levels of Cd causes reduction in photosynthesis, water uptake, and nutrient uptake. Plants grown in soil containing high levels of Cd show visible symptoms of injury reflected in terms of chlorosis, growth inhibition, browning of root tips, and finally death [29-30].

High levels of Zn in soil inhibit many plant metabolic functions; result in retarded growth and cause senescence. Zinc toxicity in plants limited the growth of both root and shoot [31-33] Zinc toxicity also causes chlorosis in the younger leaves, which can extend to older leaves after prolonged exposure to high soil Zn levels [33]. Excess of Cu in soil plays a cytotoxic role, induces stress and causes injury to plants. This leads to plant growth retardation and leaf chlorosis [34]. Exposure of plants to excess Cu generates oxidative stress and ROS [35]. Oxidative stress causes disturbance of metabolic pathways and damage to macromolecules [36].

Higher amount of Hg2+is strongly phytotoxic to plant cells. Toxic level of Hg2+ can induce visible injuries and physiological disorders in plants [37]. For example, Hg can bind to water channel proteins, thus inducing leaf stomata to close and physical obstruction of water flow in plants [38]. High level of Hg2+ interfere the mitochondrial activity and induces oxidative stress by triggering the generation of ROS. This leads to the disruption of biomembrane lipids and cellular metabolism in plants [39-41].

Toxic effects of Cr on plant growth and development include alterations in the germination process as well as in the growth of roots, stems and leaves. Hence, exposure to high level of Cr affected total dry matter production and yield of plants. Cr also causes deleterious effects on plant physiological processes such as photosynthesis, water relations and mineral nutrition. Metabolic alterations by Cr exposure have also been described in plants either by a direct effect on enzymes and metabolites or by its ability to generate ROS [42].

High amount of Pb causes inhibition of enzyme activities, water imbalance, alterations in membrane permeability and disturbs mineral nutrition [43]. Pb inhibits the activity of enzymes at cellular level by reacting with their sulphhydril groups. High Pb concentration also induces oxidative stress by increasing the production of ROS in plants [44]. Plants grown in highNi2+ containing soil showed impairment of nutrient balance and resulted in disorder of cell membrane functions. Thus, Ni2+ affected the lipid composition and H-ATPase activity of the plasma membrane as reported in Oryza sativa shoots [45].

IMPACT OF HEAVY METALS ON HUMANS

Existing research showed that heavy metals in urban soils may go into the human body through skin absorption and inhalation of dust, etc., and thus directly damage, especially children's health. They also affect the urban environmental quality and damage human health indirectly through polluting the food, water and atmosphere [1]. Cd may damage the metabolism of calcium, which will cause calcium deficiency and result in cartilage disease and bone fractures, etc. Agency for Toxic Substances Management Committee has listed Cd as the sixth most toxic substance that damages human health. Pb mainly enters human body through the digestive tract and respiratory tract, and then goes into the blood circulation in the form of soluble salts, protein complexes or ions, etc. 95% of the insoluble phosphate lead accumulates in bones.

Pb is strongly pro-organizational. It affects and damages many of the body organs and systems, such as kidney, liver, reproductive system, nervous system, urinary system, immune system and the basic physiological processes of cells and gene expression.

Cu, Zn and Ni are essential trace metals in the human body, but if the body takes excessive Cu, Zn and Ni from the outside environment, they will damage human health. Ni and Cu are tumor promoting factors, whose carcinogenesis effect has attracted global concerns. Workers who are in close contact with the nickel powder are more likely to suffer from respiratory cancer, and the content of Ni in the environment is positively correlated with nasopharyngeal carcinoma [46].

TECHNOLOGIES FOR THE RECLAMATION OF POLLUTED SOILS:

The remediation of heavy metal contaminated environments is gaining considerable momentum and is a challenging task because metals cannot be degraded and the dangers they pose are aggravated by their persistence in the environment [47].

Different approaches have been used or developed to mitigate/reclaim the heavy metal polluted soils. The physicochemical approaches include excavation and burial of the soil at a hazardous waste site, fixation/inactivation (chemical processing of the soil to immobilize the metals), leaching by using acid solutions or proprietary leachants to desorb and leach the metals from soil followed by the return of clean soil residue to the site [48], filtration, chemical precipitation, electrochemical treatment, oxidation/reduction, ion exchange, membrane technology, reverse osmosis, and evaporation recovery, have been developed for heavy metal removal [47] are being economically expensive and have disadvantages like incomplete metal removal, higher reagent, energy requirements and generation of toxic sludge.

Bioremediation which involves the use of microbes to detoxify and degrade environmental contaminants, has received increasing attention in recent times to clean up a polluted environment [49-50]. Bioremediation, being in situ treatment, provides a safe and economic alternative to commonly used physiochemical strategies[51]. However, it seems not feasible at present. On one hand, it is difficult to obtain such valuable microbes from numerous kinds of microorganisms for heavy metal bioremediation. As well as, the adaptation abilities and the remediation efficiencies of reported microorganisms are not enough for practical application. On the
other hand, the mechanism of bioremediation still needs further understanding. As it known the mechanisms may facilitate the remediation efficiencies of valuable microbes and finally enable them feasible for practical application [52].

Phytoremediation defined as the use of green plants to remove pollutants from the environment or to tend them harmless, is an insitu, solar powered remediation technology [53-55]. This technology can be applied to clean up and or/stabilize both inorganic and organic contaminants and has been considered as a promising technology for its minimal site disturbance, lower cost and higher public acceptance when compared with conventional remediation methods. However , field scale application of phytoremediation still faces several obstacles such as: slow growth and small biomass, phytotoxicity, evapotranspiration of volatile contaminants etc [55-56]. To overcome these problems microbe assisted phytoremediation can be used [53]. Numerous studies have demonstrated that rhizosphere microorganisms can enhance phytoremediation efficiently [56-57]. In comparison with rhizosphere microorganisms, endophytes interact more closely with their host plants and could more efficiently improve phytoremediation [58].

WHAT ARE ENDOPHYTES

Endophytes are bacteria or fungi that inhabit plant organs and their presence is generally inconspicuous [59-60]. The infected host tissues are at least transiently symptomless, and the microbial colonization and can be demonstrated to be internal either through histological means, by isolation from strongly surface disinfected tissues, or through direct amplification of fungal nuclear DNA from colonized plant tissue [61]. Endophytes have been widely studied in various geographic and climatic zones and were found to be ubiquitous with in all examined plants to date, and the hosts include a broad range of orders, families, genera and species [62-64]. In general endophytes enter the plant tissue through the root zone, flower, leaf and cotyledon [63] and they may either become localized at the point of entry or spread throughout the plant. In accordance with their life strategies, endophytes can be either ‘obligate’ or ‘facultative’. Obligate endophytes are strictly dependent on the host plant for their growth and survival and their transmission to other plants occurs vertically or via vectors [64]. Facultative endophytes have a stage in their life cycle in which they exist outside host plants [64-65].

In the plant endophyte symbiosis, endophytes receive carbohydrates from plants in return, they can improve plant resistant to biotic and abiotic stresses [66-67]. It has been well demonstrated that endophytes can not only mediate interactions between host plants and their competitors, herbivores and pathogens [68-69]. But they can also affect the diversity and structure of the plant community [70-71]. Moreover recent studies and demonstrations have demonstrated that many endophytes are metal resistant, able to degrade organic contaminants and endophytes have been successfully used in phytoremediation [72-73].

Fungi are known to tolerate and detoxify metals by several mechanisms including valence transformation, extra and intracellular precipitation and active uptake [74]. The high surface to volume ratio of microorganisms and their ability to detoxify metals are among the reasons that they are considered as potential alternative to synthetic resins for remediation of dilute solutions of metals and solid wastes [75-76]. Fungi posses the biochemical and ecological capacity to decrease the risk associated with metals, metalloids and radionuclide’s either by chemical modification or by influencing chemical bioavailability. Furthermore the ability of fungi from extended mycelial networks makes them well suited for bioremediation processes. The application of filamentous fungi can be a promising method or a valuable complement in situation of bacterial malfunction, in which bacterial cells fail to form the mycelia network to react with contaminants. The method is especially useful for circumstances, in which contaminants are physically inaccessible to unicellular organisms or pollution is too serious to maintain bacterial survival [77].

ENDOPHYTES ASSISTED PHYTOREMEDIATION OF HEAVY METALS

Although heavy metals are toxic to plants it has been demonstrated that many plants are metal tolerant and that some of them are metal hyperaccumulators [78-79]. Plants that are especially good at concentrating the pollutants are termed hyperaccumulators. A metal hyperaccumulator is defined as a plant that can concentrate the metals to a level of 0.1% for nickel, cobalt, copper, and lead, 1% for zinc, and 0.01% for cadmium [80]. For example, Pteris vittata (Chinese brake fern) efficiently hyperaccumulates arsenic in its fronds which can be effectively harvested [81]. Arsenic is a lethal poison that is released into the environment from natural processes in certain geographical areas and through the use of arsenic-based chemicals. Pteris vittata can effectively remove this metalloid from soil. For example, in soil contaminated with arsenic at a concentration of 97 ppm, the older fronds of the fern had arsenic concentrations of up to 3894 μg per gram of tissue. Less than 168 μg arsenic per gram was found in the root tissue. More than 95% of the arsenic removed from the soil by the fern was translocated to the aboveground biomass. Unfortunately, this plant species grows well only in warm, humid environments with mild winters (N. Peck, pers. comm.). Another hyperaccumulator is Thlaspi caerulescens, which can concentrate cadmium, a highly toxic and probably carcinogenic metal [82] in the above-ground tissues at concentrations 1000 times higher than the normal toxic concentration of only 1 ppm [83]. The mechanism of uptake of cadmium and zinc by this member of the Brassicaceae family has been well studied and involves a highly expressed metal transporter [84]. The zinc/cadmium pumping ATPase was recently purified directly from T. caerulescens and was shown to transport both zinc and cadmium [85].

The hyperaccumulator-associated endophytes could be metal resistant, due to long-term adaptation to the high concentration of metals accumulated in the plants [86]. Many metal resistant endophytes were isolated from hyperaccumulating plants such as Alyssum bertoloni, Alnus firma, Brassica napus, Nicotiana tabacum, Thlaspi caerulescens, T. goesingense and Solanum nigrum. In recent years many metal resistant endophytes have also been isolated from non – hyperaccumulating plants such as Arabis hirusta, Acacia decurrens, and Symplocos paniculata [87]. The metal resistant endophytic fungi are Microsporaopsis, Mucor, Phoma, Alternaria, Peyronellae, Steganosporium and Aspergillus.
In a study by [88] Aspergillus niger and Pencillium sp have promising bioadsorption capacity of Cr, Ni and Cd from single and multi-metal solutions and highlighted possible exploitation of the filamentous fungi of metal polluted habitat. Endophytic fungus Microsphaeropsis sp isolated from cadmium hyperaccumulator Solanum nigrum L. a high biomass yield when cultured in vitro. Endophytic Fungi Microsphaeropsis sp. LSE10 was utilized as a biosorbent for the detoxification of cadmium [47].

In another study by [89] indicates that fungal population isolated from heavy metal contaminated sites has the ability to resist higher concentration of metals. A comparative level of metal resistance was also shown by filamentous fungi originated from unpolluted sites. The tolerance and resistance of the isolates depended much more on the fungus tested than on the site of its isolation. This variation may be explained by the development of tolerance and adaptation of the fungi to heavy metals. Aspergillus sp. and Fusarium sp. was the most resistance to all the metals tested, which make them promising candidates for further investigations regarding their ability to remove metals from contaminated environment. The present study indicates that isolated fungi of contaminated water can be used as bioremediation agents.

In recent research [90] The two isolates arsenic tolerant fungal strains Aspergillus flavus (ASC1) and Aspergillus.niger (ASB3) These two strains are capable of removing 50%-76 % of arsenic from different arsenic enriched medium, simultaneously also tolerant to different other heavy metals (Cd, Pb, Hg, Zn and Cr). In near future these two fungal strains will be effective in arsenic removal planning from arsenic contaminated sites. According to [91] the Cd, Pb, Zn resistant endophytic fungi Lasiodiplodia sp. MXSF31 was isolated from metal accumulating Portulaca oleracea and re-isolated from the shoots and roots of inoculated rape. The endophytic fungus showed high biosorption and bioaccumulation capacities of Cd, Pb and Zn from the metal contaminated solutions and enhanced the metal extraction efficacy of rape in soils contaminated by multiple metals. Because of the broad host range, the endophytic nature and resistant to multiple metals, endophytic fungi from plants accumulating multiple metals should be valuable microorganism resources for bioremediation of water and soils contaminated by multiple heavy metals.

**CONCLUSION**

Heavy metals release to the environment has been increasing continuously as a result of industrial activities and technological development and poses a significant threat to environment, public and soil health, the metal cannot be degraded to harmless products and hence persist the environment indefinitely. Several methods have been devised for the treatment and removal of heavy metal in contaminated soils. Biological approach has the great potential that contributed for the bioaccumulation of heavy metals by endophytic fungi assisted phytoremediation. The endophytic fungus showed high biosorption and bioaccumulation capacities of Cd, Pb, Cr, Ni, Hg and Zn from metal-contaminated soils and enhance the metal extraction efficacy. Because of the broad host range, the endophytic nature and resistance to multiple metals endophytic fungi from plants accumulating multiple metals should be valuable microorganism resources for bioremediation of soils contaminated by multiple heavy metals.

**RECOMMENDED FUTURE RESEARCH**

Most of the works were mainly focused on endophytic bacteria but little is known about endophytic fungi. Hence, more work should be conducted on endophytic fungi with metal resistance and their ability to assist in phytoremediation. More studies should be conducted to understand the diversity and ecology of endophytes associated with plants growing in heavy metal contaminated soils. Although plants associated endophytes degrade a wide range of contaminants, but many compounds are degraded slowly or are not degraded at all. Hence more engineered endophytes with appropriate biodegradative capabilities should be produced.
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