5. DISCUSSION

As a consequence of industrial progress and urbanization, heavy metal pollution has become one of the most serious environmental problems during recent years. In nature, heavy metals such as lead (Pb), mercury (Hg), cobalt (Co), cadmium (Cd), nickel (Ni), zinc (Zn), copper (Cu) and chromium (Cr) are present in very small amounts in soil and water bodies. But at certain concentration, they can be toxic to plants, animals, human beings and aquatic life. Pollution of heavy metals is important because they are non-biodegradable and indestructible. It is therefore, necessary to remove such metals from the environment (Ezaka and Anyanwu, 2011). As the heavy metals have toxic effects on human, animal and plant health as discussed in the section of introduction and review of literature it is essential to minimize heavy metal concentration.

The microbes play a major role in the bio-geochemical cycling of toxic heavy metals and also in cleaning up or remediating metal-contaminated environments. The improvisation of effective and cheaper methods for the remediation of heavy metals is therefore warranted. There are several approaches for heavy metal removal from the environment such as electrochemical treatment, ion exchange, precipitation, reverse osmosis, evaporation and sorption for heavy metal removal techniques because they can have lower cost and higher efficiency at low metal concentrations (Zaki et al. 2014). Microbial remediation or bioremediation has the advantages of removal of large amounts of heavy metal efficiently at a low cost. The microbe related technologies provide an alternative to conventional method of removal of the heavy metals as well as its recovery (Pandit et al. 2013). Among the microorganisms, fungi are very important for bioremediation due to their mycelial nature and well documented ability to accumulate metals of all kinds (Gadd, 1993). Nevertheless, some plant-associated fungi which can assist plants to accumulate higher amount of metals without increasing phytotoxity (Weyens et al. 2009). 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 (Valls and de Lorenzo, 2002).
Isolation of endophytic fungi from *Boswellia ovalifoliolata*, an endemic plant of Tirumala hills

Endophytic fungi which colonize inner plant tissue have been found to be associated with every plant species so far investigated (Padhi and Tayung, 2013; Rao and Sathish, 2015). Microbial endophytes are an eclectic group of microorganisms having the capability to chemically colligate the bridge between microbes and associated medicinal plants due to their relatively high metabolic versatility. The search for potent endophytic fungi has been focused on the isolation of species from unexplored niches and medicinal plants. In this backdrop, the present work has been initiated to exploit hidden potential of endophytic fungi from Eastern Ghats of India because of its rich microbial diversity which has been studied only a limited extent. The Tirumala hills represent rich flora with enormous species diversity as well as endemic taxa and are therefore recognized as Seshachalam biosphere reserve (Krishnaveni and Sirinivasa Rao, 2000; Arokiyaraj *et al*. 2008). Despite the reports of ethnomedicinal plants of this region, the biodiversity and the endophytic microbes of this region remain unexplored. Therefore, in the present investigation, *B. ovalifoliolata* representing endemic medicinal taxa was subjected to isolation of fungal endophytes.

In the present study, we first analysed the isolation of endophytic fungi from *B. ovalifoliolata* and then determined the best endophytic fungal candidates for phytoremediation of soil contaminated with the heavy metals such as Co, Cd, Cu and Zn. A total of 145 endophytic fungi belonging to 15 species were isolated and identified based on morphological and unique phenotypic characters. The identified species are *Coniochaeta* sp., *Blastomyces* sp., *Botryosphaeria* sp., *Chaetomium* sp., *Xylaria* sp., *Phomopsis* sp., *Trichoderma* sp., *Gliocladium* sp., *Trichophyton* sp., *Alternaria* sp., *Eutypella* sp., *Cladosporium* sp., *Malbranchea* sp., *Microsphaeropsis* sp. and *Fusarium* sp. Among the identified taxa, *Fusarium* was most dominant genus with relative colonization frequency of 4%, followed by *Eutypella* sp. (3.75%) and *Chaetomium* sp. (3.5%). The results were consistence with other endophytic fungal studies from several medicinal plants (Zakaria *et al*. 2010; Xu *et al*. 2011; Mishra *et al*. 2012; Zhang and Yao, 2015).

Eighteen species of endophytic fungi were isolated from bark, stem and leaf segments of five medicinal plant species growing within the Kudremukh range in the
Western Ghats of India. The dominant species were *Curvularia clavata*, *C. lunata*, *C. pallescens* and *F. oxysporum*. The greatest number of endophytic fungal species were found within *Callicarpa tomentosa* (11 species), whereas *Lobelia nicotinifolia* harboired the lowest number of fungal endophytes (5 species) (Raviraja, 2005). The study provides evidence that fungal endophytes are host and tissue specific.

Nearly sixty fungal endophytes belonging to 14 genera, out of which 31 endophytes (51.66%) were obtained as filamentous forms and 29 of them (48.33%) as yeast colonies. Species of *Curvularia*, *Fusarium*, *Alternaria* and *Penicillium* were isolated as dominant and host specific endophytes (Mohanta et al. 2008).

**Screening of endophytic fungal isolates against Cobalt (Co), Cadmium (Cd), Copper (Cu) and Zinc (Zn) metal resistance**

Our preliminary screening results revealed that five endophytic fungi *Xylaria* sp., *Cladosporium* sp., *Chaetomium* sp., *Eutypella* sp. and *Fusarium* sp. showed higher resistance against Co, Cd, Cu and Zn at 25 ppm. The colony growth was decreased with increase in the concentration of heavy metals from 50 to 600 ppm. Screening test revealed that maximum radial growth was noted in *Eutypella* sp. as 5.8 cm for Co(50ppm), 5.5 cm for Zn (50ppm), 4.6 cm for Cu (50ppm) and 4.6 cm for Cd (50ppm). In case of *Fusarium* sp. 5.7 cm for Co (50ppm), 5.2 cm for Zn (50ppm), 4.0 cm for Cu (50ppm) and 3.5 cm for Cd (50ppm) was observed. The maximum tolerance index was observed in *Eutypella* sp. against Co at 600 ppm, Cu at 600 ppm, Zn at 600 ppm and Cd at 400 ppm and *Fusarium* sp. for Co at 600ppm, Cu at 400 ppm, Zn at 600 ppm and Cd at 400 ppm.

Most studies have been undertaken on filamentous fungal strains including the members from the genera *Aspergillus*, *Fusarium*, *Humicola*, and *Nannizzia* have been reported to possess resistance against heavy metals (Iram et al. 2013; Ezzouhri et al. 2009; Valix et al. 2001). Recently, several studies have reported a similar trend among endophytic fungi being able to resist several heavy metals such as Cu, Zn and Cd (Hong et al. 2010; Salvadori et al. 2013; Deng et al. 2014). Joshi et al. (2011) reported 76 fungal isolates from sewage, sludge and industrial effluents. Four identified fungi screened for their tolerance to four heavy metals (Pb, Cd, Cr and Ni). By increasing the concentration of heavy metals
there was a decrease in the number of fungi showing tolerance to heavy metals. The fungi Aspergillus terreus, Trichoderma viride, Trichoderma longibrachiatum and Aspergillus niger showed tolerance to Pb, Cd, Cr and Ni. Sharon crane et al. (2010) found that 6 ectomycorrhizal fungi exposed to Hg. Radius of Amanita muscaria expanded slowly, Laccaria laccata, Pisolithus tinctorius and Suillus decipiens radius expanded quickly, Cenococcum geophilum and Piloderma bicolour showed slow growth. Biomass production of Laccaria laccata and Pisolithus tinctorius was reduced in presence of Hg. Amanita muscaria and Suillus decipiens fungi biomass production was not affected in presence of Hg and accumulated more Hg than Pisolithus tinctorius and Laccaria laccata.

Recently, several studies revealed that (Fazli et al. 2015) Aspergillus versicolor and Terichoderma sp. showed highest minimum inhibitory concentration and tolerance index to Cd where as Microsporum sp. and Cladosporium sp. was sensitive to Cd and mycelial growth was suppressed due to Cd toxicity. In a study by Jenny choo et al. (2015) ninety three endophytic fungi were isolated from Nypa fruticans. Preliminary screening revealed that 8 fungi showed resistance against Cu, Cr ,Pb and Zn. By increasing the concentration of heavy metals the growth of the isolates was decreased due to heavy metal toxicity. Eight fungal isolates were closely related to Pestalotiopsis sp. and showed tolerance against heavy metals Cu, Cr ,Pb and Zn. Rogelio et al. (2012) isolated Scleroderma citrinum from mining sites and Pisolithus tinctorius strains Pt1 and Pt2 from unpolluted sites. The biomass production of Scleroderma citrinum was increased in presence of Cd while Pt1 and Pt2 biomass was reduced in presence of Cd. Tolerance index of Scleroderma citrinum was higher when compared to Pt1 and Pt2.

In a study by Zafar et al. (2007), fungal isolates from waste water treated soil belongs to genera Aspergillus, Penicillium, Alternaria, Geotrichum, Fusarium, Rhizopus, Monilia and Trichoderma. MIC values suggest that the resistance level against individual metals was dependent on the isolates. Two isolates of the Aspergillus and Rhizopus showed highest level of tolerance against Cu, Cd, Cr, Co and Ni. Maximum biosorption of Cr and Cd ions was found in Aspergillus and Rhizopus.

A considerable amount of intra- and interspecific variability has been observed among the responses of ectomycorrhizal fungi to metals for example, a wide range of Zn
tolerance indices was reported among several strains of *Suillus luteus*, *Suillus bovines* and *Rhizopogon luteolus* using increase in fungal dry weight as a measure of tolerance (Colpaert et al. 2005). In a study by Blaudez et al. (2000b) when several strains of ectomycorrhizal fungi representing five species were screened using several metals the toxicity of Cd and Cu varied greatly among isolates of *Paxillus involutus*, as did the toxicity of Cd, Cu and Ni among isolates of *S. luteus*. Also, groups of strains of *S. luteus* and *Pisolithus tinctorius* demonstrated greater overall tolerance to Cu, Cd and Zn than *P. involutus*, while several strains of *P. involutus* were less affected by Ni than other ectomycorrhizal fungi.

Metal tolerance and metal resistance have come to be used almost synonymously. While tolerance signifies "the ability of an organism to survive metal toxicity by means of intrinsic properties and environmental modification of toxicity", resistance is "the ability of an organism to survive metal toxicity by means of a mechanism produced in direct response to the metal species encountered, for example, synthesis of metallothioneins" (Gadd, 1993). Microorganisms yield to the stress conditions and make suitable provisions for survival or attempting to resist the stress (Beales, 2004). They develop resistance mechanisms to avert the toxicity of metals. Resistance may be expressed in terms of phenotypic or genotypic changes. For most organisms, this tolerance can be pushed to maximum limits if the cell is provided with sufficient opportunity to sense and adapt to the extreme environment.

Metal toxicity, studied through the growth response of fungi in media with added metals, has been mostly studied as a complex physiological process which is in direct relation to the ability of fungi to colonize the substrate, to spread in the ecosystem and to exploit its resources (Baldrian and Gabriel, 2002; Colpaert and Assche, 1992). It has been reported that the mechanisms of filamentous fungal tolerance to heavy metal include the extracellular precipitation, cell-wall binding, efflux of intracellular heavy metal ions, intracellular chelation by intracellular ligands, sub cellular compartmentation and the protective role of antioxidant systems (Ma et al. 2011; Li et al. 2012).

The difference in metal tolerance may be due to the presence of various strategies of resistance mechanism exhibited by the fungi (Iram et al. 2013). Fungal resistance to heavy metals develops through various mechanisms such as active transport of metal ions outside the cell, masking metals by chelation, enzymatic transformation of metal ions, creating
vacuoles in which metal ions are gathered and immobilization in the form of polyphosphates, increased production of melanin and other pigments, and production of specific metal binding compounds inside the cell (Balamarugan et al. 2006).

It has been reviewed that the fungal detoxifying function and environmental tolerance are a particularly complex and poorly understood phenotype and many of the tolerance phenotypes are polygenic that involve distributed genes in the genome (Gong et al. 2009). Research on arbuscular mycorrhizal fungi has also indicated that the expression of genes for heavy metal tolerance varies with the heavy metals in different strains (Hildebrandt et al. 2007). Thus, we presumed that the different heavy metal tolerance of conspecific/ interspecific isolates reported in the present study might be the result of this, or there could be an unrevealed heterogeneity of the group masked by morphological and ITS sequence similarities.

**Identification of potential fungal endophytes by 18S rRNA sequence analysis**

In the present study the amplification of 18S rDNA of highly metal tolerant *Fusarium equiseti*, *Eutypella scoparia* revealed a nucleotide sequence homology of 99 % with closely related sequences retrieved from the NCBI GenBank. The Phylogenetic tree was constructed using the sequences of close relatives. Partial 18S rRNA sequences of the two isolates were submitted to NCBI Gen Bank and obtained the accession numbers for the two heavy metal resistant endophytic fungi viz., *Fusarium equiseti* (KT804648) and *Eutypella scoparia* (KT750889).

Our results are in line with previous reports the Cd, Pb and Zn resistant strain was identified as *Lasiodiplodia* sp. (99 % similarity) based on the ITS1-5.8S-ITS2 sequence analysis (GenBank accession number: JX308282) Zujun Deng et al. (2014).

The fungal endophytes *A. Tenuissima*, *C. Globosum*, *C. Lobatum*, *Dendryphion sp.*, *E. Nigrum*, *Colletotrichum sp.* , *H. Fuscoatra*, *Peyronellaea sp.*, *Plectosphaerella sp.* were identified based on ITS sequence analysis and the accession number of representative isolates as well as their most closely related species in GenBank (Xinya et al. 2016).
The nuclear ribosomal RNA (rRNA) cistron has been used for fungal identification for more than 20 years. The eukaryotic rRNA cistron consists of the 18S, 5.8S, and 28S rRNA genes transcribed as a unit by RNA polymerase I. Post transcriptional processes split the cistron, removing two internal transcribed spacers (ITS). These two spacers, including the 5.8S gene, are usually referred to as the ITS region (Schardl, 2001). The 18S rRNA gene is commonly used in phylogenetics, and although its homolog (16S) is often used as a species diagnostic for bacteria, it has fewer hypervariable domains in fungi. The 28S rRNA gene sometimes discriminates species on its own or combined with the ITS. Eukaryotic rRNA genes (known as rDNA) are found as parts of repeat units that are arranged in tandem arrays, located at the chromosomal sites known as nucleolar organizing regions (NORs) (Faeth and Fagan, 2002). Each repeat unit consists of a transcribed region (having genes for 18S, 5.8S and 28S rRNAs and of the external transcribed spacers ETS1 and ETS2 and a non-transcribed spacer (NTS) region. In the transcribed region, ITS are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary. Universal PCR primers designed from highly conserved regions flanking the ITS and its relatively small size (600-700 bp) enable easy amplification of ITS region due to high copy number (up to 30000 per cell) of rDNA repeats. This makes the ITS region an interesting subject for evolutionary and phylogenetic investigations (Faeth and Fagan, 2002).

**Scanning Electron Microscope (SEM) analysis to study the morphological changes in heavy metal treated fungi.**

Scanning Electron Microscope (SEM) studies revealed that there were remarkable changes in colony morphology of *E. scoparia* and *F. equiseti* under Co, Cd, Cu and Zn stress when compared with control. In response to Co stress hyphae became diverse in shape, tightly packed and shortly septated. Under Cu treatment the morphology of the hyphae was modified, mycelial shrinkage and agglomeration was observed. In Cd stress mycelia became rough, clustered and metal ions appeared as spot like particles on the surface. When treated with Zn aerial walls of mycelia became swollen, tight and deformation was observed.
A similar effect was observed in (Szczepan Zapotoczny et al. 2007) *Acremonium pinkertoniae* which is exceptionally tolerant to high concentration of copper. Morphology changes were observed under scanning electron microscope *A. Pinkertoniae* mycelia grown at excess Cu was changed significantly in comparison with mycelia grown under control condition. Conidia formation was inhibited due to Cu toxicity. Mycelia became tightly packed and shortly septated, hyphae changed in to chains of spherical spore like structures.

Scanning electron microscope images in control without heavy metal treatment the surface of the raw biomass was smooth and uniform with regular and plain structure. The surface of Cd and Cu loaded biomass was changed when compared with control. The surface of Pb loaded biomass was much rougher. The metal ions as spot-like particles distributed on the surface of the Cd and Pb loaded fruiting body, while extra flake-like substances distributed on the surface of the Cu loaded biomass (H. Huang et al. 2012).

*Fusarium solani* was found to tolerate a number of heavy metals and others, such as Pb, Cu, Hg, As, Cr, Al, Ni, Fe, Co, Mn, Zn and Li. Certain morphological changes, such as, increase in number of spores, thickened cell wall, bulbous hyphae and changes in the shape and size of the cultures in presence of metals were observed during growth of the culture in response to metal. Pigment production also played a role in higher tolerance to metals (Kowshik and Nazareth, 2000). Similarly, the changes in the morphology of fungi in presence of toxic concentrations of metals were observed by other workers (ATeribasi and Yetis, 2001; Vankuyk, 2004; Katarzyna, 2004).

Scanning electron microscope it is noted that *G. Cylindrosporus* fungi Pb stress caused substantial changes in hyphal morphology in contrast with mycelial morphology of control. Tight, twisting, looping of individual hyphae and formation of intertwined hyphal strands under Pb stress in *G. Cylindrosporus* (Ban Y et al. 2012).

The microbes which can resist metal go through a range of morphological and ultra structural changes. After intake of the metal, the toxic metal ion form complexes with cellular membrane. This causes the loss of its integrity and impairs its function. (Yilmazer and Saracoglu, 2009). It was reported that the morphology and physiology of the cell changes with increased concentration of metal (de Siloniz et al., 2002). In case of fungi,
mycelia become short, dense, and broken in the metal treated strains in comparison to the control strains. Improved aggregation of the fungal hyphae can be one of the morphological strategies in response to toxic metals (Gadd, 2007). Due to aggregation of hyphae the exposed surface area reduces and facilitates high local concentrations of extracellular products (organic acids and siderophores), metal precipitating agents, polysaccharides and pigments with metal binding abilities (Dutton and Enans, 1996). Twisting and looping of individual hyphae and formation of intertwined hyphal strands in response to cadmium stress and decreased overall mycelial length in response to Cd and Cu stress has been reported (Gadd, 2001).

**Fourier Transform Infra-Red (FTIR) spectroscopic studies to determine the functional groups involved in heavy metal absorption.**

In the present study FTIR analysis revealed that the resistance of *E. scoparia* towards multiple heavy metals (Co, Cd, Cu and Zn) is attributed due to the presence of functional groups such as flavonoids, polyphenolic compounds, polysaccharides and proteins and the resistance of *F. equiseti* is due to the presence of functional groups flavonoids, proteins, alkaloids and polysaccharides.

Other workers have also reported that endophytic fungi *Microsphaeropsis* sp. LSE10 was isolated from cadmium hyperaccumulator *S. nigrum* L. It showed higher biomass yield and was resistant to Cd. FTIR analysis the Carboxyl, amino, sulphonate and hydroxyl groups on endophytic fungi LSE10 were responsible for biosorption of Cd (Xiao Xiaoe *et al.* 2010). From FTIR analysis it is revealed that biosorption process of endophytic fungi *Lasiodiplodia* sp. was due to the functional groups of hydroxyl, amino, carbonyl and benzene ring on the cell wall (Zujun Deng *et al.* 2014).

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 sulphate which have high metal binding capacities and are believed to have a significant potential for metal binding (Davis *et al.* 2003).

Metal binding in fungi involves functional biomolecules. Initial cell surface binding of metals is considered to occur with proteins, lipids and different polysaccharides like
glucans, mannan, chitin and chitisan present on the cell wall (Korn and Northcote, 1960; Ruiz-Herrera, 1992). For the binding of metals during the biosorption, the multilaminate, microfibrillar cell wall structures containing a large number of functional groups as carbonyl, hydroxyls, amides are known to be responsible (Tobin et al. 1990; Akar et al. 2005). Role of different functional groups as well as cell wall components in total intake of metal ions by fungal biomass has been reported earlier by a host of researchers (Zhou and Banks, 1993;). FTIR spectral analysis suggested that OH, -NH, -C=O (broad and sharp peak) are the key binding sites for metals on the surface layer of tested fungal strains. Some other functional groups viz. CH, CH2, C-N, COO are also reflected to be involved in metal adsorption as indicated by the observed peak shifting in the regions denoting these functional groups in strains treated with metals. Similar shifting in peaks in fungal biomass treated with heavy metals have been reported earlier by a host of researchers (Das and Guha, 2009; Xu et al. 2012; Damodaran et al. 2013). First two research groups studied the peak shifts in filamentous fungi treated with Cr, while Damodaran et al. (2013) observed the effects of Cd, Pb, Zn, Cu and Cr treated groups of fungi. Use of FTIR to detect the presence of both primary and secondary stress factors have been cited earlier (Qian and Krimm, 1994; Yang et al. 1999; and Shi et al. 2002).

**Evaluating the effect of heavy metal resistant endophyte on growth and physiology of *M. dubia*.**

The findings of the study suggest that heavy metal tolerant endophytic fungi *E. scoparia* association with *M. dubia* significantly enhanced the number of leaves, shoot length, root length, shoot fresh biomass, root fresh biomass, shoot dry biomass and root dry biomass in endophyte inoculated plants (T2, T3, T4, T5) when compared with endophyte un-inoculated plants (T6, T7, T8, T9) under Co, Cd, Cu and Zn stress. We observed that endophyte association activated the chlorophyll, carbohydrate, starch and protein content which was significantly higher in endophyte inoculated plants (T2, T3, T4, T5) as compared to endophyte un-inoculated plants (T6, T7, T8, T9) under Co, Cd, Cu and Zn stress condition. Very little has been known about endophytic fungi *E. scoparia* and its role in host plant resistant to metal stress. The findings of the present study suggest that the endophyte *E.*
**Scoparia** not only improve plant biomass, chlorophyll, carbohydrate, starch and protein content but also resist to the toxic effect of metal contamination.

Similar work was reported by A.R. Khan *et al.* (2016) endophytic fungi were isolated from *Solanum nigrum*. The *S. nigrum* plants were inoculated with *Glomerella truncata* PDL-1, and *Phomopsis fukushii* PDL-10 fungi. The endophytic fungi inoculated plants exhibited increased chlorophyll content when compared with non-inoculated plants.

Nonetheless, few studies also support the results that the most tolerant endophyte *Paraphaeosphaeria*, was selected to inoculate corn seedlings and observed that the endophytic fungi enhanced the Cd translocation from root zone to aerial parts under high Cd stress, promoted plant growth. These findings indicated *S. variegata* harbours an endophytic fungal flora showing a high genetic diversity as well as a high level of metal resistance to Cd that has potential values in cadmium cycling and restoration of plant, soil and water system (Hongmei *et al.* 2015). The inoculation of endophytic fungi *Lasiodiplodia* sp. increased the biomass of *Brassica napus* L., translocation factor of Cd and the extraction amount of Cd by rape in the Cd and Pb contaminated soils (Zujun Deng *et al.* 2014).

In a study by Mi Shen *et al.* (2013) sixteen endophytic fungal strains were isolated from heavy metal contaminated sites. All isolates were identified to be the same species of *Peyronellaea* based on rDNA ITS sequence analysis. The heavy metal tolerance indexes of isolates decreased with increasing concentrations of Pb and Zn but for J934 and J97 isolates heavy metal tolerance and growth was stimulated. Endophyte inoculation increased the Pb, Zn and Cd content in the shoot and root of maize.

Endophytic yeast CBSB78 was isolated from rape. The strain was identified as *Cryptococcus* sp. based on the ITS1–5.8S–ITS2 sequence analysis (Zujun Deng *et al.* 2012). The endophytic yeast CBSB78 was resistant to Cd, Pb, Zn and Cu at different concentrations. When the strain *Cryptococcus* sp. was inoculated to *Brassica alboglabra* it increased the dry weight also increase the extraction amounts of Cd, Pb and Zn by *B. alboglabra*.

According to Abdul Latif Khan and In Jung Lee, (2013) endophytic fungi *Pencillium funiculosum* was isolated from soybean plant. The endophytic fungi *P. funiculosum* was resistant to Cu and Cd and showed higher metal accumulation. The heavy metal resistant
endophytic fungi (*Pencillium funiculosum*) association with soybean plants significantly increased the shoot length, shoot fresh biomass and root fresh biomass when compared with non-inoculated endophyte plants under Cu stress. Chlorophyll and protein content was significantly higher in endophyte inoculated plants as compared to non-inoculated endophyte plants under Cu stress condition. Cu tolerance rate was significantly higher in endophyte inoculated plants compared with non-inoculated endophyte plants.

Similar work by Mohsen *et al.* (2010) *Neotyphodium* endophytes infected in two grass species *Festuca arundinacea* and *Festuca pratensis* under Cd stress showed increased shoot, root and total biomass than endophyte free plants. Cd accumulation was higher in shoot and root of endophyte infected plants (*F. Pratensis* and *F. arundinacea*) compared with non-infected plants. Cd accumulation was higher in roots compared to shoot. The endophyte infected plants had higher potential to remove Cd from contaminated soil than non-infected plants.

Arbuscular mycorrhizal (*Glomus intraradices*) fungi was inoculated to *Medicago truncatula* under Cd, Pb and Zn stress (Paul Olivier *et al.* 2009). *Glomus intraradices* inoculated plants the shoot and root biomass was increased compared to non-inoculated plants. Cd and Zn content in shoot was increased in *Glomus intraradices* fungi inoculated plants when compared with non-inoculated plants.

Arbuscular mycorrhizal fungi inoculated *Medicago Sativa* L. plants showed significant increase in plant growth and biomass under Cd and Zn toxicity compared to non mycorrhizal inoculated plants. Chlorophyll content was higher in mycorrhizal inoculated plants compared to non inoculated plants (Sadie Kanwal *et al.* 2015). Previous studies have indicated that the association of fungal endophytes can significantly enhance plant growth, including biomass as well as production yield (Ahemad and Kibret, 2014; Hoffman *et al.* 2013).

**Inductively Coupled Plasma Mass Spectroscopy (ICPMS) analysis of heavy metals in endophyte inoculated and endophyte un-inoculated plant and soil**

The study showed that Co content in the shoots (17.2ppm) and roots (20.1ppm) of *M. dubia* inoculated with *E. scoparia* was significantly increased when compared with endophyte un-inoculated plants. Similarly Cd content in the shoots (6.4ppm) and roots
(8.3ppm) of *M. dubia* inoculated with endophytic fungi were significantly increased when compared with endophyte un-inoculated plants. Endophyte inoculated plants had significantly higher Cu content in shoots (9.3 ppm) and roots (11.2 ppm) than endophyte un-inoculated plants. Zn content of shoot (14.3ppm) and root (19.8ppm) of endophyte inoculated plants was significantly higher than endophyte un-inoculated plants.

In endophyte inoculated soil the Co content (540.3 ppm) was significantly decreased when compared with endophyte un-inoculated soil (561.8 ppm). Under Cd stress, endophyte inoculated soil the Cd content was significantly decreased (372.9 ppm) when compared with endophyte un-inoculated soil (385.7 ppm). Under Cu stress endophyte un-inoculated soil Cu content was significantly increased (581.5ppm) when compared with endophyte inoculated soil (563.3ppm). Under Zn stress, in endophyte un-inoculated soil Zn content was significantly increased (562.7 ppm) when compared with endophyte un-inoculated soil (543.9 ppm). The endophyte-plant relationship was a resembling model for endophyte assisted phytoremediation of heavy metal contaminated soils.

These findings are in agreement with those of previous studies (Anzhi *et al.* 2011) in which endophyte infection of host *Lolium arundinaceum* significantly increased the biomass under Cd stress condition. Endophytic infection increased Cd accumulation in *L. arundinaceum* and Cd transport from root to shoot was significantly higher when compared with endophyte free plants.

Similar effect was found in *Festuca arundinacea* and *Festuca pratensis* inoculated with Neotyphodium spp. fungal endophytes showed higher production of biomass and Cd accumulation than plants not inoculated with endophytes in Cd-contaminated soils (Soleimani *et al.* 2010a,b).

The inoculation of endophyte *Sphingomonas* SaMR12 improved the accumulation of cadmium and zinc in host plants (Chen, Shen, *et al.* 2014; Chen, Zhang, *et al.* 2014). Janouskova *et al.* (2006) found that Arbuscular mycorrhizal fungi are able to alleviate the unfavourable effects of Cd on plant growth by the process of phytostabilization. Compared with non-mycorrhizal plants, significantly lower amounts of Cd were found in the
mycorrhizal plants because Arbuscular mycorrhizal hyphae were able to accumulate 10–20 times higher rates of Cd relative to the plant roots.

It has been suggested that the growth-stimulated endophytic fungi under heavy metal stress were potential for phytoremediation and might be utilized as biosorbents for the detoxification of heavy metals (Li et al. 2012b; Deng et al. 2014).

The heavy metal content in the host plant tissues may be increased or reduced by inoculation of fungi (Shahabivand et al. 2012; Sousa et al. 2012), depended on the fungal species, and the types and concentrations of heavy metals. Zn content of the shoots and roots of maize inoculated with J934 was significantly increased compared with the un-inoculated treatments at the highest concentration of Zn (P < 0.05). Similarly, the Cd content in the shoots of maize inoculated with J934 and the Cd content in the roots of maize inoculated with L516 were significantly increased at the highest concentration of Cd (P<0.05).

It has been demonstrated that heavy metal tolerant fungi can provide a number of benefits to both the soil and the plant. They can enhance the efficiency of the phytoremediation process directly by altering the metal accumulation in plant tissues and indirectly by promoting plant growth and shoot and root biomass (Zarei et al. 2010; Miransari, 2011; Orlowska et al. 2011; Rajkumar et al. 2012).

Heavy metal resistant fungal endophytes possess well developed physiological and molecular mechanisms comprised of extracellular or intracellular detoxification system which are regulated properly to maintain heavy metals concentration during uptake in various cellular organelles (Zhao et al. 2015).

The mechanisms of metal mobilization and immobilization in fungi include heterotrophic solubilization, cell-wall adsorption, extracellular binding by polysaccharides, intracellular sequestration by metal-lothioneins and phytochelatins, vacuolar localization, etc (Gadd, 2000).

A possible mechanism might be that the fungal mycelium in the plant binds more heavy metals, and thus reduced the metal toxicity on the plant (Colpaert et al. 2011). In addition, ectomycorrhizal fungi can enhance the solubility of heavy metals and thus improve
heavy metal uptake by producing iron chelators, siderophores, organic acids and various degrading enzymes (Rajkumar et al. 2012).

It has been reported that the mechanisms of filamentous fungal tolerance to heavy metal include the extracellular precipitation, cell-wall binding, efflux of intracellular heavy metal ions, intracellular chelation by intracellular ligands, subcellular compartmentation, and the protective role of antioxidant systems (Ma et al. 2011; Li,Wei, et al. 2012).

During symbiotic interactions, the fungal hyphal network functionally extends the root system of the host plant in symbiosis with specific fungus, which has thus the potential to take up heavy metals from an enlarged soil volume (Gohre and Paszkowski, 2006; Ren et al. 2011). With the endophytic nature and resistance to multiple metals, E. scoparia should be a valuable microorganism resource for bioremediation of contaminated soils. The endophyte-plant relationship was a resembling model for endophyte assisted phytoremediation of heavy metal contaminated soils.