TO STUDY BIOREMEDIATION AND TOXICITY OF LEAD AND ARSENIC THROUGH ENTOMOPATHOGENIC AND ANTAGONISTIC FUNGI

Thesis

by

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CERTIFICATE-I

This is to certify that the thesis titled," **To Study Bioremediation and Toxicity of Lead and Arsenic through Entomopathogenic and Antagonistic fungi"** submitted in partial fulfillment of the requirements for the award of the degree **Master of Science** in the discipline of **Environmental Science** of Dr Yashwant Singh Parmar University of Horticulture And Forestry, (Nauni) Solan (HP)- 17230 is a bonafide research work carried out by **Ms. Shivani** daughter of Sh. Krishan pal under my supervision and that no part of this thesis has been submitted for any other dergree or diploma.

This assistance and help received during the course of investigation have been fully acknowledged.

Place: Nauni, Solan
Date: 25/10/2018

(Dr Anil Sood)
Major Advisor

CERTIFICATE-II

This is to certify that the thesis entitled, "**To Study Bioremediation and Toxicity of Lead and Arsenic through Entomopathogenic and Antagonistic Fungi**" submitted by **Ms. Shivani (F-2016-29-M)** D/o Mr. Krishan Pal to Dr Yashwant Singh Parmar University of Horticulture and Forestry, Nauni-173 230, Solan (HP), India in partial fulfillment of the requirements for the award of degree of M.Sc. Environmental Science has been approved by the Advisory Committee after an oral examination of the student in collaboration with the external examiner.

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Needless to say, errors and omissions are mine.

Place: Nauni, Solan
Date: 25/10/2018 **(Shivani)**

CONTENTS

ABBREVIATIONS

%	:	Per cent
&	:	And
/	:	Per
<	:	Less than
=	:	Equals to
>	:	Greater than
±	:	Plus-Minus sign
×	:	Multiply
≤	:	Less-than or equal to
µM	:	Micromolar
As	:	Arsenic
BOD	:	Biological Oxygen Demand
C	:	Carbon
Cd	:	Cadmium
cm	:	Centimeter
cm3	:	Centimeter cube
Cr	:	Chromium
CRD	:	Completely Randomised Design
Cu	:	Copper
Dr	:	Doctor
DW	:	Dry Weight
g	:	Gram
H_2O_2	:	Hydrogen peroxide
$HClO_4$	:	Perchloric acid
$HgCl_2$	:	Mercuric chloride
HNO_3	:	Nitric acid
Hrs	:	Hours
i.e.	:	That is
ICAP	:	Inductively Coupled Atomic Plasma
In vitro	:	Under Laboratory Conditions
Kg	:	Kilogram
M	:	Meters
M.W.	:	Molecular Weight
mg kg-1	:	Milligram per kilogram
ml	:	Millilitres
mm	:	Millimetre
mmh-1		Millimetre per hour
mM	:	Millimolar
Ni	:	Nickel
No.	:	Number
0C	:	Degree Celsius
Pb	:	Lead
pH	:	Potential of Hydrogen
ppm	:	Parts per million
psi	:	Pound square inch
spp.	:	Species
Viz.	:	Namely
Zn	:	Zinc

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Chapter-1

INTRODUCTION

Environmental pollution with heavy metals is increasing day by day and has become a major global concern because of its toxicity and threat to human life and environment (Mythili and Kartikeyan, 2011). Some heavy metals such as Co, Cu, Fe, Mn, Mo, Ni, V and Zn are required in minute quantities by organisms, however excessive amounts of these elements can become harmful to organisms. Lead (Pb), cadmium (Cd), mercury (Hg) and arsenic (As) are widely dispersed in the environment. These elements have no beneficial effects in humans and there are no known homeostasis mechanisms for them, thus are regarded as the "main threats" as they are very harmful to both plants and animals (Draghici *et al.,* 2010).

Pollutants of the biosphere with toxic metals have accelerated dramatically since the beginning of industrial revolution (Nriogo, 1979). According to Forstner and Prosi (1979), the harmful effects of heavy metal as pollutant resulted from incomplete biological degradation. The severity depends upon the concentration, accumulation and also bio-available forms. Human activities have contributed to elevated and toxic levels of these metals when compared to those contributed from geogenic or lithorlogical processes (Pam *et al.,* 2013). The various human activities through which heavy metals get into the environment includes smelting, mining, burning of fossil fuels such as coal, petrol, kerosene oil, discharging agricultural, industrial and domestic waste, discharge from auto exhausts, using pesticides containing compounds of heavy metals. Excessive levels of trace metals may also occur by geographical phenomena like volcanic eruptions, weathering of rocks, leaching into rivers, lakes and oceans due to action of water (Sankhla *et al,* 2016).

Aquatic animals, algae and hydrophytic weeds absorb heavy metals through their roots, shoots, leaves or through their body and function as a carrier (Jackson, 1998). As the macrophytes die and decay, the accumulated metal sink in the bottom sediment increases the concentration of metals. When agricultural soils are polluted, these metals are taken up by plants and consequently accumulate in their tissues and make them available to affect the environment through food chain (Sankhla *et al,* 2016).

Arora *et al*. (2008) reported that heavy metals have the ability to accumulate in different parts of human body. Heavy metal concentrations have the characteristics of having long biological half-lives as well as resistant to degradable processes and exhibit their chemical toxicity in soluble water. For this reason, the heavy metals are considered as a threat to human and other organisms even though when present in low concentrations.

Among various metals, lead and arsenic are considered potentially important environmental pollutants. Since these metals are non biodegradable, they persist and accumulate over a long period in the soils and vegetation resulting to serious environmental pollution. This calls for an increasing concern because the pollution may eventually result in negative influence on plants, animals and humans through food chain (Mtunzi *et al.*, 2015).

Lead is a dangerous element; it is harmful even in small amounts. Lead enters the human body in many ways; it can be inhaled in dust from lead paints or waste gases from leaded gasoline. High concentrations of lead in the body can cause death or permanent damage to the central nervous system, brain and kidneys. This damage commonly results in behavior and learning problems, memory and concentrations problems, high blood pressure, hearing problems, slow growth, digestive problems, muscle and joint pain (Sankhla *et. al*, 2016).

Arsenic is the twentieth most abundant element on earth and its inorganic forms such as arsenite and arsenate compounds are lethal to the environment and living creatures. Chronic As exposure leads to skin lesions, hyper pigmentation, keratosis, diabetes and cardiovascular disease. “Blackfoot disease” is a severe form of peripheral vascular disease, in which the blood vessels in the lower limbs are severely damaged, resulting eventually in progressive gangrene, discoloration and blackening of extremities. It had been observed in Taiwan which was caused by drinking water from deep artesian wells high in As (Chiou *et al.,* 2001). Millions of people in Bangladesh had been poisoned by consuming groundwater contaminated with high levels of As, sometimes as high as 800 ug/L (Anawar *et al*., 2011).

Bioremediation is an option that offers the possibility to destroy or render harmless various contaminants using natural biological activity. As such, it uses relatively low- cost, low technology techniques which generally have public acceptance and can often be carried out on site. Bioremediation is the use of living organisms, primarily microorganisms, to

degrade the environmental contaminants into less toxic forms. It uses naturally occurring bacteria and fungi or plants (phyto-remediation) to degrade or detoxify substances hazardous to human health or the environment (Mueller, 1995). At present, bioremediation is the most effective management tool to manage the polluted environment and recover contaminated environment.

Miransari (2011) reported that hyper accumulating plants and arbuscular mycorrhiza (AM) fungi have some unique abilities, which make them suitable to treat heavy metals. This new technology held promise for removing heavy metals and hydrocarbon contaminants from the land by channeling them to the fruit bodies for removal (Okhuoya, 2011).

Myco-remediation is a process of using fungi to return an environment contaminated by pollutants to a less contaminated state (Asiriuwa *et al*., 2013). Fungi are a diverse group of organisms and are ubiquitous in the environment. Fungi are capable to grow under environmental conditions of stress like environment with low pH values, poor in nutrients and with low water activity. Myco-remediation is a cost effective technique, a natural process and does not usually produce toxic by-products and a permanent solution as a result of complete mineralization of the contaminants in the environment (Thenmozhi *et al*., 2013). Most studies have revealed that mushrooms can bio-accumulate heavy metals from metal contaminated soils and they are purifier of oil contaminated soils, for example *Galverina vittiformis* can remove Cu, Cd, Cr, Pb and Zn (Singh and Gauba, 2014).

Entomopathogenic fungus is a fungus that can act as a parasite of insects and kills or seriously disables them. The entomopathogenic fungi particularly *Beauveria bassiana* and *Metarhizium anisopliae* are most promising biocontrol agents against a great number of agricultural insect pests and they also have ability to inhibit growth of several phytophagous fungi. They are widely used for the bioremediation of heavy metal contaminated soils (Akello *et al.*, 2009). Entomopathogenic fungi have ability to uptake heavy metals and metabolise them. Some copper forms like copper hydroxide and copper oxychloride are less toxic to *Beauveria bassiana*, with respect to reduced fungal growth, sporulation and conidial germination and are most accumulated by this species (Martins *et al*., 2012). *Beauveria bassiana* fungi is able to reduce copper levels in soil to some extent, highest per cent reduction 21.89 % is observed in 200 ppm copper.

Antagonistic fungi are those fungi which suppress or interfere with the normal growth and activity of a plant pathogen. These organisms are used for pest control and are referred to as “Biological Control Agents”. *Aspergillus* and *Trichoderma* species are antagonistic fungi which are used for remediation of heavy metals in soil. Faster metabolic rates, anti-microbial metabolites and physiological conformation are key factors which chiefly contribute to antagonism of these fungi. Fungal strains like *Aspergillus awamori, A. flavus, Trichoderma viride* are tolerant to heavy metals like Pb, Cd, Cr and Ni and able to tolerate up to 400 ppm concentration of these heavy metals (Joshi *et al*., 2011).

Keeping in view the above facts, the present study was undertaken with the following objectives:

i) To study toxicity of Pb and As for entomopathogenic and antagonistic fungi under *in-vitro* conditions.

ii) To study bioremediation of Pb and As through entomopathogenic and antagonistic fungi under *in- vitro* conditions.

Chapter-2

REVIEW OF LITERATURE

Heavy metals are natural constituents of the earth's crust but human activities have drastically altered their geochemical cycles and biochemical balance. Heavy metal pollution is responsible for several environmental problems such as decreased soil microbial activity, fertility and yield losses as well as risks to the human health. To improve soil fertility, management of heavy metal contaminated sites is becoming more challenging for bioremediation. Bioremediation is a cleanup technology making use of living organisms or microorganisms (bacteria, fungi, algae etc.) for treating environment contaminants particularly heavy metals in soil, ground water and industrial waste. Many entomopathogenic fungi which are promosing biocontrol agents against several insect pests and fungal pathogens have potential for removal of heavy metals. Keeping these points in mind, the literature pertaining to the present investigations is reviewed under the following headings:

2.1 To study toxicity of Pb and As for entomopathogenic and antagonistic fungi under *in-vitro* conditions

2.2 To study bioremediation of Pb and As through entomopathogenic and antagonistic fungi under *in- vitro* conditions

2.1.1 TO STUDY TOXICITY OF Pb AND As FOR ENTOMOPATHOGENIC AND ANTAGONISTIC FUNGI UNDER *in-vitro* CONDITIONS

Somashekar *et al*. (1983) assessed the comparative toxicity of heavy metals such as Cu, Co, Ni and Zn to some plant pathogenic and non-pathogenic fungi. The growth of mycelia in substrate treated with metals remained dose dependent and at higher concentrations total inhibition was noticed. Sporulation was delayed in all cases, although there occurred a vigourous growth of mycelia after a few days of incubation.

Sharouny *et al*. (1988) examined the changes in the composition of the soil mycoflora after soil treatment with 500, 2000 and 5000 ppm of mercury, zinc, lead, copper, nickel and cadmium on Czapek's glucose agar at intervals up to15 weeks. Treatment of soil with mercuric chloride and lead nitrate significantly reduced the fungal population and counts for

soil treated with zinc or copper sulphate and with nickel or cadmium chloride counts were increased on some treatments and reduced on others. When heavy metals incorporated into the isolation medium, they depressed the total count of fungi, as well as affecting individual species.

Levinskaite (2001) examined the influence of cadmium, nickel and zinc on the development of the fungi *Penicillium atramentosum* and *P. funiculosum.* All tested stages i.e. spore germination, germ tube growth rate and conidiogenesis were affected. Both fungi at their tube emergence stage were most susceptible to the metals than the other periods of their development. *P. funiculosum* was more resistant towards the tested metals at all stages of development.

Turnau *et al*. (2002) studied the role of ecto- mycorhizal fungi in zinc waste restoration in Poland and found that fungal species viz. *Chroogomphus rutilus, Suillus luteus* and *Rhizopogon roseolus* were symbiotic associator with *Pinus sylvestris and P. nigra* and *Lactarius pubescens, L. torminosus and Leccinum scabrum*, respectively. Pines (*Pinus sylvestris and P. nigra)* and birch were able to grow in sites contaminated with zinc due to the symbiotic association.

Oremland *et al.* (2002) isolated a facultative chemoautotrophic bacterium, strain MLHE-1 from arsenite-enriched bottom water from Mono Lake, California, that oxidized As (+3) [more toxic] anaerobically to As (+5) [less toxic], using nitrate as terminal electron acceptor. This organism was also able to grow heterotrophically with acetate as carbon and energy sources and oxygen (aerobic growth) or nitrate (anaerobic growth) as terminal electron acceptors.

Baldrian (2003) suggested that white rot fungi (*Daedalea quercina, Ganoderma applanatum, Stereum hirsutum* and *Schizophyllum commune*) require trace amounts of essential heavy metals such as Cd, Mn, or Zn for their growth, but these metals are toxic when present in excess. Toxic heavy metals can inhibit the growth, cause morphological and physiological changes and affect the reproduction of *Basidiomycetes.* Fungal species and strains differ in their sensitivity towards metals and in the protection mechanisms involved. The toxicity of some heavy metals such as Hg, Cu and Ni has been used for the development of antifungal preservatives.

Yilmaz *et al.* (2003) conducted a study on edible, inedible and poisonous macrofungi collected around the Balkykesir- Manisa highway, Turkey from two different areas (roadside and background area) in 1998-1999. Cu, Zn, Fe, Mn, Co, Cd, Ni and Pb contents were determined by atomic absorption spectrophotometer in 256 samples belonging to 24 macrofungi species. According to mean dry weight Mn, Co, Fe and Cd contents were high in *Omphalotus olearius,*which is a poisonous macrofungus species compared to the others; however, Fe levels were also extremely high. The lowest Cu, Mn and Fe contents were found in *Laetiporus sulphureus* (an edible macrofungus). The highest Pb and Zn contents determined in *Lycoperdon perlatum* were 6.5 mg/kg and 274 mg/kg, respectively. The contents of Ni and Cd seemed to be lower near the road.

Fomina *et al*. (2005) conducted a study on *Beauveria caledonica* and found it as highly tolerant to toxic metals and solublized cadmium, copper, lead and zinc, converting them into oxalates. In a study by Romero *et al.* (2006), fungal strain *Talaremyces helices* (efficient to degrade biphenyl) was treated with high copper levels and become tolerant to cobalt, lead and cadmium when cultured in their presence. This strain produced 4-hydroxy-BP as the only metabolite. The growth rates of fungus were initially inhibited at 150 ppm, being the no treated strains more sensitive. With 400 ppm Cu^{2+}, the no training biotypes develop, whereas the treated ones exhibited growth even at 600 ppm Cu^{2+}. The comparative toxicity of the studied metals to non trained *T. helices* followed the sequence $Co^{2+}>Cd^{2+}>Cu^{2+}>Pb^{2+}$, but their relative toxicities were independent of the ion sizes and membrane penetration, as their no hydrated ionic radii are 0.069, 0.072, 0.114 and 0.072, respectively.

Mateos *et al.* (2006) proposed that *Corneybacterium glutamicum,* which is gram-positive with a thick cell wall, be used to accumulate As (3+) [when As (+5) is reduced to As (+3)] as a means to clean up As in water. *C. glutamicum* can tolerate up to 12 mM As (+3) and more than 400 mM As (+5).

Ebong *et al.* (2008) determined contents of Ni, Cu, Pb, Mn, Cd and Zn in edible fungal sporocarps viz., *Armillariella mellea, Pleurotus sapidus* and *Agaricus biosporus* in soil from the Niger delta wetlands, Nigeria. A species-dependent bioaccumulating potential was observed. *Armillariella mellea* had the highest content of Zn and Pb i.e. 74.92 and 1.21ug/g respectively, while *Pleurotus sapidus* had the lowest bioaccumulating potential of 0.66, 0.95 and 19.45 ug/g respectively for Ni, Pb and Cu. Generally, the heavy metal

accumulating potential decreased in the trend: Zn>Mn>Cu>Ni>Pb>Cd and were inferior to the FAO/WHO (1976) dietry standards. The presence of detectable amounts of Pb and Cd in the study may be a pointer to health risk associated with excessive consumption of *Agaricus biosporus*, particularly during harvest periods and should be avoided.

Mondal *et al.* (2008) reported that three strains of bacteria *Ralstonia eutropha*, *Pseudomonas putida* and *Bacillus indicus* showed that these were able to remove arsenic (67%, 60% and 61%, respectively) from waste water.

Chitpirom *et al.* (2009) in Thailand, isolated arsenic resistant bacteria from tannery effluent and agricultural soils that belonged to *Klebsiella, Pseudomonas, Comamonas* and *Enterobacter* genera with the MIC of 40 mM (arsenite) and 400 mM (arsenate).

Ezzouhri *et al.* (2009) isolated and screened thirty-six microorganisms (fungi and yeasts strains) from heavy metal contaminated site at Morocco, Spain belonging to genera *Aspergillus, Penicillium, Fusarium, Alterneria* and *Geotrichum* for their resistance to heavy metals viz. Pb, Cu, Zn, Cr and Cd. Majority of the isolates were resistant to Pb, Cr, Cu and Zn, but in Cd contained sites only the fungus *Penicillium* sp. was able to grow. Minimum inhibitory concentration (MICs) for Pb2+, Cr6+, Cu2+ and Zn2+ were also determined. *Aspergillus* and *Penicillium* isolates were the most tolerant to the heavy metal exhibited strong growth, often exceeding the control (isolates grown in agar medium without heavy metals). Their MIC ranged from 20-25 mM for lead, followed by 15-20 mM both for Cu, and Zn and 10-15 mM for Cr. These fungi have shown a high level of resistance to all metal tested, which makes them attractive potential candidates for further investigations regarding their ability to remove metals from contaminated waste waters.

Sohailbani (2011) isolated number of heavy metal tolerant fungi from the municipal waste water treatment plant from Riyadh, Saudi Arabia. *Aspergillus niger* and *Penicillium chrysogenum* showed tolerance to copper, cadmium, lead upto 800 ppm concentration. *A. niger* had maximum resistance while *P. chrysogenum* was least tolerant particularly for cadmium. Both fungal species were able to adapt to high concentration of heavy metal and had potential for use in bioleaching of heavy metals.

Bukhari *et al.* (2012) studied the effect of heavy metals viz. sodium arsenate, lead nitrate and mercuric chloride at 100, 200, 500, 1000uM concentrations on *in-vitro* growth

profiles of the mutant strains of *Duddingtonia flagrans* (nematode trapping fungus) and *Verticillium chlamydosporium* (egg parasitic fungus) in Chattisgarh, India. No apparent inhibitory effect of sodium arsenate and lead nitrate on both of the mutant strains of *D. flagrans* and *V. chlamydosporium* was observed, whereas, mercuric chloride inhibited the growth. The study revealed that the mutant strains of *D. flagrans* and *V. chlamydosporium* could easily be deployed in the environment contaminated by industrial toxicants like arsenic, lead, mercury.

Sharma *et al.* (2012) conducted a study on five edible macro fungi namely *Lentinus sajor-caju, L. conatus, L. torulosus, L. cladopus and L. squarrosulus* in Northern India for the presence of toxic heavy metals viz., arsenic, lead, silver, mercury and antimony. All the species had negative results for the presence of heavy metals. Although higher concentration of cadmium and mercury were noted in wild species than cultivated species of mushrooms.

Jacob *et al.* (2013) isolated filamentous fungi viz., *Aspergillus, Fusarium* and *Penicillium* from sea water located near industrial areas in Manglore, Karnataka and screened them for resistance to Pb and Se. All the isolated strains were tolerant to lead and selenium (upto 20 mg/l) but the level of tolerance varied in the isolates from the same source. *Aspergillus* and *Penicillium* isolates were the most tolerant to lead and selenium and exhibited strong growth which make it an attractive potential candidate for further investigations.

Mejdad (2013) studied the effect of Cu, Mg and Zn on the fungal pathogens viz., *Aspergillus niger, Candida albicans* and *Cryptococcus neoformans* which were collected from the waters of Shatt al-Arab River, Iraq under laboratory conditions in liquid and solid media. In liquid media, Cu, Zn ions inhibited growth of *A. niger* whereas, Mg ion increased the growth, Copper ion (40ppm) increased growth of *C. neoformans* as compared with control. The metals decreased the colony number of fungal strains, whereas, Mg increased the number of colonies of *A. niger* during 24 hrs in solid medium.

Hassn *et al.* (2014) studied the effect of heavy metal ions viz., Cd, Cu, Fe, Pb and Zn on the growth and sporulation of the entomopathogenic fungi *Isaria javanica* (Syn. *Paecilomyces javanicus*) under laboratory conditions in Iraq, added into culture media in three concentrations: A-Conc. corresponding to the mean content of that metal in soils of Duhok, Northern of Iraq, B- Conc. 10 times higher and C-100 times higher than the mean

ones. The fungus had low sensitivity to metal present in the medium at the A concentration, whereas it was unable to grow on the media with highest concentration (C). Cu and Zn strongly inhibited fungal growth over 10 time higher level of individual metals in the soil.

Ramesh *et al.* (2014) isolated heavy metal tolerant filamentous fungi *Aspergillus species* from automobile industry waste disposal area, Tamilnadu, India and evaluated its isolates for metal tolerance and growth efficiency in the presence of heavy metals viz. Cr, Ni, Zn and Fe. The growth rates of *Aspergillus* species were slightly higher at 0.5% of all the metals but the concentration increased from 0.5-3% growth rates significantly declined with no growth at 2.5%(Cr), 55%(Fe), 2.5% (Ni and Pb).

Min *et al*. (2015) reported that *Schizopora paradoxa KUC8140* is a white rot wood degrader which has been reported in Korea as tolerance of heavy metals and polycyclic aromatic hydrocarbons and dye de-colorization activity, make this strain a potential candidate for mycoremedation.

Iram and Abrar (2015) isolated heavy metal resistant fungi from waste water treated soil samples of Hudiara drain, Lahore. Biosorption capacity of *Aspergillus flavus* and *Aspergillus niger* highly tolerant fungus was checked against Cu (ll) and Pb (ll), respectively. The biosorption capacity of *A. flavus* was 20.75-93.65 mg/g for Cu (ll) with initial concentration 200-1400 ppm, whereas the biosorption capacity of *A. niger* for Pb (ll) ranged from 3.25-172.25 mg/g with same range of initial concentration.

Tanneru and Gawai (2016) subjected five test *fungi Aspergillus flavus, Aspergillus niger, Cladosporium herbarum, Curvularia lunata and Trichoderma viride* to grow in mercury containing basal medium and level of tolerance was determined at 10, 20, 30, 40 and 50 ppm concentration. Dry weight and total protein content in their culture filtrate were estimated. It was observed that mercury was highly toxic to the fungi, it caused inhibition of growth. Maximum reduction in growth was observed in *Cladosporium herbarum and Curvularia lunata*. Sporulation was found to decrease in the presence of mercury. Maximum decrease in the protein content was observed at 50 ppm concentration.

Oladipo *et al*. (2018) reported that *Trichoderma ghanense* exhibited high tolerence to Cd, Cu, Pb and As heavy metals at different concentrations than *Fomitopsis meliae* isolated from gold and gemstone mining sites.

2.1.2 TO STUDY BIOREMEDIATON OF Pb AND As THROUGH ENTOMOPATHOGENIC AND ANTAGONISTIC FUNGI UNDER *in-vitro* CONDITIONS

Microbial metal bioremediation is an efficient strategy due to its low cost, high efficiency and eco-friendly nature. Microbial populations are known to affect the mobility of heavy metals and availability to the plant through the release of chelating agents, acidification, phosphate solubilisation and redox changes (Abu Shanab *et al*., 2003). Use of microbes for metal removal has been reported to be more efficient than chemical and physical methods, since they have evolved resistance mechanisms to avoid metal toxicity. Besides this, they transform the metals to reduce (even increases) their availability for them as well as for other organisms.

Kapoor *et al*. (1998) evaluated the potential of the fungus *Aspergillus niger* collected from the American Type Culture Collection (ATCC) Rockville, USA to remove heavy metals viz., Pb, Cd, Cu and Ni ions from waste water. The study showed that fungal biosorption had a potential to be used in the removal of heavy metal ions from waste waters.

Ropek and Para (2003) reported the toxicity of heavy metal ions to entomopathogenic fungi. Derivatives of starch dialdehyde viz., dihydrazone (DHZ), dithiosemicarbazone (DTSC) and Dioxime (DOX) were used to form water-insoluble complexes with heavy ions which provided protection to entomopathogenic fungus *Paecilomyces farinosus* (Polish strain) from the toxic effects of heavy metal ions. The metal complexes of DHZ, DTSC and DOX were non-toxic to *Paecilomyces farinosus* and could protect entomopathogenic fungi from the toxic effects of metal ions.

Karunasagar *et al*. (2003) studied the sorption capacities of *Aspergillus niger* against mercury. Both live and inactivated microbial biomass has been used in studies for removal of toxic metal ions. The study revealed that *A. niger* or other related fungi are attractive biological materials, which could have potential use in decontaminating materials polluted by mercury compounds.

Ridven (2003) reported that fungi *Penicillium purpurogenum* has bioadsorption capacity of heavy metal Pb, Cd, As and Hg and found metal accumulation in order Pb (II)> Cd(II)> Hg(II)>As(III).

Bishoil and Garima (2004) reported that the common filamentous fungi can sorb heavy metals viz., Zn, Cd, Pb, Fe, Ni, Ag from aqueous solutions to varying extents. Fungal biosorption largly depends on parameters such as pH, metal ion and biomass concentration, physical or chemical pretreatment of biomass and presence of various ligands in solution. When compared with commercial ion exchange resins, carbons and metal oxides, fungal derivatives generally perform well. Biomass can be regenerated by using various eluants. The availability of variety of biomass and their metal binding potential makes it a economical and sustainable option for developing effluent treatment process for removal and recovery of heavy metals.

Samal *et al.* (2004) reported that among different algal strain blue green algal species *Oscillatoria-Lyngbya* mixed culture showed maximum efficiency in removing 64% Arsenic (V) and 60% Arsenic (III) after 21 days incubation from 0.1 mg/L arsenic (III) enriched medium.

Jabbour and Barbercheck (2009) studied the effects of soil management on entomopathogenic fungi viz., *Metarhizium anisopliae, Beauveria bassiana, Isaria fumosorosea* and *Isaria farinosa* for organic production of food grains rotation in Central Pennsylvania. *M. anisopliae* detection was negatively associated with soil moisture, organic matter and Zn, S and Cu concentration in the soil and it was the most important fungal species.

Su *et al*. (2010) conducted studies on bioaccumulation and biovolatilisation of Arsenic contaminated soil in Chenzhou city, China. They reported that highest level of Arsenic (2500 µg) was accumulated by *Penicillin janthinellum* after 10 days and by *Fusarium oxysporum* (304.06 µg) after 15 days. *Trichoderma aserellum* and *Fusarium oxysporum* showed superior abilities for the absorption and accumulation of Arsenic.

Iskander *et al.* (2011) obtained isolates of filamentous fungi from sediment of the Langat River, Selangor, Malaysia and screened them for their tolerance and uptake capability of copper (Cu) and lead (Pb). Isolates viz., *Aspergillus niger, Aspergillus fumigates, Trichoderma asperellum, Penicillium simplicissimum, Penicillium janthinellum. A. niger* and *P. simplicissimum* were able to survive at 1000 mg/L of Cu(II) concentration, while only *A. niger* was able to survive at 5000 mg/L Pb concentration. *A. niger, P. simplicissimum* and *T.*

asperellum have a better uptake capacity for Pb compared to Cu and they showed promising bio-sorption of Cu and Pb.

Microorganisms including fungi have been reported to exclude heavy metals from waste water through bioaccumulation and biosorption at low cost and in eco-friendly way. Upto 76 fungal isolates including *Aspergillus awamori, A. flavus* and *Trichoderma viride* tolerant to heavy metals like Pb, Cd, Cr and Ni were isolated from sewage, sludge and industrial effluents containing heavy metals. The majority of the fungal isolates were able to tolerate up to 400 ppm concentration of these metals (Joshi *et al*., 2011).

Shrivastava *et al*. (2011 a) conducted study on *Trichoderma* spp. which has highest arsenic tolerate ability. This fungus was effectively removed from arsenic contaminated agriculture soils. They also found that *Trichoderma* strain *FA-06* was able to sporulate well on the arsenate exposure as compared to other T*richoderma* strains.

Maity *et al.* (2011) reported that bacterial cultures were efficient in arsenic removal. They investigated As (V) reduction characteristics of two different bacteria namely *Pseudomonas stuzeri* and *Bacillus cereus* and found 500 ppb As (V) was completely reduced to As (III) by *Bacillus cereus* and *Pseudomonas stutzeri* in 114h and 120h, respectively.

Gautam *et al.* (2011) reported two arsenic tolerant strains of fungi ASC1 and ASB 3 (isolated from Dhapa soil) in Kolkata. These two strains were identified as *Aspergillus flavus* (ASC1) and *A. niger* (ASB3), ASC1 was more arsenic tolerant than ASB3, as Minimum Inhibitory Concentration (MIC) of arsenic for ASC1 was estimated to be around 2000 ppm, whereas, for ASB3 it is 1200 ppm. Similarly ASC1 is found to be resistant against Cd, Pb and Cr, while ASB3 is resistant for Hg and Zn.

Srivastava *et al.* (2011 b) detailed arsenic removal potential of *Trichoderma* and *Aspergillus* sp. isolated from arsenic contaminated sites of West Bengal. These species were identified tolerant up to 5000 mg/L of arsenic.

Edith *et al*. (2012) isolated *Beuveria bassiana* from vineyard soil of Switzerland that was treated for centuries with copper based pesticide agents and it suddenly developed resistance towards high concentrations of copper ions, thus making it suitable agent for bioremediation.

Martin *et al.* (2012) evaluated the relationships between copper tolerance, solubilization and bioaccumulation in the entomopathogen *Beauveria bassiana* exposed to Bordeaux mixture copper hydroxide and copper oxychloride. Bordeaux mixture was highly detrimental to fungus as it inhibited the growth totally at the recommended dose. Whereas, copper hydroxide and copper oxychloride were found to be less toxic, reducing fungus growth, sporulation and conidial germination in an average of 29, 30 and 58 per cent, respectively. These two copper forms were the easiest to solubilize, to participate and the most accumulated by *Beauveria bassiana.*

Smridhi and Usha (2012) conducted studies on bioremediation of tannery effluent contaminated soil of Coimbatore, India. The soils were found to have higher pH and large amount of total suspended and total dissolved solids, minerals and metals like Na, K, Cr, Zn and Cu. The isolated *Bacillus sp*. was found to reduce 85.90% of Cr from the medium after 96 hours. The results also showed that the isolated Bacillus sp. has capacity to remove other heavy metals (Ni, Cr, Cu, Zn and Cd) in the tannery effluent. The metal removing capacity increased with increase in concentration of heavy metals.

Sharaf and Alharbi (2013) isolated seven fungi species belonging to three genera viz. *Aspergillus candidus, Aspergillus carneus, Aspergillus flavipes, Aspergillus flavus* var *columnaris, Aspergillus unguis, Cephalosporium curtipes* and *Cylindrophora hoffmanni* from soil of the industrial city at Al-Madinah Al-Munawarah Saudi Arabia. The soils have a long-term application of untreated industrial effluents. The isolated species were *A. candidus, A. carneus, A. flavipes, A. flavus var columnaris, A. unguis, C. curtipes* and *C. hoffmanni. Aspergillus*, the most dominant, accounted for 95.1% of the total count and was represented by five species. *A. candidus* was the most prevalent species on the isolation plates (37.2 % of the total count) followed by *A. flavus* and *A. unguis*. The isolated fungi were investigated for their potential to remove heavy metals from waste waters effluent of tanning leather industry. The metals had high significant metal sorption capacity which was species and metal dependent. Almost all the fungi showed more affinity to Pb^{2+} than Cr^{6+} and Sr^{2+}. The most dominant *A. candidus* on the isolation plates exhibited the highest activity for biosorption of heavy metals.

Shazia *et al.* (2013) conducted a biosorption study on the various isolates of highly tolerant fungal species, *Aspergillus fumigates* which were isolated from polluted soil in kasur district, Pakistan against metals viz. lead (Pb), chromium (Cr), Cadmium (Cd), Nickel (Ni),

Copper (Cu) and Zinc (Zn) at 200ppm, 400ppm, 600ppm and 800ppm, respectively. The highest biosortion value (76.07) was exhibited by *A. fumigates* isolate K3 for Pb, followed by Cu (69.6) and Cr (40.0) at 800 ppm metal concentration.

Babu *et al*. (2014) have successfully isolated *Trichoderma virens* named *PDR-28* and found MICs (Minimum inhibitory concentrations) for 1300mg of Arsenic, 2500mg of Pb indicated the heavy metal adaptation stress. Difference in tolerance may reflect different adaptation strategies or mechanisms involving permeability barriers, Intra and extracellular sequestration, efflux pumps, enzymatic detoxification and metal speciation.

Shukla and Vanker (2014) studied the role of *Trichoderma* species in bioremediation of hexavalent chromium present in tannery effluents and reported through Fourier Transform Infrared Spectroscopy (FT-IR) analysis that chromium binding sites in the fungal cell surface were carboxyl and amino groups (most likely). The fungal surfaces showed efficient biosorption for chromium in Cr^{6+} oxidation state. Best results for sorption were obtained at 5.5-5.8 pH, at low or high pH values; Cr (VI) uptake was significantly reduced.

Chang (2015) studied the role of arsenite oxidase enzyme to convert arsenite (more toxic form) to arsenate (less toxic) in some bacteria like *Alcaligenes faecalis*, *Pseudomonas arsenitoxidans* and *Centribacterium arsenoxidans*, which are chemolithotrophic arsenic oxidizing bacteria.

Chen *et al*. (2017) found that plant *Oryza sativa* L. is resistant to both forms of arsenic i.e. arsenite and arsenate and can be used for their phytoremediation on contaminated sites.

Chapter-3

MATERIAL AND METHODS

The present study entitled "**To study bioremediation and toxicity of lead and arsenic through entomopathogenic and antagonistic fungi**" was carried under laboratory conditions of Department of Environmental Sciences, Dr. Y S Parmar University of Horticulture and Forestry, Nauni-Solan, H.P. during 2016-2018. Material used and methodology employed during the present investigations are discussed in this chapter.

3.1 COLLECTION OF FUNGAL STRAINS

The pure culture of fungal strains viz. *Aspergillus niger*, *Trichoderma harzianum*, *Beauveria bassiana* and *Metarhizium anisopliae* were collected from Department of Plant Pathology and Department of Entomology, Dr Y S Parmar University of Horticulture and Forestry, Nauni-Solan, H.P. The fungal strains were then sub-cultured under laboratory conditions of Department of Environmental Science, Dr. Y S Parmar University of Horticulture and Forestry, Nauni-Solan, H.P. on potato dextrose agar media.

3.2 PREPARATION OF POTATO DEXTROSE MEDIA

The Potato Dextrose Agar media contained the following ingredients:

Peeled potato	250g
Dextrose	20g
Agar agar	20g
Distilled water	1L (Final volume)

Potatoes were properly washed, peeled and cut into small pieces. The potatoes were boiled in a 500 ml beaker in distilled water until they got soft. Soft pieces of potatoes were strained through muslin cloth. In another beaker, 500 ml distilled water was heated to simmering state, dextrose and agar agar were added slowly with continuous stirring. After proper dissolving, the mixture of potato extract was added to it in order to make the final volume to 1L. The media was sterilized in autoclave at 121^{o} C and 15 p.s.i. pressure for 20 minutes to avoid any contamination. Slants of media were prepared in test tubes for further inoculation and stored in refrigerator. The fungal mycelium from the previously maintained

slants was transferred to petriplates which were then kept in the B.O.D incubator (25±1^{o}C) for growth and sporulation of the fungus.

3.3 POISON FOOD TECHNIQUE

The heavy metals lead (Pb) and arsenic (As) were used at selected doses (0 ppm, 1 ppm, 5ppm and 10 ppm) and their inhibition effects on mycelia growth of the test fungi were evaluated with the help of Poison Food Technique (Falck, 1907).

Double strength potato dextrose agar medium was prepared in distilled water and sterilized in autoclave (15 lb p.s.i.). Double strength concentration of tested heavy metal was also prepared in sterilized distilled water. Heavy metal suspension was then added separately to equal quantities of double strength potato dextrose agar medium and then poured into petri plates. The plates were solidified and then inoculated with equal size bits (0.5 mm) of the vigorously growing fungal culture. A control treatment with no heavy metal concentration was also maintained side by side in which only sterilized distilled water was added to double strength medium. Each treatment was replicated thrice and incubated at 25±1^{o}C in B.O.D. incubator. Observations on radial growth of the fungal colonies were recorded by measuring the colony diameter at 24 h interval until the control plates were fully covered with the mycelium.

3.3.1 Growth of the fungi at different tested concentrations of heavy metals

The growth rate rg (mmh^{-1}) for each treatment was calculated as per the method given by Verma, (1997) and is described as under :

$$rg = \frac{cd_2 - cd_1}{t_2 - t_1}$$

Where;

rg = rate of mycelia growth (mmh^{-1})

cd_2 = mean diameter of colony at time t_2

cd_1 = mean diameter of colony at time t_1

t_2 = time when mean diameter of colony was cd_2

t_1 = time when mean diameter of colony was cd_1

Each treatment was replicated thrice and subjected to statistical analysis.

3.3.2 Per cent Growth Inhibition

The colony diameter in each treatment was measured on every day after inoculation and the per cent inhibition of each tested fungi was calculated by the following formula (Vincent, 1927):

$$PI = \frac{C-T}{C} \times 100$$

Where;

PI= Growth inhibition (%)

C= Diameter of colony in control (mm)

T= Diameter of colony in treatment (mm)

Each treatment was replicated thrice and subjected to statistical analysis.

3.3.3 Mycelium dry weight (mg):

The agar mycelia contents of each petri plate were scrapped and placed in a beaker of rapidly boiling distilled water for 4 minutes. The contents of beaker were then filtered by using a Whatman no. 42 filter paper which was pre-dried for one day at 70^{o}C and pre-weighed, placed in Buchner funnel. Two 250 ml samples of rapidly boiling, distilled water were used to rinse the resultant mycelia mat on the filter paper. After the final wash, the sample was aspirated for 30 seconds (Sutton and Starzyk, 1972) dried at 70^{o}C in oven for 24 hrs and then dry weight (mg) was taken on electronic weighing balance.

3.4 SERIAL DILUTION TECHNIQUE:

The disc of 0.5 mm was cut from the petri plate of pure culture of fungi with the help of sterile cork borer and dispensed into the test tube containing 9 ml sterilized distilled water. The test tube was stirred on magnetic shaker for 15 minutes for proper mixing of the fungal mycelia strands with sterilized distilled water. One ml solution from the prepared suspension was transferred to the next test tube containing 9 ml of sterilized distilled water. The steps were repeated until the dilution of 10^{-3} and 10^{-5} were achieved.

The stock suspension of different fungal isolates viz. *A. niger, T. harzianum, B. bassiana, M. anisopliae* were standardized to $1x10^{3}$ and $1x10^{5}$ spores/ml with the help of haemocytometer.

3.4.1 Spore germination technique

About 0.5 ml solution from test tube containing $1x10^3$spores/ml of test fungi was transferred into the cavity glass slide containing 0.5 ml of tested concentration of heavy metal under aseptic conditions. These slides were kept on glass rods placed above two folds of filter paper and then moistened. Three replications were maintained for each concentration. The control was prepared with sterilized distilled water. Similarly this procedure was followed for $1x10^5$spores/ml, also. These petri plates were then incubated at 25±1 ^oC for 24 hrs and then observed for number of spores germinated. Finally, the per cent spore germination was calculated with the help of following formula (Nisa *et al.*, 2011).

$$\textbf{Spore germination (\%)} = \frac{\text{Number of spores germinated}}{\text{Total number of spores counted}} \times 100$$

3.5 UPTAKE OF HEAVY METALS BY MYCELIUM:

The dried fungal biomass was weighed and heavy metal concentration was estimated in 0.20 g by digestion with nitric acid and perchloric acid (3:1) ratio as per the standard procedure of Singh *et al*. (2005). The digested fungal biomass was filtered through Whattman filter no. 42 and the volume of filterate was made to 50 ml in volumetric flask. The heavy metal concentration in filterate was estimated by Atomic Absorption Spectrophotometer. All the experiments were conducted in three replications and data was analyzed statistically.

The heavy metal uptake through fungi was calculated by the following formula (Iskander *et al*., 2011):

$$\textbf{Q}_\textbf{e}\textbf{ (mg/g)} = [(C_i - C_f)/w] \times V$$

Where;

Q_e = Concentration of heavy metal accumulated by fungal biomass

C_i = Initial Concentration of heavy metal (ppm)

C_f = Final concentration of heavy metal (ppm)

V = Volume of the medium (ml)

w = Dry weight of the fungal biomass (g)

STATISTICAL ANALYSIS

The data recorded on various parameters was subjected to statistical analysis under CRD (Completely Randomized Design) and appended in tables I-XII. Analysis of Variance (ANOVA) was worked out and critical difference at 5 per cent level of significance was calculated as suggested by Cochran and Cox (1963).

Chapter-4

RESULTS AND DISCUSSION

The results of present investigation entitled **"To study bioremediation and toxicity of lead and arsenic through entomopathogenic and antagonistic fungi"** have been presented and described in this chapter under the following heads.

4.1 Effect of different doses of Pb and As on radial growth (mmh^{-1}) of fungi.

4.2 Effect of different doses of Pb and As on per cent growth inhibition of fungi.

4.3 Effect of different doses of Pb and As on spore germination percentage of fungi.

4.4 Effect of different doses of Pb and As on mycelium dry weight of fungi.

4.5 Effect of different doses of Pb and As on heavy metal uptake of fungi.

4.1 EFFECT OF DIFFERENT DOSES OF Pb and As ON RADIAL GROWTH (mmh^{-1}) OF FUNGI

4.1.1 Effect of different doses of Pb and incubation period on rate of radial growth (mmh^{-1}) of fungi.

The data recorded on the effect of different doses of lead (Pb) on rate of radial growth (mmh^{-1}) of the entomopathogenic (*Metarhizium anisopliae* and *Beauveria bassiana)* and antagonistic (*Aspergillus niger* and *Trichoderma harzianum)* fungi at different incubation hours is presented in Table 4.1. The data on rate of radial growth for all the fungi was recorded after 24, 48, 72, 96 and 120 hrs (5 days) of incubation. Maximum mean radial growth of 0.98 mmh^{-1} was observed in T_1 (*Aspergillus niger* at 0 ppm) which was statistically different from all other treatments. It was followed by T_5 (*Trichoderma harzianum* at 0 ppm, 0.84 mmh^{-1}) which was also significantly different from other treatments. It was followed by T_9 (*Beauveria bassiana* at 0 ppm, 0.74 mmh^{-1}) which was at par with T_2 (*Aspergillus niger* at 1 ppm, 0.72 mmh^{-1}) and T_6 (*Trichoderma harzianum* at 1 ppm, 0.70 mmh^{-1}). Minimum rate of radial growth 0.15 $mmh^{-1}$was observed in T_{16} (*Metarhizium anisopliae* at 10 ppm).

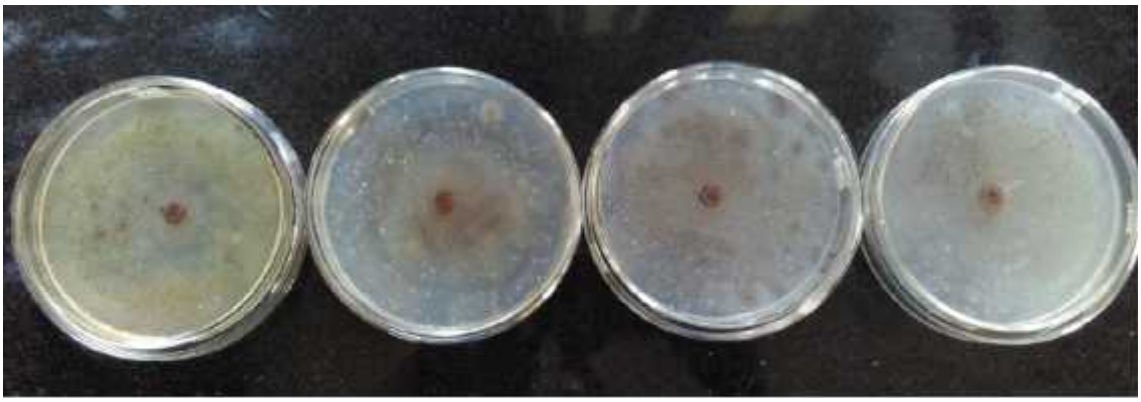
Potential of *Aspergillus versicolor* to tolerate high concentration of Pb (upto 100 ppm) have been reported by Rasool and Irum (2014) who observed two times more growth of fungus in treatment as compare to control. The minimum radial growth rate of *Metarhizium anisopliae* fungus is in agreement with the findings of Hussein *et al*. (2011) who reported

least bio-sorption capacity of *Metarhizium anisopliae* due to the difference in cell wall structure. In the present study, there was decrease in the radial growth rate with increase in the concentration of lead. At lower concentrations, all metal ions present could interact with the binding sites and thus the bio-sorption percentage was higher than those at higher ion concentrations. At higher concentrations, lower adsorption may have occurred due to the saturation of adsorption sites. These findings are in congruence with those of Gabr *et al.,* 2008; Lu *et al*., 2006 and Pardo *et al*., 2003.

Irrespective of fungi, maximum radial growth (0.74mmh^{-1}) was observed after 48 hrs of incubation, which was statistically different from all other treatments. It was followed by radial growth after 72 hrs (0.67mmh^{-1}) and 96 hrs (0.52mmh^{-1}) of incubation. Whereas, minimum radial growth rate of 0.30 mmh^{-1}was observed after 120 hrs of incubation. There was increase in radial growth from 24-48 hrs (0.45-0.74 mmh^{-1}), maximum radial growth rate was observed at 48 hrs, after that there was a gradual decrease in radial growth rate from 72-120 hrs (0.67-0.30 mmh^{-1}). The result on growth rate of fungi at different concentrations of lead with respect to incubation period is similar to trend of bell shaped growth curve of microbes. Growth curve initially shows slight increase in growth indicating microbes are entering in lag phase, then exponential increase of growth is observed in log phase, then cells enter in stationary phase in which rate of growth of living cells is equal to rate of dying cells, in decline phase there is decrease in growth rate of microbes, because rate of dying cells is more than rate of dividing cells. As reported by Huang *et al.* (1990), increased bio-sorption has been observed during the lag period or early stages of growth and decline as culture reached stationary phase. *A. niger*, *T. viride* and *P. spinulosum* showed a similar uptake pattern.

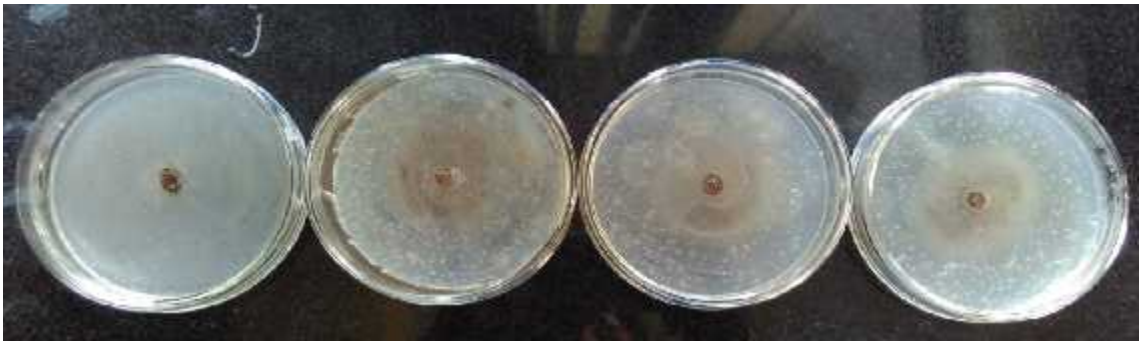
Radial growth rate at different concentrations of Pb observed for *A. niger* was higher as compared to all other fungi at different concentrations. Akar and Tunali (2006) reported high affinity of *Aspergillus niger* to remove lead (Pb) and copper (Cu) than *Trichoderma asperellum* and *Penicillium simplicissimum*. The order of average rate of radial growth (mmh^{-1}) for all the fungi when grown in the absence of lead was *Aspergillus niger* (0.98) >*T. harzianum* (0.84) > *B. bassiana* (0.74) > *M. anisopliae* (0.44).

The mean radial growth rate decreased with increase in lead concentration (from 0-10 ppm) for each fungus i.e. 0.98-0.50 mmh^{-1}; 0.84-0.44 mmh^{-1}; 0.74-0.32 mmh^{-1}; 0.44-0.15 mmh^{-1} for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae*, respectively. The results



0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of Pb on *Aspergillus niger*



0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of As on *Aspergillus niger*

Plate 1.

are in accordance with the findings of Baldrian (2003) who observed that *A. niger* was found to accumulate more of lead in the mycelia mat as the concentration of heavy metal increased. *A. niger* was observed to grow even at 100 mg/L concentration without any inhibition.

Table 4.1 Effect of different doses of Pb and incubation period on radial growth (mmh^{-1}) of fungi

Treatments		Incubation period					Mean (mmh^{-1})
		24	48	72	96	120	
T_1	(*Aspergillus niger*, 0 ppm)	0.84	1.34	1.24	0.86	0.63	**0.98[a]**
T_2	(*Aspergillus niger*, 1 ppm)	0.53	0.90	0.90	0.74	0.53	**0.72[c]**
T_3	(*Aspergillus niger*, 5 ppm)	0.46	0.78	0.70	0.57	0.49	**0.60**
T_4	(*Aspergillus niger*, 10 ppm)	0.49	0.54	0.64	0.46	0.39	**0.50**
T_5	(*Trichoderma harzianum*, 0 ppm)	0.76	1.14	1.08	0.74	0.46	**0.84[b]**
T_6	(*Trichoderma harzianum*, 1 ppm)	0.59	1.07	0.84	0.65	0.34	**0.70[c]**
T_7	(*Trichoderma harzianum*, 5 ppm)	0.53	0.99	0.70	0.47	0.17	**0.57**
T_8	(*Trichoderma harzianum*, 10 ppm)	0.40	0.78	0.58	0.35	0.10	**0.44**
T_9	(*Beauveria bassiana*, 0 ppm)	0.70	0.97	0.97	0.69	0.38	**0.74[c]**
T_{10}	(*Beauveria bassiana*, 1 ppm)	0.69	0.78	0.70	0.62	0.31	**0.62**
T_{11}	(*Beauveria bassiana*, 5 ppm)	0.43	0.68	0.58	0.57	0.13	**0.48**
T_{12}	(*Beauveria bassiana*, 10 ppm)	0.21	0.51	0.57	0.24	0.04	**0.32**
T_{13}	(*Metarhizium anisopliae*, 0 ppm)	0.36	0.43	0.47	0.57	0.36	**0.44**
T_{14}	(*Metarhizium anisopliae*, 1 ppm)	0.26	0.38	0.27	0.28	0.09	**0.28**
T_{15}	(*Metarhizium anisopliae*, 5 ppm)	0.14	0.29	0.31	0.30	0.05	**0.21**
T_{16}	(*Metarhizium anisopliae*,10 ppm)	0.13	0.22	0.19	0.15	0.02	**0.15**
Mean		**0.45 [d]**	**0.74 [a]**	**0.67 [b]**	**0.52 [c]**	**0.30 [e]**	

$CD_{0.05}$

Treatment = 0.049

Incubation period = 0.027

Treatment × Incubation period = 0.109

4.1.2 Effect of different doses of As and incubation period on radial growth rate (mmh^{-1}) of different fungi

The data recorded on the effect of different doses of arsenic (As) on rate of radial growth (mmh^{-1}) of the antagonistic (*Aspergillus niger* and *Trichoderma harzianum*) and entomopathogenic (*Metarhizium anisopliae* and *Beauveria bassiana*) fungi at different incubation hours is presented in Table 4.2. Irrespective of incubation period, maximum mean radial growth rate of 0.73 mmh^{-1} was observed in T_5 (*T. harzianum* at 0 ppm) which was at par with T_6 (*T. harzianum* at 1 ppm, 0.72 mmh^{-1}) and T_7 (*T. harzianum* at 5 ppm, 0.66 mmh^{-1}) but significantly different from other treatments. It was followed by T_1 (*A. niger* at 0 ppm, 0.65 mmh^{-1}) and T_2 (*A. niger* at 1 ppm, 0.61 mmh^{-1}). Minimum mean radial growth rate of 0.13 $mmh^{-1}$was observed in T_{16} (*M. anisopliae* at 10 ppm).

From the data presented in Table 4.2, it is evident that maximum radial growth rate of fungi was recorded in their respective control (0 ppm) i.e. when they were grown without

arsenic and there was decrease in radial growth rate with increase in the concentration of arsenic i.e. 0.65-0.48 mmh^{-1}; 0.73-0.41 mmh^{-1}; 0.60-0.16 $mmh^{-1}$and 0.40-0.13 mmh^{-1} for *A. niger, T. harzianum, B. bassiana and M. anisopliae,* respectively. The data showed that the metal bio-sorption decreased with increase in metal ion concentration. The decrease in percentage of bio-sorption may be attributed to a lack of sufficient free sites for metal bio-sorption. The present findings are in concordance with the findings of Zouboulis *et al.* (2004).

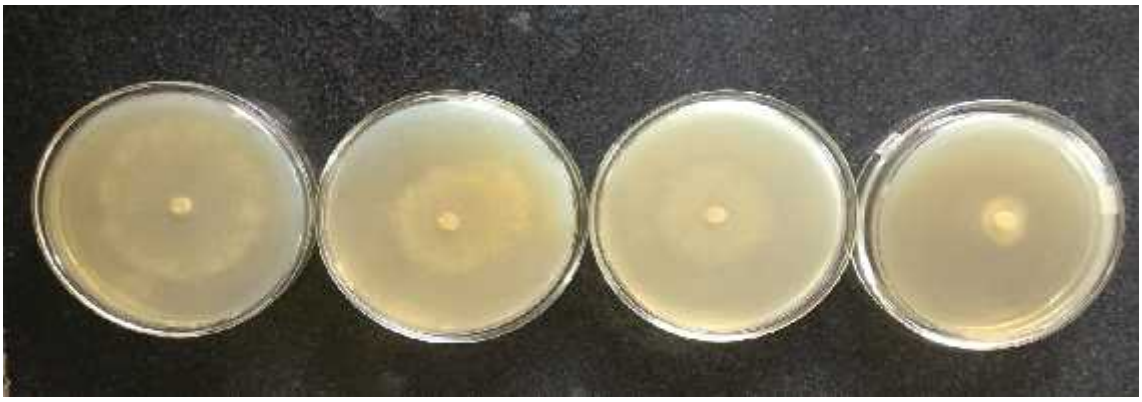
Table 4.2 Effect of different doses of As and incubation period on radial growth rate (mmh^{-1}) of fungi

Treatments		Incubation period					Mean (mmh^{-1})
		24	48	72	96	120	
T_1	(*Aspergillus niger*, 0 ppm)	0.88	1.11	1.11	0.08	0.04	**0.65** [b]
T_2	(*Aspergillus niger*, 1 ppm)	0.68	1.11	1.04	0.14	0.06	**0.61** [b]
T_3	(*Aspergillus niger*, 5 ppm)	0.72	1.04	0.50	0.33	0.03	**0.53** [c]
T_4	(*Aspergillus niger*, 10 ppm)	0.61	0.88	0.39	0.28	0.24	**0.48** [c]
T_5	(*Trichoderma harzianum*, 0 ppm)	0.82	1.20	0.97	0.54	0.10	**0.73** [a]
T_6	(*Trichoderma harzianum*, 1 ppm)	0.89	0.97	0.85	0.76	0.11	**0.72** [a]
T_7	(*Trichoderma harzianum*, 5 ppm)	0.49	1.07	0.93	0.72	0.08	**0.66** [a]
T_8	(*Trichoderma harzianum*, 10 ppm)	0.50	0.97	0.22	0.18	0.15	**0.41**
T_9	(*Beauveria bassiana*, 0 ppm)	0.83	1.24	0.39	0.36	0.17	**0.60** [b]
T_{10}	(*Beauveria bassiana*, 1 ppm)	0.78	1.03	0.46	0.25	0.15	**0.51** [c]
T_{11}	(*Beauveria bassiana*, 5 ppm)	0.63	1.03	0.42	0.17	0.07	**0.46** [c]
T_{12}	(*Beauveria bassiana*, 10 ppm)	0.00	0.25	0.21	0.17	0.01	**0.16**
T_{13}	(*Metarhizium anisopliae*, 0 ppm)	0.60	0.46	0.40	0.36	0.28	**0.40**
T_{14}	(*Metarhizium anisopliae*, 1 ppm)	0.00	0.74	0.36	0.21	0.17	**0.29**
T_{15}	(*Metarhizium anisopliae*, 5 ppm)	0.00	0.83	0.21	0.14	0.11	**0.26**
T_{16}	(*Metarhizium anisopliae*,10 ppm)	0.00	0.00	0.35	0.18	0.14	**0.13**
Mean		**0.53** [b]	**0.64** [a]	**0.54** [b]	**0.46**[c]	**0.21** [d]	

$CD_{0.05}$

Treatment	=	0.072
Incubation period	=	0.040
Treatment × Incubation period	=	0.162

Irrespective of fungi, maximum mean radial growth (0.64mmh^{-1}) was observed after 48 hrs of incubation which was statistically different from all other treatments. It was followed by radial growth rate, observed after 72 hrs (0.54 mmh^{-1}), which was at par with the rate of radial growth after 24 hrs (0.53 mmh^{-1}). Minimum radial growth rate (0.21mmh^{-1}) was observed after 120 hrs of incubation. The growth trend of fungi with respect to incubation time is similar to growth trend in bell shaped growth curve of microbes. Further, no radial growth was observed in T_{14} (*M. anisopliae* at 1 ppm), T_{15} (*M. anisopliae* at 5 ppm), T_{16} (*M. anisopliae* at 10 ppm) after 24 hrs of incubation.



0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of Pb on *Beauveria bassiana*

0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of As on *Beauveria bassiana*

Plate 2.

Radial growth rate observed for all the fungi from 24 to 120 hrs of incubation period at 0 ppm concentration was 0.88-0.04 mmh^{-1}, 0.82-0.10 mmh^{-1}, 0.83-0.17 mmh^{-1}, 0.60-0.28 mmh^{-1} for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae*, respectively. The order for all the fungi grown without arsenic was *T. harzianum* (0.73 mmh^{-1}) >*A. niger* (0.65 mmh^{-1}> *B. bassiana* (0.60 mmh^{-1}) >*M. anisopliae* (0.40 mmh^{-1}). The results of present study are in confirmation with the findings of Maheshwari and Murugesan (2011) who reported high arsenic removal potential of *Aspergillus candidus and Aspergillus flavus,* respectively. Similarly Srivastava *et al*. (2011 a) reported detailed arsenic removal potential of *Trichoderma* and *Aspergillus species* isolated from arsenic contaminated sites of West Bengal. They had identified about ten species of *Aspergillus* and *Trichoderma* which were tolerant up to 5000 mg/L of arsenic.

4.2 EFFECT OF DIFFERENT DOSES OF Pb AND As ON PER CENT GROWTH INHIBITION OF FUNGI

4.2.1 Effect of different doses of Pb and incubation period on per cent growth inhibition of fungi

The data on effect of different doses of Pb on percent growth inhibition of fungi over their respective control recorded after 24, 48, 72, 96 and 120 hrs of exposure is presented in Table 4.3. Irrespective of the incubation period, maximum mean per cent growth inhibition of 50.96 per cent was observed in T_{16} (*M. anisopliae* at 10 ppm Pb) which was found statistically different from all other treatments. It was followed by T_{15} (*M. anisopliae* at 5 ppm Pb, 43.25 %) which was also significantly different from other treatments. Minimum per cent growth inhibition of 6.68 percent was observed at T_2 (*A. niger* at 1 ppm Pb) which was at par with T_3 (*A. niger* at 5 ppm Pb, 7.54%), T_4 (A. niger at 10 ppm, 9.37%), T_6 (*T. harzinum* at 1 ppm Pb, 7.84%) and T_7 (*T. harzinum* at 5 ppm Pb, 8.76%). These findings are in accordance with those of Joshi *et al*. (2011) who reported growth inhibition of some of the fungal isolates at high concentrations of heavy metals.

Whereas, irrespective of the fungi, maximum per cent growth inhibition of 32.93 per cent was observed after 24 hrs of incubation period which was found to be statistically different from all other treatments. It was followed by 16.98 per cent growth inhibition recorded after 48 hrs, followed by 12.37 per cent growth inhibition observed after 72 hrs and 9.38 per cent of growth inhibition obtained after 96 hrs of incubation period. Minimum per cent growth inhibition of 6.40 per cent was obtained after 120 hrs of incubation. At 24 hrs of incubation, 100.00% growth inhibition was recorded T_{14} (*M. anisopliae* at 1 ppm), T_{15} (*M.*

anisopliae at 5 ppm) and T_{16} (*M. anisopliae* at 10 ppm). The present findings find support from the results of Zafar *et al*. (2007) who reported minimum inhibitory concentration (MIC) of 0.6-9.0 mg/g of copper for *Aspergillus* and *Trichoderma.*

Table 4.3: Effect of different concentration of Pb and incubation period on per cent growth inhibition of fungi

Treatments		Incubation period					Mean (%)
		24	48	72	96	120	
T_1	(*Aspergillus niger*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_2	(*Aspergillus niger*, 1 ppm)	12.56 (20.75)	8.46 (16.91)	6.22 (14.44)	3.95 (11.47)	2.20 (8.53)	**6.68 (14.42)**
T_3	(*Aspergillus niger*, 5 ppm)	18.37 (25.37)	9.38 (17.83)	6.32 (14.56)	2.77 (9.58)	0.87 (5.36)	**7.54 (14.54)**
T_4	(*Aspergillus niger*, 10 ppm)	19.00 (25.83)	13.18 (21.28)	6.55 (14.82)	4.15 (11.75)	3.97 (11.50)	**9.37 (17.04)**
T_5	(*Trichoderma harzianum*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_6	(*Trichoderma harzianum*, 1 ppm)	15.14 (22.89)	10.75 (19.13)	6.41 (14.66)	3.03 (10.02)	2.24 (8.60)	**7.84 (15.32)**
T_7	(*Trichoderma harzianum*, 5 ppm)	16.78 (24.18)	11.87 (20.15)	7.61 (16.00)	4.54 (12.30)	2.22 (8.57)	**8.76 (16.40)**
T_8	(*Trichoderma harzianum*, 10 ppm)	26.10 (30.71)	14.31 (22.22)	10.46 (18.86)	4.70 (12.51)	3.91 (11.40)	**11.40 (18.73)**
T_9	(*Beauveria bassiana*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_{10}	(*Beauveria bassiana*, 1 ppm)	25.09 (30.05)	22.94 (28.61)	22.25 (28.13)	12.41 (20.62)	7.34 (15.72)	**18.44 (25.08)**
T_{11}	(*Beauveria bassiana*, 5 ppm)	43.65 (41.34)	31.99 (34.43)	23.16 (28.76)	23.14 (28.74)	9.54 (17.99)	**25.85 (29.80)**
T_{12}	(*Beauveria bassiana*, 10 ppm)	50.25 (45.13)	34.59 (36.01)	34.37 (35.88)	27.21 (31.43)	21.40 (27.55)	**33.56 (35.20)**
T_{13}	(*Metarhizium anisopliae*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_{14}	(*Metarhizium anisopliae*, 1 ppm)	100.00 (89.96)	14.85 (22.65)	6.83 (15.15)	5.51 (13.57)	3.42 (10.65)	**26.12 (30.40)**
T_{15}	(*Metarhizium anisopliae*, 5 ppm)	100.00 (89.96)	43.45 (41.22)	33.00 (35.05)	24.96 (29.96)	14.88 (22.68)	**43.25 [b] (43.78)**
T_{16}	(*Metarhizium anisopliae*,10 ppm)	100.00 (89.96)	55.89 (48.37)	34.79 (36.13)	33.74 (35.50)	30.41 (33.46)	**50.96 [a] (48.68)**
Mean		**32.93 [a] (33.51)**	**16.98 [b] (27.03)**	**12.37 [c] (18.79)**	**9.38 (15.62)**	**6.40 (12.80)**	

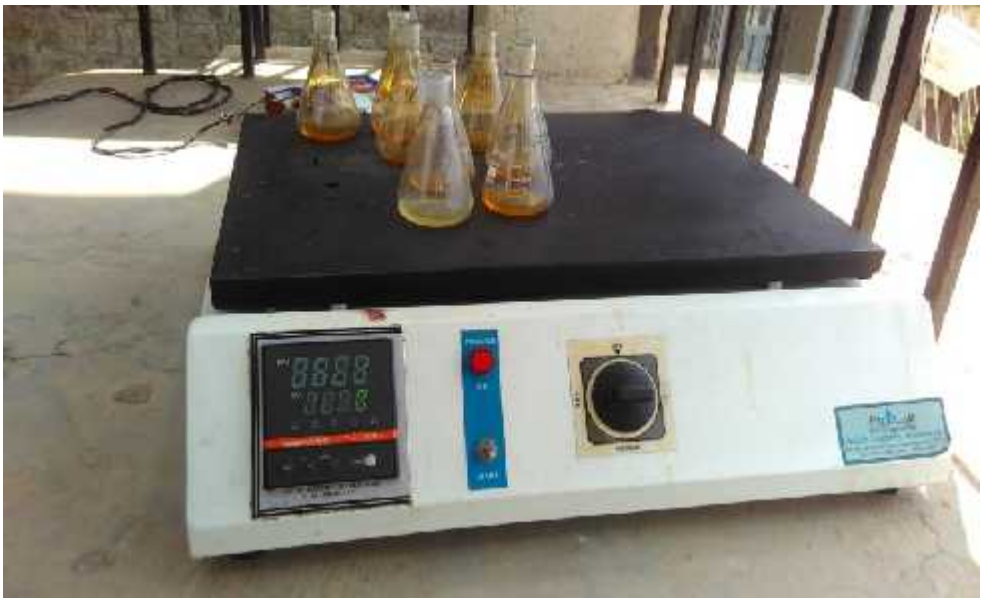
**Values in parenthesis are arc sin transformations*

$CD_{0.05}$

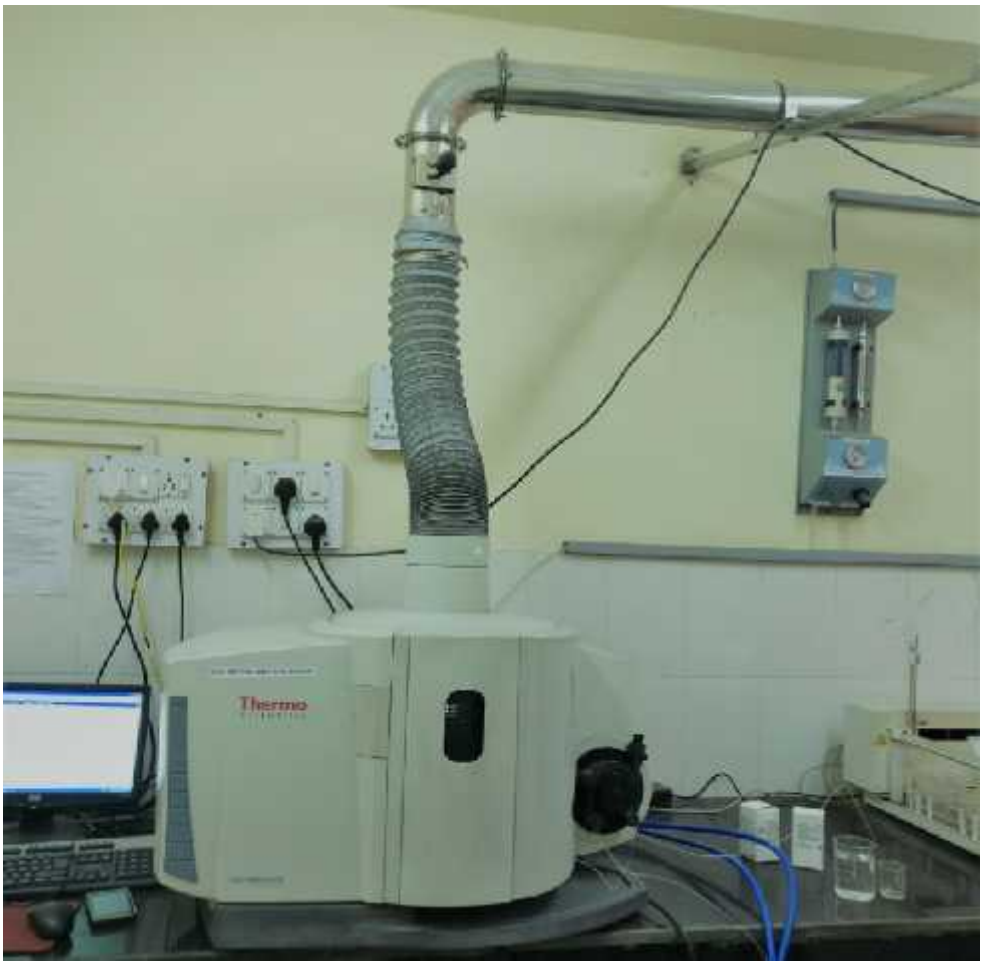
Treatment	=	4.40
Incubation period	=	2.75
Treatment × Incubation period	=	7.92

4.2.2 Effect of different concentrations of As and incubation period on per cent growth inhibition of fungi

The data recorded on effect of different doses of As on per cent growth inhibition of entomopathogenic and antagonistic fungi over their respective control after24, 48, 72, 96 and



Digestion of fungal mycelia



Estimation of Pb and As by ICAP- 6300 Duo

Plate 3.

120 hrs of incubation is presented in Table 4.4. Irrespective of incubation period maximum mean percent growth inhibition of 73.33 per cent was observed in T_{16} (*M. anisopliae*, at 10 ppm) which was at par with T_{12} (*B. bassiana* at 10 ppm, 72.57 %). Minimum mean per cent growth inhibition of 6.37 per cent was observed in T_{10} (*B. bassiana* at 1 ppm) which was statistically different from all other treatments.

Table 4.4 Effect of different doses of As and incubation period on per cent growth inhibition of fungi

Treatments		Incubation period					Mean (%)
		24	48	72	96	120	
T_1	(*Aspergillus niger*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_2	(*Aspergillus niger*, 1 ppm)	42.14 (40.46)	16.88 (24.26)	15.17 (22.91)	12.73 (20.89)	11.73 (20.02)	**19.73 (14.42)**
T_3	(*Aspergillus niger*, 5 ppm)	50.74 (45.41)	37.26 (37.61)	29.73 (33.03)	23.93 (29.28)	22.72 (28.46)	**32.88 (14.54)**
T_4	(*Aspergillus niger*, 10 ppm)	59.33 (50.36)	38.58 (38.38)	38.28 (38.21)	33.92 (35.61)	31.99 (34.43)	**40.42 [b] (17.04)**
T_5	(*Trichoderma harzianum*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_6	(*Trichoderma harzianum*, 1 ppm)	22.01 (27.97)	16.17 (23.70)	12.66 (20.83)	9.72 (18.16)	7.96 (16.38)	**13.70 (21.72)**
T_7	(*Trichoderma harzianum*, 5 ppm)	28.24 (32.09)	17.29 (24.56)	17.10 (24.42)	13.58 (21.62)	11.14 (19.49)	**17.47 (24.70)**
T_8	(*Trichoderma harzianum*, 10 ppm)	47.34 (43.46)	29.97 (33.18)	29.11 (32.64)	25.11 (30.06)	13.58 (21.62)	**29.02 (32.58)**
T_9	(*Beauveria bassiana*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_{10}	(*Beauveria bassiana*, 1 ppm)	8.21 (16.65)	7.87 (16.29)	7.00 (15.34)	5.18 (13.15)	3.59 (10.93)	**6.37 (14.62)**
T_{11}	(*Beauveria bassiana*, 5 ppm)	45.30 (42.29)	16.81 (24.20)	16.42 (23.90)	12.74 (20.91)	5.52 (13.59)	**19.36 (26.10)**
T_{12}	(*Beauveria bassiana*, 10 ppm)	100.00 (89.96)	75.40 (60.24)	71.68 (57.83)	71.67 (57.82)	44.12 (41.61)	**72.57 [a] (58.40)**
T_{13}	(*Metarhizium anisopliae*, 0 ppm)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	**0.00 (0.00)**
T_{14}	(*Metarhizium anisopliae*, 1 ppm)	100.00 (89.96)	16.55 (24.00)	14.33 (22.24)	5.50 (13.56)	2.63 (9.33)	**27.80 (31.81)**
T_{15}	(*Metarhizium anisopliae*, 5 ppm)	100.00 (89.96)	44.86 (42.04)	37.42 (37.70)	30.09 (33.25)	12.74 (20.91)	**45.02 b (42.13)**
T_{16}	(*Metarhizium anisopliae*,10 ppm)	100.00 (89.96)	100.00 (89.96)	56.57 (48.76)	55.26 (48.00)	54.85 (47.77)	**73.33 a (58.89)**
Mean		**43.95 [a] (41.51)**	**26.11 [b] (30.71)**	**21.59 [c] (27.68)**	**18.72 [c] (25.63)**	**13.95 (21.89)**	

**Values in parenthesis are arc sin transformations*

CD $_{0.05}$

Treatment	=	5.20
Incubation period	=	3.73
Treatment × Incubation period	=	10.04

Similar results on increase in growth inhibition with increase in concentration of heavy metals are reported by Gola *et. al*. (2016). The results obtained in the present study on mycelia growth inhibition of *B. bassiana* and *M. anisopliae* are in agreements with the findings of Tkaczuk (2005) who reported that metals of Cu & Zn in the medium caused a significant reduction of fungal colonies of entomopathogenic fungus *B. brongniartii* and *P. fumosoroseus.*

There was increase in mean growth inhibition of each fungus over their respective control with increase in arsenic (As) concentration i.e. 19.73-40.42 (*A. niger*), 13.70-29.02 (*T. harzianum*), 6.37-72.57 (*B. bassiana*) and 27.80-73.33 (*M. anisopliae*), respectively. Irrespective of fungi, with increase in incubation hours decrease in per cent growth inhibition was observed. Maximum, mean per cent growth inhibition of 43.95 per cent was observed after 24 hrs of incubation, which was found to be statistically different from all other treatments. It was followed by 26.11 per cent inhibition after 48 hrs, 21.59 per cent inhibition after 72 and 18.72 per cent growth inhibition after 96 hrs of incubation. These values were at par with each other. While, minimum mean per cent growth inhibition 13.95 per cent was observed after 120 hrs of incubation. Thus with increase in the exposure period of fungi to arsenic, there was decrease in per cent growth inhibition i.e. 43.95-13.95% from 24-120 hrs of incubation. At 24 hrs of incubation period maximum percent growth inhibition (100.00 per cent) was recorded in T_{12} (*B. bassiana* at 10 ppm), T_{14} (*M. anisopliae* at 1 ppm), T_{15} (*M. anisopliae* at 5 ppm) and T_{16} (*M. anisopliae* at 10 ppm), respectively.

4.3 EFFECT OF DIFFERENT DOSES OF Pb AND As ON DRY WEIGHT OF FUNGI

4.3.1 Effect of different doses of Pb and As on dry weight of fungi

The data on effect of different doses of lead and arsenic after 120 hrs of growth on dry weight (mg) of entomopathogenic and antagonistic fungi is presented in Table 4.5. In case of lead, the dry weight of fungi in various treatments varied from 283.33-56.67 mg. Among all the treatments, the maximum dry weight of 283.33 mg was observed in T_1 (*A. niger* at 0 ppm), which was at par with T_2 (*A. niger* at 1 ppm, 280.00 mg), T_3 (*A. niger* at 5 ppm, 236.67 mg) and T_5 (*T. harzianum* at 0 ppm, 275.00 mg). More accumulation of lead in the mycelia mat of *A. niger* with increase in the concentration was reported by Rasool and Irum (2014). They reported the order of uptake of heavy metals by *A. niger* which was in the following way lead > copper > cadmium. Similarly Iram and Abrar (2015) had also reported

Aspergillus sp. as good adsorbing medium for metal ions having high adsorption yields for the treatment of soils containing copper and lead ions. Iskandar *et al*. (2011) had also reported the similar results i.e. growth of *A. niger* was higher as compared to *T. asperellum* at all concentration of lead and the dry biomass obtained for *A. niger* was higher than that of *T. asperellum*. They also reported that with increase in lead concentration there was decrease in microbial growth. Minimum dry weight of 56.67 mg was observed in T_{16} (*M. anisopliae* at 10 ppm Pb) which was at par with T_{15} (*M. anisopliae* at 5 ppm Pb, 60.00 mg), T_{14} (*M. anisopliae* at 1 ppm Pb, 77.17 mg) and T_{12} (*B. bassiana* at 10 ppm Pb, 93.33 mg).

There was decrease in fungal weight from 283.33-150.00, 275.00-87.67, 146.67-93.33 and 106.67-56.67 for *A. niger, T. harzianum, B. bassiana and M. anisopliae* with increase in concentration of lead from 0-10 ppm, respectively. Velmurugan *et al*. (2009) had also reported reduction in the biomass of fungus *Penicillium sp.* with increase in the concentration of heavy metals, Se and Pb. The fungi could be ordered as *A. niger* > *T. harzianum* > *B. bassiana* > *M. anisopliae* with respect to dry weight in response to different doses of lead.

The data on effect of different doses of arsenic on dry weight (mg) of entomopathogenic and antagonistic fungi recorded up to 120 hrs of exposure to arsenic is also presented in Table 4.5. Among all the treatments maximum dry weight of 240.00 mg was observed in T_5 (*T. harzianum* at 0 ppm), which was at par with T_1 (*A. niger* at 0 ppm, 223.33 mg) and T_6 (*T. harzianum* at 1 ppm, 216.67). The results are in reference to the findings of Adeyemi (2009) who reported that accumulation of arsenic in the fungal biomasses of *Trichoderma versicolor* was greater than *A. niger*. The minimum dry weight of 54.67 mg was observed in T_{16} (*M. anisopliae* at 10 ppm) which was at par with T_{15} (*M. anisopliae* at 5 ppm, 70.00 mg). In general, there was decrease in dry weight of all the four fungi with increase in concentration of arsenic. The dry weight for fungi decreased from 223.33-86.67 mg, 240.00-140.00 mg, 146.67-103.33 mg and 106.67-54.67 mg for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae* with increase in concentration of arsenic from 0 ppm to 10 ppm respectively. The fungi can be ordered as *T. harzianum* > *A. niger* > *B. bassiana* > *M. anisopliae* with respect to average dry weight when exposed to graded doses of arsenic. From the Table 4.5, It can be concluded that *A. niger* has highest mean dry weight at 10 ppm concentration (118.34 mg), so it is able to tolerate both heavy metals (Pb and As) upto 10 ppm.

Table 4.5 Effect of different doses of Pb and As on dry weight (mg) of fungi after 120 hrs

Treatments		Dry weight (mg)	
		Pb	**As**
T_1	**(*Aspergillus niger*, 0 ppm)**	283.33	223.33
T_2	**(*Aspergillus niger*, 1 ppm)**	280.00	164.33
T_3	**(*Aspergillus niger*, 5 ppm)**	236.67	122.67
T_4	**(*Aspergillus niger*, 10 ppm)**	150.00	86.67
T_5	**(*Trichoderma harzianum*, 0 ppm)**	275.00	240.00
T_6	**(*Trichoderma harzianum*, 1 ppm)**	234.00	216.67
T_7	**(*Trichoderma harzianum*, 5 ppm)**	116.00	203.33
T_8	**(*Trichoderma harzianum*, 10 ppm)**	87.67	140.00
T_9	**(*Beauveria bassiana*, 0 ppm)**	146.67	146.67
T_{10}	**(*Beauveria bassiana*, 1 ppm)**	126.67	136.67
T_{11}	**(*Beauveria bassiana*, 5 ppm)**	110.00	120.00
T_{12}	**(*Beauveria bassiana*, 10 ppm)**	93.33	103.33
T_{13}	**(*Metarhizium anisopliae*, 0 ppm)**	106.67	106.67
T_{14}	**(*Metarhizium anisopliae*, 1 ppm)**	77.17	96.67
T_{15}	**(*Metarhizium anisopliae*, 5 ppm)**	60.00	70.00
T_{16}	**(*Metarhizium anisopliae*,10 ppm)**	56.67	54.67
Mean		**152.49**	**139.48**

$CD_{0.05}$

Treatment (Pb) = 48.60

Treatment (As) = 36.31

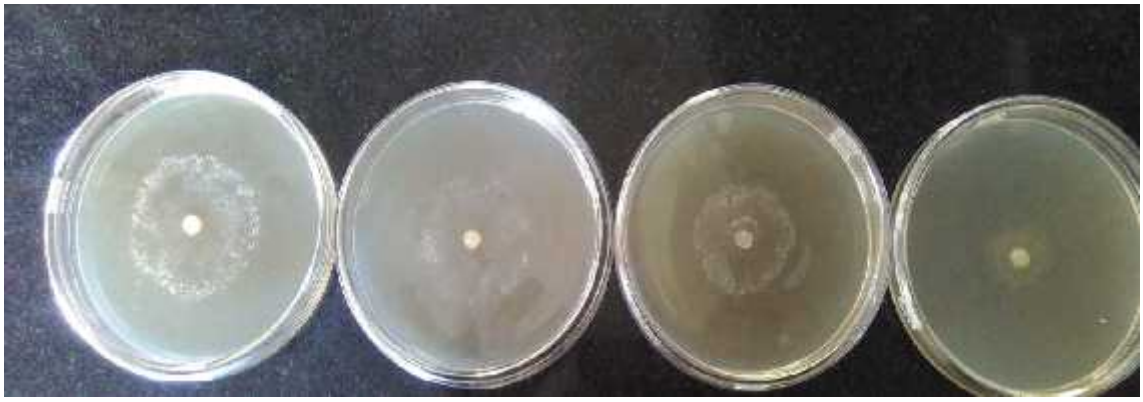
4.4 EFFECT OF DIFFERENT DOSES OF Pb AND As ON GERMINATION (%) OF SPORES AND REDUCTION POTENTIAL OF FUNGI

4.4.1 Effect of different doses of Pb and spore concentration on germination (%) of spores and reduction potential of fungi

The data recorded on the effect of different doses of Pb on spore germination percentage of antagonistic (*A. niger* and *T. harzianum*) and entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) after 24 hrs of incubation at two different doses of the fungi is presented in Table 4.6

(A) 1×10^3 spores/ml

Maximum spore germination of 79.66 per cent was observed in T_1 (*A. niger* at 0 ppm) which was statistically different from all other treatments. It was followed by T_5 (*T. harzianum* at 0 ppm, 71.67 %) which was also statistically, different from other treatments. Minimum spore germination percentage (9.66%) was observed in T_{16} (*M. anisopliae* at 10



0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of Pb on *Metarhizium anisopliae*

0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of As on *Metarhizium anisopliae*

Plate 4.

ppm) which was at par with T_{12} (*B. bassiana* at 10 ppm, 11.33%). In general, the spore germination percentage, for all the four fungi decreased with increase in concentration of lead i.e. maximum spore germination was recorded at the respective control of all the fungi i.e. 79.66 per cent for *A. niger* at 0 ppm which reduced to 46.33 per cent at 10 ppm of lead, 71.67 per cent for *T. harzianum* at 0 ppm which decreased to 32.66 per cent at 10 ppm lead, 55.00 per cent for *B. bassiana* at 0 ppm which reduced to 11.33 per cent at 10 ppm of lead and 44.00 per cent for *M. anisopliae* at 0 ppm reduced to 9.66 per cent at 10 ppm of lead.

Reduction potential for all the fungi also increased with the increase in concentrations of heavy metals. It increased from 20.00-42.50 %, 8.33-54.16%, 27.27-80.00%, 36.36-77.27% for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae,* respectively. Less is the reduction potential, more strong is fungus to tolerate concentration of heavy metals, and more is the value of reduction potential week is the fungus to tolerate heavy metals. Lowest reduction potential was observed in T_6 (*T. harzianum* at 1 ppm, 8.33 %) so it is a strong fungi and has high ability to tolerate Pb whereas, maximum reduction potential was observed in T_{12} (*B. bassiana* at 10 ppm, 80.00%) so it is a week fungi and has less ability to tolerate Pb.

(B) 1×10^5 spores/ml

The effect of lead (Pb) on the spore germination at 1×10^5 spores/ ml is presented in Table 4.6. The maximum spore germination percentage of 82.00 per cent was observed in T_1 (*A. niger* at 0 ppm) which was at par with T_5 (*T. harzianum* at 0 ppm, 80 %). Minimum spore germination percentage 13.33 per cent was recorded in T_{16} (*M. anisopliae* at 10 ppm) which was statistically different from all other treatments. The present studies, on the effect of lead, on the spore germination of fungi find support from the findings of Tkaczuk (2005), where the conidia of *P. neoaphidis* were unable to germinate in the presence of chromium, copper, lead and zinc ions in the medium at a concentration that was 100 times higher than the mean one.

In general, the spore germination percentage for all the four fungi decreased with increase in lead concentration i.e maximum spore germination was recorded at the respective control of all the fungi i.e. 82.00 per cent for *A. niger* at 0 ppm which reduced to 51.33 per cent at 10 ppm lead, 80.00 per cent for *T. harzianum* at 0 ppm which reduced to 37.33 per cent at 10 ppm of lead, 67.66 per cent for *B. bassiana* at 0 ppm which reduced to 22.66 per

cent at 10 ppm lead and 52.66 per cent for *M. anisopliae* at 0 ppm which reduced to 13.33 per cent at 10 ppm lead.

Reduction potential for all the fungi also increased with the increase in concentrations of Pb. It increased from 9.75-37.80 %, 13.75-53.75%, 10.29-66.00%, 33.96-75.47% for *A. niger, T. harzianum, B. bassiana, M. anisopliae,* respectively. Less is the reduction potential, more strong is fungus to tolerate concentration of heavy metals, and more is the value of reduction potential week is the fungus to tolerate heavy metals. Lowest reduction potential was observed in T_2 (*A. niger* at 1 ppm, 9.75%) which indicated that it is a strong fungi and has high ability to tolerate Pb whereas, maximum reduction potential was observed in T_{16} (*M. anisopliae* at 10 ppm, 75.47%) which showed that it is a week fungi and has less ability to tolerate Pb.

Table 4.6 Effect of different doses of Pb and spore concentration on germination (%) of spores and reduction potential of fungi

Treatments		Germination % of spores		Reduction Potential (%)	
		1×10^3 spores/ml	**1×10^5 spores/ml**	**1×10^3 spores/ml**	**1×10^5 spores/ml**
T1	**(*Aspergillus niger*, 0 ppm)**	79.66 (63.17)	82.00 (64.87)	0.00 (0.00)	0.00 (0.00)
T2	**(***Aspergillus niger*, 1 ppm)	64.00 (53.11)	74.33 (59.54)	20.00 (26.55)	9.75 (18.19)
T3	**(***Aspergillus niger*, 5 ppm)	53.66 (47.08)	68.00 (55.53)	32.50 (34.74)	17.00 (24.34)
T4	**(***Aspergillus niger*, 10 ppm)	46.33 (42.88)	51.33 (45.75)	42.50 (40.67)	37.80 (37.92)
T5	**(***Trichoderma harzianum*, 0 ppm)	71.67 (57.82)	80.00 (63.41)	0.00 (0.00)	0.00 (0.00)
T6	**(***Trichoderma harzianum*, 1 ppm)	65.66 (54.11)	69.00 (56.14)	8.33 (16.77)	13.75 (21.76)
T7	**(***Trichoderma harzianum*, 5 ppm)	44.33 (41.73)	54.66 (47.66)	38.88 (38.56)	31.00 (33.82)
T8	**(***Trichoderma harzianum*, 10 ppm)	32.66 (34.84)	37.33 (37.65)	54.16 (47.37)	53.75 (47.13)
T9	**(***Beauveria bassiana*, 0 ppm)	55.00 (47.85)	67.66 (55.32)	0.00 (0.00)	0.00 (0.00)
T10	**(***Beauveria bassiana*, 1 ppm)	40.33 (39.41)	61.33 (51.53)	27.27 (31.47)	10.29 (18.70)
T11	**(***Beauveria bassiana*, 5 ppm)	26.33 (30.86)	47.33 (43.45)	52.70 (46.53)	30.88 (33.75)
T12	**(***Beauveria bassiana*, 10 ppm)	11.33 (19.66)	22.66 (28.42)	80.00 (63.41)	66.00 (54.31)
T13	**(***Metarhizium anisopliae*, 0 ppm)	44.00 (41.54)	52.66 (46.51)	0.00 (0.00)	0.00 (0.00)
T14	**(***Metarhizium anisopliae*, 1 ppm)	27.66 (31.72)	35.33 (36.46)	36.36 (37.07)	33.96 (35.63)
T15	**(***Metarhizium anisopliae*, 5 ppm)	18.00 (25.09)	23.33 (28.87)	59.09 (50.22)	56.60 (48.77)
T16	**(***Metarhizium anisopliae*,10 ppm)	9.66 (18.11)	13.33 (21.41)	77.27 (61.50)	75.47 (60.29)
Mean		**43.15 (41.04)**	**52.52 (46.43)**		

**Values in parenthesis are arc sin transformations*

$CD_{0.05}$

Treatment (1×10^3 spores/ml) = 2.45

Treatment (1×10^5 spores/ml) = 3.64

High spore germination percentage was observed in 1×10^5 spores/ml dose than 1×10^3 spores/ml, this is due to the selection pressure, spores have become resistant to toxicity of different concentrations of Pb.

4.4.2 Effect of different doses of arsenic and spore concentration on germination (%) of spores and reduction potential of fungi

The data recorded on the effect of different doses of As on spore germination percentage of antagonistic fungi (*A. niger* and *T. harzianum*) and entomopathogenic fungi (*M. anisopliae* and *B. bassiana*) after 24 hrs of incubation at two different doses is presented in Table 4.7

(A) 1×10^3 spores/ml

The influence of arsenic, on the spore germination of 1×10^3 spores/ml dose indicated maximum spore germination of 68 per cent was observed in T_5 (*T. harzianum* at 0 ppm) which was statistically different from all other treatments. Minimum spore germination percentage (15.00%) was observed in T_{16} (*M. anisopliae* at 10 ppm As) and it was statistically different from all other treatments. In general, the spore germination percentage for all the four fungi decreased with increase in concentration of arsenic i.e. maximum spore germination was recorded at respective control of all the fungi i.e. 68.00 per cent for *T. harzianum* at 0 ppm which reduced to 41.00 per cent at 10 ppm of arsenic, 64.66 per cent for *A. niger* at 0 ppm which reduced to 40.33 per cent at 10 ppm arsenic, 60.66 per cent for *B. bassiana* at 0 ppm which reduced to 22.00 per cent at 10 ppm arsenic and 44.67 per cent for *M. anisopliae* at 0 ppm which reduced to 15.00 per cent at 10 ppm arsenic.

Reduction potential for all the fungi also increased with increase in concentrations of heavy metals. It increased from 9.23-38.46 %, 14.70-39.70%, 22.95-63.93%, 20.00-66.66% for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae,* respectively. Less is the reduction potential, more strong is fungus to tolerate concentration of heavy metals, and more is the value of reduction potential week is the fungus to tolerate heavy metals. Lowest reduction potential was observed in T_2 (*A. niger* at 1 ppm, 9.23%) which showed that it is a strong fungi and has high ability to tolerate As whereas, maximum reduction potential was observed in T_{16} (*M. anisopliae* at 10 ppm, 66.66%) there by indicating that it is a week fungi and has less ability to tolerate As.

(B) 1×10^5 spores/ml

The influence of arsenic on the spore germination of 1×10^5 spores/ml dose indicated maximum spore germination of 75.00 per cent in T_5 (*T. harzianum* at 0 ppm) which was found to be statistically different from all other treatments. Minimum spore germination percentage of 23.33 per cent was observed in T_{16} (*M. anisopliae* at 10 ppm As) which was statistically different from all other treatments.

Table 4.7 Effect of different concentration of As and spore concentration on germination (%) of spores and reduction potential of fungi

Treatments		Germination % of spores		Reduction Potential (%)	
		1×10^3 spores/ml	1×10^5 spores/ml	1×10^3 spores/ml	1×10^5 spores/ml
T1	**(*Aspergillus niger*, 0 ppm)**	64.66 (53.51)	67.67 (55.32)	0.00 (0.00)	0.00 (0.00)
T2	**(***Aspergillus niger*, 1 ppm)	59.00 (50.16)	62.67 (52.32)	9.23 (17.68)	7.35 (15.72)
T3	**(***Aspergillus niger*, 5 ppm)	44.00 (41.54)	60.67 (51.14)	32.30 (34.62)	10.00 (18.43)
T4	**(***Aspergillus niger*, 10 ppm)	40.33 (39.41)	48.33 (44.03)	38.46 (38.31)	29.00 (32.57)
T5	**(***Trichoderma harzianum*, 0 ppm)	68.00 (55.53)	75.00 (59.98)	0.00 (0.00)	0.00 (0.00)
T6	**(***Trichoderma harzianum*, 1 ppm)	58.00 (49.58)	68.00 (55.53)	14.70 (22.54)	9.33 (17.78)
T7	**(***Trichoderma harzianum*, 5 ppm)	48.00 (43.84)	53.00 (46.70)	29.40 (32.82)	29.00 (32.57)
T8	**(***Trichoderma harzianum*, 10 ppm)	41.00 (39.80)	56.33 (48.62)	39.70 (39.04)	25.33 (30.21)
T9	**(***Beauveria bassiana*, 0 ppm)	60.66 (51.14)	65.00 (53.71)	0.00 (0.00)	0.00 (0.00)
T10	**(***Beauveria bassiana*, 1 ppm)	46.66 (43.07)	60.66 (51.14)	22.95 (28.61)	6.00 (14.17)
T11	**(***Beauveria bassiana*, 5 ppm)	32.33 (34.64)	51.33 (45.75)	47.50 (43.55)	21.53 (27.63)
T12	**(***Beauveria bassiana*, 10 ppm)	22.00 (27.96)	40.67 (39.61)	63.93 (53.07)	37.00 (37.45)
T13	**(***Metarhizium anisopliae*, 0 ppm)	44.67 (41.92)	55.66 (48.23)	0.00 (0.00)	0.00 (0.00)
T14	**(***Metarhizium anisopliae*, 1 ppm)	36.33 (37.05)	48.66 (44.22)	20.00 (26.55)	12.50 (20.70)
T15	**(***Metarhizium anisopliae*, 5 ppm)	24.00 (29.32)	33.33 (35.25)	46.66 (43.07)	41.00 (39.80)
T16	**(***Metarhizium anisopliae*,10 ppm)	15.00 (22.78)	23.33 (28.87)	66.66 (54.711)	58.90 (50.11)
Mean		**44.04 (41.56)**	**54.00 (47.28)**		

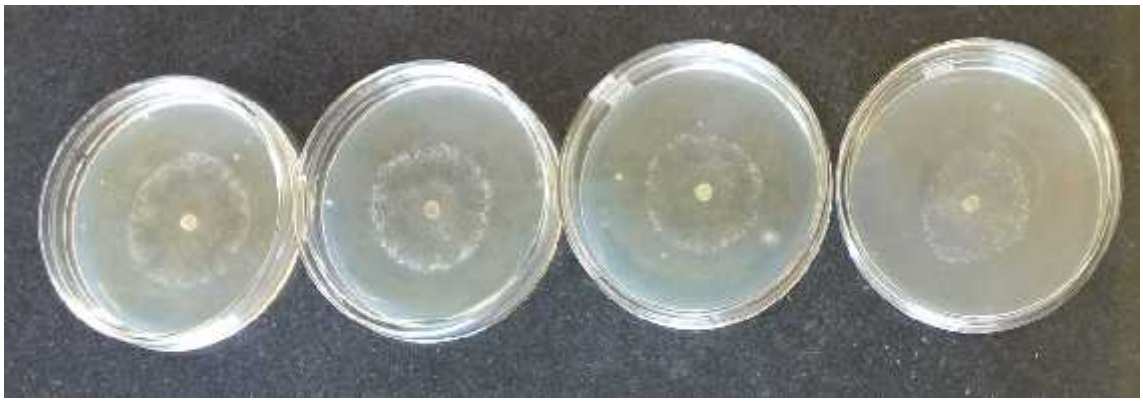
**Values in parenthesis are arc sin transformations*

$CD_{0.05}$

Treatment (1×10^3 spores/ml) = 1.64

Treatment (1×10^5 spores/ml) = 2.73

In general the spore germination percentage for all the four fungi decreased with increase in concentration of arsenic i.e. maximum spore germination was recorded at respective control of all the fungi i.e. 75.00 per cent for *T. harzianum* at 0 ppm which reduced to 56.33 per cent at 10 ppm of arsenic, 67.67 per cent for *A. niger* at 0 ppm which reduced to 48.33 per cent at 10 ppm arsenic, 65.00 per cent for *B. bassiana* at 0 ppm which reduced to



0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of Pb on *Trichoderma harzianum*

0 ppm **1 ppm** **5 ppm** **10 ppm**

Effect of different doses of As on *Trichoderma harzianum*

Plate 5.

40.67 per cent at 10 ppm arsenic and 55.66 per cent for *M. anisopliae* which reduced to 23.33 per cent at 10 ppm arsenic. The present study can be supported from the findings of

Reduction potential for all the fungi also increased with increase in concentrations of arsenic. It increased from 7.35-29.00 %, 9.33-25.33%, 6.00-37.00%, 12.50-58.90% for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae,* respectively. Less is the reduction potential, more strong is fungus to tolerate concentration of heavy metals, and more is the value of reduction potential week is the fungus to tolerate heavy metals. Lowest reduction potential was observed in T_{10} (*B. bassiana* at 1 ppm, 6.00%) thereby stating that it is a strong fungi and has high ability to tolerate Pb whereas, maximum reduction potential was observed in T_{16} (*M. anisopliae* at 10 ppm, 58.90%) which showed that it is a week fungi and has less ability to tolerate Pb.

High spore germination percentage was observed in 1×10^5 spores/ml dose than 1×10^3 spores/ml in different concentrations of arsenic. This is due to the selection pressure spores have become resistant to toxicity of arsenic.

4.5 EFFECT OF DIFFERENT DOSES OF Pb AND As ON HEAVY METAL UPTAKE OF FUNGI.

4.5.1 Effect of different doses of Pb on heavy metal uptake (mg/g) of fungi.

The data on uptake of lead (Pb) by mycelium of the respective fungi up to 120 hrs of exposure to different levels of lead is presented in Table 4.9. Maximum uptake of Pb (0.30 mg/g) was observed in T_{12} (*B. bassiana* at 10 ppm). No lead (Pb) content was recorded in T_1 (*A. niger*), T_5 (*T. harzianum*), T_9 (B. *bassiana*), T_{13} (*M. anisopliae*), at 0 ppm lead concentration i.e. in the mycelium of fungi when grown in the absence of lead. Minimum uptake was observed in T_{14} (*M. anisopliae* at 1 ppm, 0.02 mg/g).

The role of fungi in bioremediation of heavy metals through mycelium has been reported by many workers (Messaccesi *et al*., 2002 and Kurniati *et al*., 2014). Further, the role of entomopathogenic fungi viz., *B. bassiana*, *M. anisopliae*, *Penicillium fusarium* to accumulate and tolerate significant amounts of metals and accumulation of high metal contents (cadmium, copper, iron, lead, zinc) in the fruiting bodies and mycelium of these fungi is reported (Nordgren *et al*., 1985 and Gadd, 1993).

Table 4.9 Effect of different concentrations of Pb and As on uptake by fungi

Treatments		vUptake (mg/g)	
		Pb	**As**
T_1	**(*Aspergillus niger*, 0 ppm)**	0.000	0.000
T_2	**(*Aspergillus niger*, 1 ppm)**	0.037	0.040
T_3	**(*Aspergillus niger*, 5 ppm)**	0.074	0.130
T_4	**(*Aspergillus niger*, 10 ppm)**	0.106	0.180
T_5	**(*Trichoderma harzianum*, 0 ppm)**	0.000	0.000
T_6	**(*Trichoderma harzianum*, 1 ppm)**	0.080	0.121
T_7	**(*Trichoderma harzianum*, 5 ppm)**	0.110	0.200
T_8	**(*Trichoderma harzianum*, 10 ppm)**	0.201	0.324
T_9	**(*Beauveria bassiana*, 0 ppm)**	0.000	0.000
T_{10}	**(*Beauveria bassiana*, 1 ppm)**	0.138	0.013
T_{11}	**(*Beauveria bassiana*, 5 ppm)**	0.260	0.050
T_{12}	**(*Beauveria bassiana*, 10 ppm)**	0.300	0.104
T_{13}	**(*Metarhizium anisopliae*, 0 ppm)**	0.000	0.000
T_{14}	**(*Metarhizium anisopliae*, 1 ppm)**	0.020	0.010
T_{15}	**(*Metarhizium anisopliae*, 5 ppm)**	0.030	0.060
T_{16}	**(*Metarhizium anisopliae*,10 ppm)**	0.040	0.070
Mean		**0.087**	**0.081**

$CD_{0.05}$

Arsenic = 0.015

Lead = 0.021

From the Table 4.9 (a) it was observed that *B. bassiana* has highest uptake of Pb (0.175 mg/g) than other fungi and the uptake of fungi is in the order of *B. bassiana* > *T. harzianum* > *A. niger* > *M. anisopliae.* High uptake of Pb by *B. bassiana* than *M. anisopliae* has been reported by Hussain *et al*. (2011). According to him *B. bassiana* has shown uptake of Pb up to 83.33 mg/g while *M. anisopliae* has shown uptake up to 66.66 mg/g which could be due to difference in cell wall structure. Potential of *B. bassiana* to precipitates heavy metals like Zn and Pb by releasing oxalic acid and degrade them also has been reported by Purchase *et al*. (2008).

Table 4.9 (a): Interaction effect of different doses of Pb and fungi on uptake (mg/g)

Fungus	Pb Uptake (mg/g)				Mean
	0 ppm	**1 ppm**	**5 ppm**	**10 ppm**	
Aspergillus niger	0.000	0.037	0.074	0.106	**0.054**
Trichoderma harzianum	0.000	0.080	0.110	0.201	**0.098**
Beauveria bassiana	0.000	0.138	0.260	0.300	**0.175**
Metarhizium anisopliae	0.000	0.020	0.030	0.040	**0.023**
Mean	0.000	**0.069**	**0.119**	**0.162**	

$CD_{0.05}$

Fungus = 0.041

Concentration = 0.050

Fungus × Concentration = 0.070

4.5.2 Effect of different doses of As on heavy metal uptake (mg/g) of fungi.

The data on uptake of As by mycelium of the respective fungi up to 120 hrs of exposure (at harvest) to different levels of arsenic is presented in Table 4.9. Maximum Arsenic (As) uptake 0.324 was recorded in T_8 (*T. harzianum* at 10 ppm). No arsenic (As) content was recorded in T_1 (*A. niger*), T_5 (*T. harzianum*), T_9 (*B. bassiana*), T_{13} (*M. anisopliae*), at 0 ppm arsenic concentration i.e. in the mycelium of fungi when grown in the absence of arsenic. Minimum (0.010 mg/g) arsenic content was recorded in the T_{14} (mycelium of *M. anisopliae* at 1 ppm).

Table 4.9 (b) Interaction effect of different doses of As and fungus on uptake (mg/g)

Fungus	As Uptake (mg/g)				Mean
	0 ppm	1 ppm	5 ppm	10 ppm	
Aspergillus niger	0.000	0.040	0.130	0.180	**0.088**
Trichoderma harzianum	0.000	0.121	0.200	0.324	**0.161**
Beauveria bassiana	0.000	0.013	0.050	0.104	**0.042**
Metarhizium anisopliae	0.000	0.010	0.060	0.070	**0.035**
Mean	**0.000**	**0.046**	**0.110**	**0.170**	

$CD_{0.05}$

Fungus	=	0.036
Concentration	=	0.049
Fungus × Concentration	=	0.050

The data obtained in Table 4.9 (b) showed that *T. harzianum* had highest uptake (0.161 mg/g) for As than other fungi. Thus fungi could be order as *T. harzianum* > *A. niger* > *B. bassiana* > *M. anisopliae.* This present studies find support from the findings of Oladipo *et al*. (2018) who reported *Trichoderma ghanense* and *Rhizopus microspores* exhibited tolerence to Cd, Cu, Pb, As and Fe at all concentrations with tolerance index > 1.

Chapter-5

SUMMARY AND CONCLUSION

The present investigations entitled **"To study bioremediation and toxicity of lead and arsenic through entomopathogenic and antagonistic fungi"** were carried under laboratory conditions of the Department of Environmental Science, College of Forestry, Dr. Y S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh during the year 2017-2018. The important findings of the present investigation are summarized as under:

Effect of different doses of Pb showed that maximum mean radial growth of 0.98 mmh^{-1} was observed in T_1 (*A. niger* at 0 ppm). Minimum radial growth of 0.15 mmh^{-1} was observed in T_{16} (*M. anisopliae* at 10 ppm). With increase in the incubation period from 24-120 hrs (5 days), there was increase in mean radial growth rate from 24 – 48 hrs (0.45-0.74 mmh^{-1}), with gradual decrease in radial growth rate from 72-120 hrs (0.67-0.30 mmh^{-1}) following the trend of growth curve. The mean radial growth rate decreased with increase in lead concentration for each fungus as compared to their respective control.

The effect of different doses of arsenic indicated that statistically maximum mean radial growth of 0.73 mmh^{-1} was observed in treatment T_5 (*T. harzianum* at 0 ppm). Minimum mean radial growth rate of 0.13 mmh^{-1} was observed in T_{16} (*M. anisopliae* at 10 ppm). In general there was decrease in radial growth rate with increase in concentration of arsenic.

Effect of different doses of lead on per cent growth inhibition of fungi showed that irrespective of the incubation period, maximum mean per cent growth inhibition of 50.96 per cent was observed in T_{16} (*M. anisopliae* at 10 ppm). Minimum per cent growth inhibition of 6.68 per cent was observed in T_2 (*A. niger* at 1 ppm). Irrespective of the fungi, maximum per cent growth inhibition (32.93 %) was observed at 24 hrs of incubation period and minimum (6.40%) was recorded at 120 hrs of incubation.

In the presence of arsenic, irrespective of incubation period, maximum mean per cent growth inhibition (73.33%) was observed in T_{16} (*M. anisopliae* at 10 ppm) and minimum (6.37%) was observed in T_{10} (*B. bassiana* at 1 ppm). Irrespective of fungus, maximum mean

per cent growth inhibition of 43.95 per cent was observed after 24 hrs of incubation and minimum (13.95%) mean per cent growth inhibition was observed after 120 hrs of incubation. With increase in incubation period there was decrease in per cent inhibition for all the treatments under study.

In the presence of Pb, the maximum dry weight (283.33 mg) was observed in T_1 (*A. niger* at 0 ppm) and minimum (56.67 mg) was observed in T_{16} (*M. anisopliae* at 10 ppm). The fungi were ordered as *A. niger* > *T. harzianum* > *B. bassiana* > *M. anisopliae* with respect to dry weight.

In the presence of arsenic, maximum dry weight (240.00 mg) was recorded in T_5 (*T. harzianum* at 0 ppm) and minimum (54.67 mg) was observed in T_{16} (*M. anisopliae* at 10 ppm). Mean maximum dry weight was observed for T_5 (*T. harzianum* at 0 ppm, 257.50 mg). The fungi were ordered as *T. harzianum* > *A. niger* > *M. anisopliae* > *B. bassiana* with respect to dry weight. There was decrease in fungal weight with increase in concentration of Pb and As from 0 to 10 ppm for all the four fungi.

In the presence of Pb, maximum spore germination (79.66%) was observed in T_1 (*A. niger* at 0 ppm) which was statistically different from other treatments. Minimum (9.66%) was observed in T_{16} (*M. anisopliae* at 10 ppm) for the dose 1×10^3 spores/ ml of fungi. At 1×10^5 spores/ml, the maximum spore germination percentage (82.00%) was observed in T_1 (*A. niger* at 0 ppm) and minimum (13.33%) in T_{16} (*M. anisopliae* at 10 ppm). In general, the spore germination percentage for all the four fungi decreased with increase in lead concentration.

Maximum reduction potential was obtained for T_{12} (*B. bassiana* at 10 ppm, 80%) and minimum reduction potential was obtained for T_6 (*T. harzianum* at 1 ppm) for the dose 1×10^3 spores/ ml of fungi. At 1×10^5 spores/ml, the maximum reduction potential (75.47%) was observed in T_{16} (*M. anisopliae* at 10 ppm) and minimum (9.75%) in T_2 (*A. niger* at 1 ppm).

The influence of 1×10^3 spores/ml of fungi showed that maximum spore germination (68.00%) was observed in T_5 (*T. harzianum* at 0 ppm) and minimum (15.00%) was observed in T_{16} (*M. anisopliae* at 10 ppm). At 1×10^5 spores/ml, the maximum spore germination percentage (75.00%) was observed in T_5 (*T. harzianum* at 0 ppm) and minimum (23.33%)

was observed in T_{16} (*M. anisopliae* at 10 ppm). In general, the spore germination percentage for all the fungi decreased with increase in concentration of arsenic.

Maximum reduction potential was obtained for T_{16} (*M. anisopliae* at 10 ppm, 66.66%) and minimum reduction potential was obtained for T_2 (*A. niger* at 1 ppm, 9.23%) for the dose 1×10^3 spores/ ml of fungi. At 1×10^5 spores/ml, the maximum reduction potential (75.47%) was observed in T_{16} (*M. anisopliae* at 10 ppm, 58.90%) and minimum T_{10} (*Beauveria bassiana* at 1 ppm, 6.00%). The reduction potential for all the four fungi increased with increase in As concentration. It increases from 7.35-29.00%, 9.33-25.33%, 6.00-37.00%, 12.50-58.90% for *A. niger, T. harzianum, B. bassiana* and *M. anisopliae*, respectively.

Maximum lead uptake (0.30 mg/g) was recorded in T_{12} (*B. bassiana* at 10 ppm) and minimum uptake (0.04 mg/g) in T_{14} (*M. anisopliae* at 1 ppm). Irrespective of the concentration, maximum mean uptake of Pb was recorded in *B. bassiana* (0.175 mg/g). The order of fungi with respect to lead (Pb) uptake was *B. bassiana* > *T. harzianum* > *A. niger* > *M. anisopliae.*

Maximum arsenic uptake value of 0.324 mg/g was recorded in T_8 (*T. harzianum* at 10 ppm) and minimum uptake value (0.01 mg/g) was recorded in T_{14} (*M. anisopliae* at 1 ppm). Irrespective of concentrations, mean maximum uptake was recorded in *T. harzianum* (0.161 mg/g). The order of fungi with respect to uptake of arsenic was *T. harzianum* > *A. niger* > *B. bassiana* > *M. anisopliae.*

From the study, it can be concluded that though the entomopathogenic fungus *B. bassiana* had low radial growth rate, high per cent growth inhibition and low spore germination at 10 ppm concentration of lead (Pb), but it had maximum lead uptake among all the fungi and could be used for remediation of low levels of lead(<10ppm). Whereas, the antagonistic fungus *T. harzianum*, had highest radial growth, lowest per cent growth inhibition and highest spore germination and maximum uptake of arsenic (As) and could be used in remediation of low arsenic (<10 ppm). The present studies also widen the scope for studying the effect of higher concentrations of heavy metals in future to confirm the survival and bioremediation ability of these fungi.

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APPENDIX– I

Analysis of variance table for effect of different doses of Pb and incubation period on radial growth

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	7.077	2.359
Concentrations	3	5.524	1.841
Fungi × Concentrations	9	0.174	0.019
Incubation Period	4	5.762	1.44
Fungi × Incubation Period	12	1.73	0.144
Concentrations × Incubation Period	12	0.188	0.016
Fungi × Concentrations × Incubation Period	36	0.581	0.016
Error	160	0.733	0.005
Total	239	21.77	

APPENDIX– II

Analysis of variance table for effect of different doses of Pb and incubation period on radial growth

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	3.426	1.142
Concentrations	3	2.951	0.984
Fungi × Concentrations	9	1.547	0.172
Incubation Period	4	5.086	1.271
Fungi × Incubation Period	12	10.994	0.916
Concentrations × Incubation Period	12	3.772	0.314
Fungi × Concentrations × Incubation Period	36	4.815	0.134
Error	160	1.605	0.01
Total	239	34.196	

APPENDIX– III

Analysis of variance table for effect of different doses of Pb and incubation period on per cent growth Inhibition

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	23,570.16	7,856.72
Concentrations	3	22,381.68	7,460.56
Fungi × Concentrations	9	11,708.58	1,300.95
Incubation Period	4	7,606.16	1,901.54
Fungi × Incubation Period	12	26,203.02	2,183.59
Concentrations × Incubation Period	12	4,193.43	349.452
Fungi × Concentrations × Incubation Period	36	13,505.36	375.149
Error	160	8,999.61	56.248
Total	239	1,18,167.99	

APPENDIX– IV

Analysis of Variance Table for effect of different doses of As and incubation period on per cent growth Inhibition

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	14,119.67	4,706.56
Concentrations	3	92,148.68	30,716.23
Fungi × Concentrations	9	19,962.28	2,218.03
Incubation Period	4	11,068.19	2,767.05
Fungi × Incubation Period	12	19,133.34	1,594.45
Concentrations × Incubation Period	12	6,857.12	571.426
Fungi × Concentrations × Incubation Period	36	18,194.04	505.39
Error	160	11,836.91	73.981
Total	239	1,93,320.22	

APPENDIX– V

Analysis of variance table for effect of different doses of Pb on dry weight after 120 hrs

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	1,79,832.77	59,944.26
Concentration	3	81,413.18	27,137.73
Fungi × Concentrations	9	36,434.63	4,048.29
Error	32	27,072.67	846.021
Total	47	3,24,753.25	

APPENDIX– VI

Analysis of variance table for effect of different doses of As on dry weight after 120 hrs

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	86,027.56	28,675.85
Concentration	3	44,600.23	14,866.74
Fungi × Concentrations	9	10,975.69	1,219.52
Error	32	15,113.00	472.281
Total	47	1,56,716.48	

APPENDIX– VII

Analysis of variance table for different doses of Pb and spore concentration on germination per cent of spores at 1×10^3 spores/ml

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	16,034.23	5,344.74
Concentrations	3	9,336.23	3,112.08
Fungi × Concentrations	9	374.854	41.65
Error	32	94	2.938
Total	47	25,839.31	

APPENDIX– VIII

Analysis of variance table for different doses of Pb and spore concentration on germination per cent of spores at 1×10^5 spores/ml

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	5,778.23	1,926.08
Concentrations	3	10,464.23	3,488.08
Fungi × Concentrations	9	563.521	62.613
Error	32	126	3.938
Total	47	16,931.98	

APPENDIX– IX

Analysis of variance table for effect of different doses of As and spore concentration on germination per cent of spores at 1×10^3 spores/ml

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	4,414.75	1,471.58
Concentrations	3	6,383.08	2,127.69
Fungi × Concentrations	9	205.417	22.824
Error	32	186.667	5.833
Total	47	11,189.92	

APPENDIX– X

Analysis of variance table for effect of different doses of As and spore concentration on germination per cent of spores at 1×10^5 spores/ml

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	3,672.83	1,224.28
Concentrations	3	4,327.17	1,442.39
Fungi × Concentrations	9	514	57.111
Error	32	170.667	5.333
Total	47	8,684.67	

APPENDIX– XI

Analysis of variance table for effect of different doses of Pb on uptake of fungi

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	0.043	0.014
Concentration	3	0.224	0.075
Fungi × Concentrations	9	0.032	0.004
Error	32	0.023	0.001
Total	47	0.323	

APPENDIX– XII

Analysis of variance table for effect of different doses of As on uptake of fungi

Source of Variation	DF	Sum of Squares	Mean Squares
Fungi	3	0.088	0.029
Concentration	3	0.237	0.079
Fungi × Concentrations	9	0.084	0.009
Error	32	0.084	0.003
Total	47	0.493	

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Title of Thesis	**:**	**"To study bioremediation and toxicity of Lead and Arsenic through entomopathogenic and antagonistic fungi"**
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Major Field	**:**	Environmental Science
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ABSTRACT

The present investigations entitled **"To study bioremediation and toxicity of Lead and Arsenic through entomopathogenic and antagonistic fungi"** was conducted during 2017-2018 in the Department of Environmental Science, College of Forestry, Dr. Y.S. Parmar University of Horticulture and Forestry, Nauni Solan H.P. The study comprised of 4 fungal strains viz. *Beauveria bassiana* and *Metarhizium anisopliae* (entomopathogenic fungi) and *Aspergillus niger* and *Trichoderma harzianum* (antagonistic fungi) which were tested for their toxicity and bioremediation for lead and arsenic (heavy metals). The cultures of these fungi were maintained under laboratory conditions in the B.O.D incubator at 25 ± 1^0C. The effect of different doses of lead and arsenic on various fungal parameters such as radial growth, per cent growth inhibition, mycelium dry weight, germination of spores and reduction potential of fungi and heavy metal uptake by fungal mycelia was studied. The whole experiment was replicated thrice in CRD. Significantly maximum mean radial growth (0.98 mmh^{-1}) was observed in *A. niger* at 0 ppm for Pb while it was 0.73 mmh^{-1} for As in *T. harzianum* at 0 ppm. The mean radial growth decreased with increase in concentrations of Pb and As from 0 to 10 ppm. At 10 ppm concentration *M. anisopliae* showed maximum per cent growth inhibition of 50.96 per cent and 73.33 per cent for Pb and As, respectively. With increase in concentration of Pb and As per cent growth inhibition also increased for each fungi. The dry weight of *A. niger* and *T. harzianum* was higher than other fungi i.e. 283.33 mg and 240.00 mg for Pb and As respectively at 0 ppm concentration. Mycelium dry weight decreased with increase in concentration of both the heavy metals. Spore germination percentage and reduction potential for all the fungi under two doses (1×10^3 spores/ml and 1×10^5 spores/ml) was recorded against both the heavy metals (Pb and As). In all the fungi per cent spore germination decreased with increase in the concentration of Pb and As while reduction potential increased with increase in concentration of Pb and As, respectively from 0 to 10 ppm. The spore germination percentage in the presence of Pb and As was in the order of *T. harzianum* > *A. niger* > *M. anisopliae* > *B. bassiana.* Maximum Pb uptake was recorded for B. bassiana at 10 ppm (0.30 mg/g). The order of fungi with respect to Pb uptake was *B. bassiana* > *T. harzianum* > *A. niger* > *M. anisopliae.* Maximum As uptake was recorded for *T. harzianum* at 10 ppm (0.324 mg/g). The order of fungi with respect to As uptake was *T. harzianum* > *A. niger* > *B. bassiana* > *M. anisopliae*. From the study it can be concluded that though the entomopathogenic fungus *B. bassiana* had low radial growth, high per cent growth inhibition and low spore germination at 10 ppm concentration of Pb but it had maximum Pb uptake among all the fungi and could be used for remediation of low levels of Pb (<10 ppm). Whereas, antagonistic fungus *T. harzianum* had highest radial growth, lowest per cent growth inhibition, highest spore germination and maximum uptake of As and could be used in remediation of low As (<10 ppm). The present studies also widens the scope for studying the effect of higher concentrations of heavy metals in future to confirm the survival and bioremediation ability of these fungi.

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