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SCREENING OF FUNGAL ENDOPHYTES WITH DIFFERENT CONCENTRATIONS OF HEAVY METALS ISOLATED FROM *TERMINALIA PALLIDA* AN ENDEMIC PLANT OF TIRUMALA HILLS

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Abstract:
Heavy metals are effecting the environment have been increasing continuously as a result of industrial activities and technological development and possess a significant threat to human beings, plants, animals and environment. Heavy metals causes liver and lungs damage, bone generation and blood damage. The metals can be removed by various technological methods but they are costly and possess less accurate results. To overcome from these a research study was carried out to biologically approach the accumulation of heavy metals by endophytic fungi. Endophytic fungi was isolated from Terminalia Palida an endemic plant of Tirumala hills and pure cultures of endophytes were isolated and stored at 28±1°C and was screened with different concentrations of selective heavy metals and the heavy metal resistance endophytic fungi was evaluated and 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.

Keywords: *Endophytic fungi, heavy metal, endemic plant and screening.*

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INTRODUCTION:

Heavy metal pollution by industrial activities and technological development is posing significant threats to the environment and public health because of their toxicity and non biodegradability. Many physical and chemical methods were developed to remove heavy metals from the effluents but they are expensive, ineffective, toxic and produce large quality of sludge. So a novel biological method of heavy metal removal was developed by using endophytic fungi. This fungi are known to have high heavy metal resistant capacity when compared to other microorganisms. This biological technique is low cost, eco-friendly and gives accurate results even for large scale.

METHODS:

Isolation of endophytic fungi:

The fungal endophytes were isolated from *Terminalia Pallida* by using a modified method described by *Arnold et al,.* (2000) [1]. The material was thoroughly washed in sterile water, surface disinfected by soaking in 70% ethanol for 1 min and 3% Sodium Hypochlorite (NaOCl) solution for 4 min, rinsed in sterile demineralised water and 96% ethanol for 30 sec. The plant material was subsequently rinsed in sterile demineralised water. The surface sterilized plant materials were cut into small segments (leaf of 5 × 5 mm size and stems: 10 mm length) and placed on different media incorporated with streptomycin sulphate (250 mg/L) and incubated at 28±1°C with 12 h photoperiod for 3-4 days to few weeks till the growth initiated. Individual hyphal tips that emerged from the edges of each treated plant bits were transferred separately onto fresh PDA plates, and incubated in a light chamber under near ultraviolet (UV) for at least 10~15 days. Each fungal culture was checked for purity and transferred to agar slants by the hyphal tip and single spore isolation methods [2,]. Pure forty cultures of endophytic fungi were stored at 28±1°C for further screening against heavy metals.

Screening of Endophytic fungi with heavy metals:

Sterilized PDA along with different concentrations of selective heavy metals were taken ranging from 50 ppm to 600 ppm concentrations. The PDA plates were inoculated with Endophytic fungi and stored at 28±1°C for 1 week.. The MIC (in cms) was calculated and tabulated for time interval of 1 week.

Identification of Fungal endophytes:

Phenotypic identification of Fungal Endophytes:

The cultures were examined periodically and identified when isolates sporulated using morphological characteristics such as growth pattern, hyphae, color of colony on the medium, surface texture, margin character, aerial mycelium and characteristics of the spore [3]. Fungal mycelium was stained in cotton blue and mounted in polyvinyl lactic acid glycerol (PVLG) by heating at 65°C for 2-3 days and observed under microscope. An Olympus CX13 armed with interference contrast was employed for examination by light microscopy. All the endophytic fungal isolates were identified and placed in appropriate genera and species of fungi using standard taxonomic keys and monographs. Authoritative monographs were referred for identification of endophytes *Bhat 2010[4],* (Hyphomycetes, Ascomycetes); *Leslie and Summerell 2006,* (Coelomycetes). In addition, other taxonomic papers relating to particular genera and species of endophytes were also referred. For colour differentiation of cultures 'Methuen Hand book of Colour' *(Kornerup and Wanscher, 1978)* [5] was also referred.

Molecular Characterization of Fungal Endophytes:

The identity of the certain interesting/non-sporulating strains was confirmed using molecular tool i.e., analysis of 18S rRNA gene sequence [6-11].

DNA extraction: Each endophytic fungus was cultured in potato dextrose broth (PDB) at 25°C with constant shaking for 7 days. The fungal mycelia were freeze-dried and used for the genomic DNA extraction. Genomic DNA was extracted by CTAB (Cetyl trimethylammonium bromide) method. Briefly; 500 mg of fungal mycelia were crushed in liquid nitrogen to make a fine powder. The cells were lysed in 10 mL of extraction buffer (50 mM Tris–HCl pH 8.0, 50 mM EDTA, 0.7 M NaCl, 2% cetrimide, 1% SDS and 50 µl β-mercaptoethanol), mixed thoroughly and incubated at 65°C for 30 min with continuous shaking. The lysate was extracted with an equal volume of chloroform/isoamyl alcohol (24:1) and centrifuged at 10,000×*g* for 10 min at 4°C. The aqueous phase was transferred to a sterile tube; the genomic DNA was precipitated in a 2×volume of chilled isopropanol and centrifuged at 4°C for 10 min at 10,000×*g*. The resulting pellet was washed twice with 70% ethanol, air dried and dissolved in 20 µL of sterile Millipore water.

PCR (polymerase chain reaction) Amplification and Sequencing of 18S rRNA gene:

Molecular characterization of the endophytes was carried out by the acquisition of ITS ribosomal gene sequencing. Protocol outlined by White et al. (1990) was followed for polymerase chain reaction in a CG Palm Cycler (Corbett Research, Mortlake, NSW, Australia). The ITS regions of the fungi were amplified with the universal ITS primers, ITS1(5′TCCGTAGGTG AACCTGCGG 3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC 3′) using the polymerase chain reaction (PCR). The amplified fragments include ITS1, 5.8S and ITS2 region of rDNA. The PCR reaction mixture (50 µL) was prepared as follows: 50 ng of template DNA, 10X PCR buffer(10 mM Tris-HCl, 50 mM KCl, pH 8.3), 1.5 mM $MgCl_2$, 2.5mM each dNTP, 200 ng/µL of each primer, and 2U/µL of Taq DNA polymerase (Genei, Banglore)) and MilliQ water was added to complete the final volume of the reaction. The amplified products (5 µL) were visualized on 1% (w/v) agarose gel to confirm the presence of a single amplified band. The PCR products were purified using a QIA Quick Gel Extraction Kit (Qiagen, Hilden, Germany). The purified PCR product was directly sequenced using Sanger dideoxy nucleotide sequencing method at the MWGAG Biotech (Bangalore, India). The 18S rRNA gene sequences obtained in FASTA format were subjected to BLASTn (http://www.ncbi. nim.nih.gov) analysis for identification of the test fungus and then submitted to GenBank using SEQUIN program. The GenBank also provided accession numbers for submitted sequences of the respective strains.

RESULTS AND DISCUSSION:

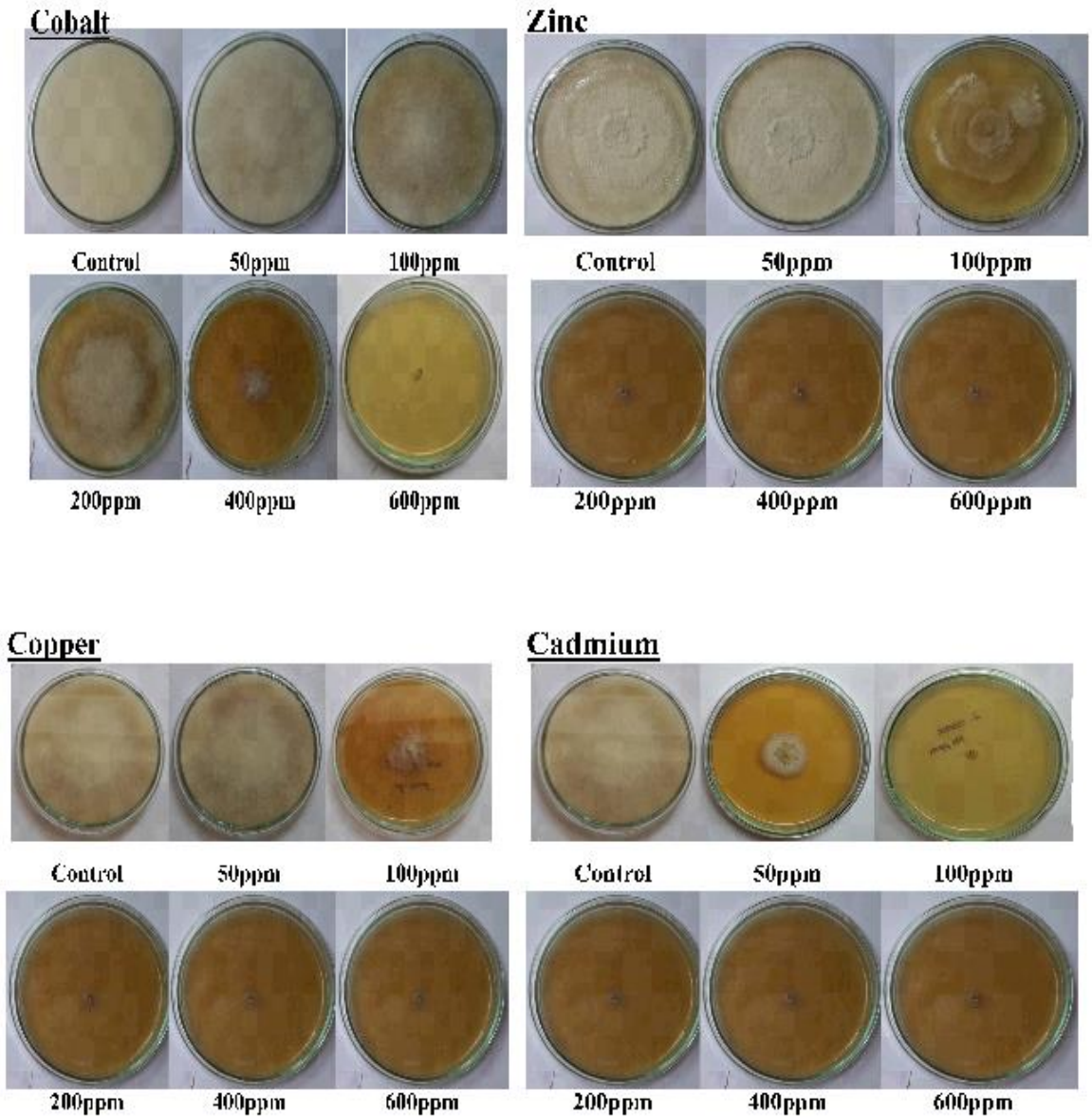
Isolation of Endophytic fungi:

Pure endophytic growth was observed from the tissues of leaf and stem by effective removal of epiphytes. A total of 40 fungal endophytes belonging to different taxa were isolated from *Terminalia Pallida* an endemic plant of Tirumala hills. Endophytic fungi are known to be highly heavy metal resistant and can be exploited as effective biosorbents due to great surface area when compared to other microorganisms.

Screening of endophytic fungal isolates for heavy metal tolerance:

Out of forty fungal endophytes isolated from endemic flora of Tirumala hills thirteen have exhibited tolerance to 50 ppm concentrations of Cobalt, Zinc, Copper and Cadmium. These isolates were further screened for their tolerance to higher concentrations of Cobalt, Zinc, Copper and Cadmium.

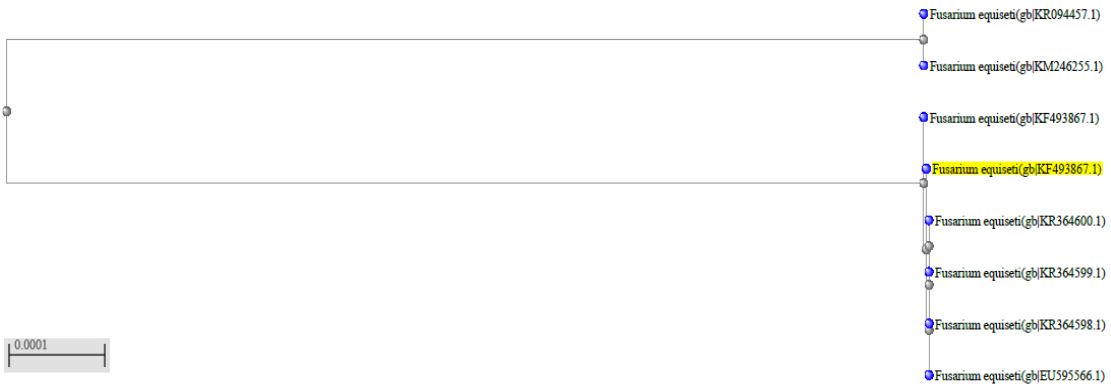
Potato Dextrose Agar medium was supplemented separately with 100ppm, 200ppm, 400ppm and 600ppm of $CoCl_2$, $ZnCl_2$, $CdCl_2$, $CuCl_2$ and sterilized at 121^0C for 15min. The culture grown on PDA supplemented with 50ppm of selected heavy metal were cut into 5mm discs and transferred to the respective plates containing different concentrations of heavy metals. The inoculated plates were incubated for two weeks at 37^0C in BOD incubator. The growth was periodically observed and the size of the colony was measured on 14^{th} day to determine the heavy metal tolerant potential of the fungal endophyte. The PDA medium without heavy metals was served as control. Out of thirteen tested fungal endophytes seven were found to have the tolerance to more than 50 ppm with a maximum of 600ppm. The growth of the colony decreased with the increase in concentration of heavy metals. When no apparent growth of the isolate was observed on the plates with an increase concentration, the metal concentration was considered as Minimum Inhibitory Concentration (MIC) of the particular heavy metal to the tested isolate. The level just below the MIC was considered as the highest metal concentration tolerated by that isolate.



Screening of *Fusarium sp* with different heavy metal concentrations namely 50ppm, 100ppm, 200ppm, 400ppm and 600ppm

Identification of Endophytic fungi:

Among thirteen fungal isolates *Fusarium* showed highest resistance towards heavy metals upto 200ppm in cobalt, 100 ppm in Zinc, 100 ppm in Copper and 50 ppm in Cadmium. The fungal colonies are white in colour and rapidly grow on PDA. *Fusarium species* is dense aerial, initially white or cream white. The colony appearance, conidia, and hyphal features of non-sporulating endophytic fungi (morphotypes) isolated from *Terminalia Pallida.* All the morphotypes isolated were then identified based on the 18S rRNA gene sequence analysis. The PCR amplification of 18S rRNA gene was done by using universal ITS1 and ITS4 primers. An amplified product was obtained for all the isolates. The fungal rDNA-ITS sequences of amplified products obtained in this study were deposited in checked in GenBank. The isolates that were obtained from this study along with the best BLAST results. Molecular identification using ITS rRNA sequences obtained from the non sporulating isolates revealed ten taxa of endophytic fungi.



CONCLUSION:

Endophytic fungi were isolated from *Terminalia Palida* an endemic plant of Tirumala hills. The forty isolated endophytic fungi were screened with 50ppm concentration of selective heavy metals out of forty thirteen isolates showed resistance to 50ppm. So, thirteen isolates were further screened to different concentrations ranging from 50ppm to 600ppm. Out of thirteen one isolate showed highest resistance to four heavy metals and was identified as *Fusarium sp.* by morphological and molecular identification. *Fusarium* showed highest resistance towards heavy metals upto 200ppm in cobalt, 100 ppm in Zinc, 100 ppm in Copper and 50 ppm in Cadmium. Further studies are necessary for the production of low cost biosorbents which is a economic, eco-friendly and efficient technique for the effective removal of heavy metals from polluted areas.

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