INTEGRATED APPROACH FOR PHYTOEXTRACTION OF Pb CONTAMINATED SOIL USING *PELARGONIUM* SPECIES

By

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I dedicate this thesis to my beloved parents who would always be a source of inspiration for me and stood beside me at every moment in my life

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LIST OF ABBREVIATIONS

Pb	Lead
PDA	Potato Dextrose Agar
EDTA	Ethylene Diamine Tetra Acetic Acid
DTPA	Diethylene Triamine Pentaacetic Acid
EDDS	Ethylene Diamine Disuccinic Acid
PGP	Plant Growth Promoting
Sp.	Species
PDB	Potato Dextrose Broth
DW	Distilled Water
UV	Ultra Violet

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Abstract

Lead being toxic and the most widely present heavy metal, causes different problems in the environment. Various technologies have been developed for the removal of Pb from soil. Microbes and synthetic chelate-assisted phytoextraction have been used for the remediation of Pb contaminated soils. The present study focused on the integration of synthetic chelates along with biological systems for enhanced phytoextraction of Pb by *Pelargonium hortorum*. Pb resistant fungal and bacterial strains capable of Pb solubilization were selected by growing them individually with five different metal chelators and the growth (optical density) of each strain was checked using U.V/Visible Spectrophotometer. Laboratory results showed that *Aspergillus flavus* & *Klebsiella quasipneumoniae subsp. Similipneumoniae* had significantly better growth in citric acid after 48 and 24 h, respectively. Disc diffusion method confirmed the growth compatibility of these two strains. The finally selected bacterial and fungal combination along with citric acid was applied to the pilot scale setup of Pb phytoextraction using *Pelargonium hortorum*. Three plots having different levels of Pb (13, 1187 and 2292 mg kg^{-1}, respectively) were transplanted by one-month old seedlings of *Pelargonium hortorum* and were provided with selected chelates combination under natural conditions for four months. The growth parameters of plants as well as Pb uptake by each plant grown in soil with the highest exposure level (2292 mg kg^{-1} Pb) were significantly higher as compared to the plants in 1187 mg kg^{-1} Pb and 13 mg kg^{-1} Pb. The results suggested that integration of biological and chemical amendment can be a better solution for plant growth and performance in high Pb contaminated soils.

Chapter 1

INTRODUCTION

1.1. Background

With an increase in industrial activities contamination of heavy metals has now become a major concern throughout the world as well as in Pakistan (Waseem et al., 2014). One of the most commonly used heavy metals is lead (Pb), which is present naturally in the earth's crust. Due to the unique properties of lead such as low melting point and resistance to corrosion, it is widely used in manufacturing processes (Flora et al., 2012). The increased use of lead in manufacturing processes has contaminated the environment. Being a purely toxic metal, lead is extremely harmful even in very small concentrations (Tiwari et al., 2013). According to US EPA, levels of lead in blood up to 10 µg dL^{-1} pose serious concerns in children. Higher lead levels in blood cause anemia, lower IQ as well as behavioral problems in children (US EPA, 2018). Increased use of lead has not only contaminated the soil but also damaged water quality and the environment. The heavy metal contamination of the food chain poses serious threats to human health (Toth et al., 2016).

Contamination of soil with lead has also affected the food quality and agriculture productivity in Pakistan. In turn, the consumption of food is posing serious effects on human health. It was found that Pb contamination in vegetables was from 1.8 to 11 mg kg^{-1} which exceeded the minimum acceptable limit set by FAO that is 0.3 mg kg^{-1} (Rehman et al., 2017). For this purpose, heavy metal removal techniques have been developed so that toxic effects of metals on environment and human health could be minimized (Dixit et al., 2015).

1.2. Sources of lead in the environment

The common sources of Pb contamination are emissions from vehicles, mining and smelting activities and the use of Pb based paints and batteries (Casas and Sordo, 2011). These sources of Pb have resulted in the contamination of soil and crops, and in turn have affected the whole food chain (Casas and Sordo, 2011). However, in Pakistan, the major sources of Pb contamination are mining and the use of polluted water for irrigation as well as vehicular emissions specifically in the area of Punjab (Khan et al., 2013, Nawab et al., 2015 and Khalid et al., 2018). The use of Pb contaminated water is not only polluting the soil, but also resulting in the accumulation of metal in our food chain and causing deleterious effects on human health (Perveen et al., 2017).

1.3. Lead as a contaminant

Lead is considered an occupational toxin because of its non-biodegradable and persistent properties (Flora et al., 2012). It is considered as one of the most toxic components of airborne particulate matter (Waseem et al., 2014). According to EPA, acceptable level of Pb in residential soil is 400 ppm for play areas and 1200 ppm for non-play areas (Agency for Toxic Substances and Disease Registry, 2017). The primary route of Pb exposure in human bodies is through respiratory tract. However, some other routes include the consumption of contaminated water and food (Mushak, 2011; Agency for Toxic Substances and Disease Registry, 2007). They cause lethal effects through acute and chronic toxicity. Some of the deleterious effects of Pb are encephalopathy, hemolytic anemia, renal dysfunction, ischemic coronary heart disease and infertility (Flora et al., 2012). Pb not only affects humans but also has severe effects on plants. Some of the effects of Pb on plants are oxidative stress, cytoplasmic enzymes inhibition, and negative effects

on plant's growth as well as on the activities of soil microbiota (Asati et al., 2016). Heavy metals not only affect the quality of soil microbial community but also its quantity. Heavy metals have greater impact on the reproduction of microorganisms by reduction in the metabolic enzymes (Chu, 2018).

1.4. Lead removal techniques

In order to remove Pb from soil, a number of physical, chemical and biological technologies have been developed. These technologies include isolation, immobilization, toxicity reduction, physical separation and extraction which are *in-situ* as well as *ex-situ* techniques. However, these approaches have different disadvantages and side effects on soil properties and biodiversity. Also, these are either not economically feasible or may further contaminate the environment through the addition of chemicals or extractants (Evanko and Dzombak, 1997; Thakur et al., 2016). It has been reported that among all the techniques used for soil remediation, biological method specifically phytoextraction is considered the best and most eco-friendly way, which involves the use of hyperaccumulator plants for heavy metals removal from soil or water (Ali et al., 2013). For the effective phytoextraction of metal, it should be easily bioavailable to the plant, which is a major limitation of this approach. Due to soil properties, Pb may not be available easily to the plant due to its adsorption to soil particles (Meyerholt, 2013). In order to enhance the process of phytoextraction and make Pb available to plants, a number of studies have been performed. Usually organic or inorganic chemicals or chelators are applied in the soil to make lead available to plants. Natural chelators commonly used are actually low molecular weight organic acids (LMWOA). They include oxalic acid, citric acid, fumaric and tartaric acid. Synthetic chelating agents used in Phytoextraction are Ethylene diamine

tetra acetic acid (EDTA), Diethylene triamine penta acetic acid (DTPA) and Ethylene diamine disuccinic acid (EDDS) (Shahid et al., 2012). Apart from the use of chelating agents, some studies have focused on the use of mycorrhizal fungus and PGP bacteria which are said to improve the metal uptake by plants, enhancing the phytoextraction (Ullah et al., 2015 and Bhargava et al., 2012).

1.5. Significance of the study

Studies reported thus far have involved either the use of microbes, fungus or chelators in the phytoremediation of heavy metals. In order to enhance the bio-availability and uptake of metals and to decrease the toxic effects of chelators on soil and water, integration of organic chelator, bacteria and fungi for the Phytoextraction of Pb has been the focus of present study. The study involves the integration of bacterial and fungal strains previously studied and isolated by Manzoor et al. (2018) and a metal chelator along with the use of *Pelargonium* species for Pb contaminated soil.

1.6. Objectives

Keeping in view the literature available and previous studies done within Environmental Biotechnology research group at Institute of Environmental Sciences and Engineering, the specific objectives of present study were:

1. Optimization of conditions for Pb phyto-extraction from spiked soil.
2. Integrated Pb phyto-extraction at pilot scale.

Chapter 2

LITERATURE REVIEW

2.1. Lead contamination in Pakistan

Contamination of heavy metals specifically lead (Pb) has now become a major concern for developing countries including Pakistan. Industrial activities including mining, smelting, manufacturing along with vehicles are releasing large amount of Pb in air, water and soil thereby contaminating the environment. From soil and plants, metal transfers to livestock and hence enter in our food chain. Pb is considered as one of the most toxic components of airborne particulate matter. It has been found that sources like contaminated food, house and respirable dust are the major causes of Pb poisoning in newborns and pregnant women (Fatmi et al., 2017).

The predicted emission levels of Pb from coal and oil industries around the world each year are 450 million kg while for natural sources, it is 30 million kg each year. The use of Pb-free fuel has decreased the concentration of lead in Islamabad, but the current levels are still high ranging from 0.002 to 4.7 $\mu g\ m^{-3}$. According to WHO air quality guidelines, the average concentration of Pb for urban air quality is between 0.15 and 0.5 $\mu g\ m^{-3}$ and hence Islamabad is facing a serious problem of air pollution (Waseem et al., 2014).

For fruits and vegetables, the maximum acceptable limit of Pb given by WHO and FAO is 0.3 $mg\ kg^{-1}$. The Pb concentration found in fruits and vegetables collected from various site of Khyber Pakhtunkhua showed an increased concentration of Pb ranging from 1.8 to 11 $mg\ kg^{-1}$ (Rehman et al., 2017).

Mining activities greatly affect the pollution status of an area. The samples of soil and plants collected form mine affected area in Shangla District, Pakistan showed high

concentration of Pb and cadmium in plants. Most of the plants exceeded the concentration of Pb as recommended by WHO while in soil, Pb concentration was highest as compared to Cadmium, which is 82 mg kg^{-1} (Nawab et al., 2015).

Another source of heavy metal contamination in crops is the use of sewage water for irrigation purposes. Irrigation of *Allium sativum* L. with municipal waste water in District Khushab resulted in the increased metal content in the crop. The concentration of Pb in soil irrigated with sewage water was 36.94 µg g^{-1} whereas 47.5 µg g^{-1} arsenic was found which exceeded permissible limit of 20 µg g^{-1}. This situation is posing serious threats to the health of locals (Khan et al., 2017). The food crops in the areas of Sialkot and Wazirabad were also irrigated with water from polluted streams which was highly contaminated with heavy metals especially Pb. The concentration of metals in crops irrigated with waste water was higher than water from tube well. Also, the HRI and accumulation factor in crops and vegetables for Pb was above permissible limits i.e. greater than 1 which can pose serious health effects (Khan et al., 2013).

2.2. Methods of soil heavy metal removal

To eliminate toxic heavy metals from the soil, several physical, chemical and biological technologies have been developed and used. However, they have different disadvantages and side effects on soil properties and biodiversity.

2.2.1. Physical methods

The physical technologies of soil heavy metal removal include:

- Soil replacement; replacing polluted soil by non-polluted soil.
- Electrokinetic remediation; separation of heavy metals in soil through electrophoresis.

- Isolation; separating the contaminated soil from non-contaminated soil by using sub-surface barriers.
- Vitrification; reducing the mobility of metals in soil by applying high temperature (Khalid et al., 2017).

2.2.2. Chemical methods

Some of the chemical methods to remove heavy metals from soil are:

- Encapsulation; contaminated soil mixed with concrete, lime or cement to reduce the contamination of soil.
- Soil washing; mixing of contaminated soil with extractants or reagents and metal is transferred to liquid phase.
- Immobilization; addition of chemical agents in soil to immobilize the metals or to decrease their bioavailability (Khalid et al., 2017).

The drawbacks associated with these remediation approaches are:

- Increased cost
- Soil compaction in case of sub-surface barriers
- Contamination of environment through treatment by the addition of chemicals
- Soil acidification
- Ex-situ process not suitable for soil with homogenous distribution of pollutants

2.2.3. Biological Methods

Biological method of heavy metal removal includes bioremediation in which microbes or phytoremediation in which plants are used for the removal of metals from soil. Among all the techniques used for soil remediation, phytoremediation is considered the best and most

eco-friendly method of heavy metal removal (Evanko and Dzombak, 1997; Thakur et al., 2016).

2.3. Phytoremediation

The word phytoremediation is a combination of two words, '*phyto*' for plant and '*remedium*'; to remove. Hence, phytoremediation is a process in which plants are used to remove heavy metals from soil or water (Ali et al., 2013).

Phytoremediation is further divided into the following processes:

1. Phytoextraction; using hyper-accumulator plants to remove metals and accumulate them in roots and shoots of plants.
2. Phytostabilization; limiting the availability and mobility of metals in the soil with the help of plants.
3. Rhizofiltration; using roots to remove heavy metals from polluted water through absorption, concentration and precipitation (Evanko and Dzombak, 1997).

The processes involved in phytoremediation have been shown in figure 2.1.

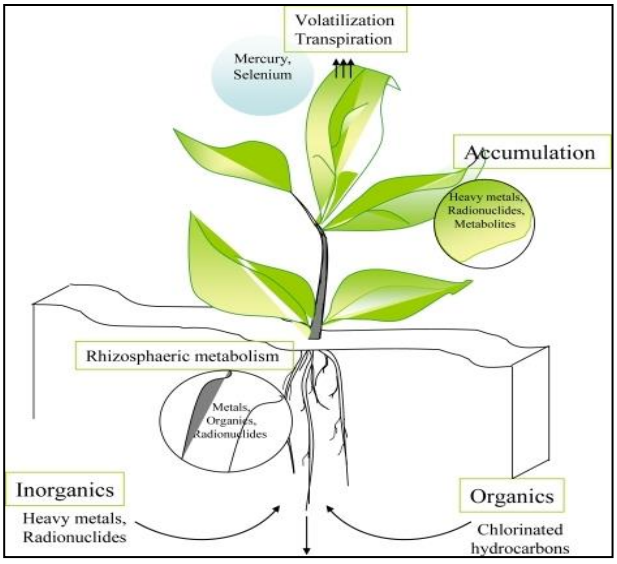

Fig.2.1. Phytoremediation remediation of heavy metals

2.4. Phytoextraction of Lead

Phytoextraction process involves the use of hyper-accumulator plants having the ability to absorb and collect high level of metals in their parts, especially shoots, compared to other plants (Thakur et al., 2016)

These plants accumulate not only essential micro elements but also non-essential elements or metals in their shoots are called hyperaccumulator plants. They are able to accumulate more than one metal with the ability to detoxify these metals. As compared to non-accumulator plants, Hyperaccumulators uptake 100 times more concentration of metals than the former one and have bio-accumulation factor greater than one (Ashraf et al., 2010). The most important aspect of an effective phytoextraction is the ability of plants to accumulate metal in their shoots as the biomass of roots is not achievable. The process of phytoextraction usually depends on the bioavailability of metal in the soil (Ali et al., 2013). Although, Pb is present naturally in the soil ranging from 50 to 400 mg kg^{-1} (US EPA, 2018). Low concentration and bioavailability of metal greatly affect the phytoextraction of metals (Ali et al., 2013).

2.4.1. Ornamental plants as hyperaccumulators of Pb

Apart from using food crops, ornamental plants have been recently used for phytoremediation purposes. Various studies involve the use of ornamental plants that act as hyperaccumulator for heavy metals. It has been found that ornamental plants possess the ability to store high levels of heavy metals which make them suitable for phytoremediation (Liu et al., 2017).

Tauqeer et al. (2016) studied the phytoremediation potential of *Alternanthera bettzickiana* by exposing the plant to several levels of Pb and Cd. The study revealed that

the plant's growth response to metal levels at the concentration of 1 mM was higher and with level of 2mM, the growth decreased. However, the uptake of metals by plants as well as shoot-to-root ratio of metals increased with an increase in the concentration of metals in the soil.

Pb phytoremediation potential of *Cornopus didymus* L. has been investigated by Sidhu et al. (2018). Different concentrations of Pb in soil have been used to check the uptake level and accumulation potential of the plant. The study showed that Pb uptake by plants increased with increasing Pb concentration i.e. 100 mg kg^{-1} to 2500 mg kg^{-1}. The 6 weeks old plants showed higher concentration of Pb both in shoots and roots than 4 weeks old plants. In roots, Pb concentration was found to be 3091 mg kg^{-1} while it was 527 mg kg^{-1} in shoots. Although the plant did not show the Pb phytoremediation potential greater than 1000 mg kg^{-1} in roots but has a potential for Pb accumulation.

Hardy limelight hydrangea (*Hydrangea paniculata)* and common sunflower (*Halianthus annuus)* have also been examined for their ability to remove heavy metals by Forte and Mutiti (2017). It has been found that *H. paniculata* is able to uptake large concentration of Pb from soil but the translocation from stem to leaves is less. On the other hand, common sunflower contained more Pb in leaves than stem.

Another ornamental plant *Chrysanthemum indicum* L. has been studied for the phytoextraction of Pb in a pot experiment by Mani et al. (2015). The study conducted in India, indicated that phytoextraction was improved by the plant when Sulfur and vermicompost were applied in combination to Pb contaminated soil. It was found that addition of elemental Sulfur and vermicompost (0.6 mg kg^{-1} and 0.8 mg kg^{-1} each) with

different levels of Pb (0, 10, 20 and 50 mg kg^{-1}), enhanced the uptake of lead by plants and hence improved the remediation efficiency.

2.4.2. Pelargonium species for phytoextraction

Various studies suggest that *Pelargonium* species, also known as "Geraniums", can be used for the bioremediation of contaminated soil. *Pelargonium*s has the ability to grow and survive on soil contaminated with various metals and can accumulate more than one metal at a time. The major benefit of using geraniums is that the contaminant can be extracted from their biomass through the plant's essential oil (Singh, 2005). Arshad et al. (2016) reported that pH of rhizospheric soil was reduced when Pelargonium cultivars (Attar of Roses) was grown. However, both cultivars Concolor and Attar of roses increased the content of dissolved organic carbon two and three times, respectively. The study suggested that Attar of roses acidified the soil by 0.4 pH units and more Pb was accumulated in its roots and shoots.

Different *Pelargonium species* have been identified as hyperaccumulator of heavy metals. Mahdieh et al. (2013) determined the capacity of *Pelargonium roseum* which accumulated 86,566 mg kg^{-1} of Pb in roots while 4,416 mg kg^{-1} Pb in shoots dry biomass of plants and is regarded as a hyperaccumulator.

2.5. Enhanced Phytoextraction of Metals

Availability of metals in soil depends upon the characteristics of metal and hence there are three categories:

- Easily bioavailable: these metals are Cd, Ni, Zn, As, Se, Cu
- Slightly bioavailable: metals include Co, Mn, Fe
- Least bioavailable: the metals are Pb, Cr, U (Prasad, 2003).

In order to cope up with the availability of metals, plants have developed methods to solubilize heavy metals present in soil. In this procedure, phyto-siderophores are released by the roots of plants which mobilize metal that are easily taken up by plants (Leung, 2013). In case of Pb, the bioavailability and mobility depend on its chemical form in the soil (Shahid et al., 2012). Pb generally exist in soil either as an inorganic component (HCO_3, CO_3^{2-}, SO_4^{2-} and Cl^-) or in the form of organic ligands such as amino acids, fulvic acids or humic acids. As Pb binds strongly with soil particles or components, it is less available for plants (Pourrut et al., 2011).

Depending upon the chemical nature of metals and their availability to plants, phytoextraction has been divided into continuous and induced phytoextraction. Continuous phytoextraction involves the natural capability of hyperaccumulator plants to uptake heavy metals in their tissues. While induced phytoextraction involves the addition of substances such as metal chelating agents or microbes to enhance the process (Leung, 2013).

2.6. Approaches for enhancing metal phytoextraction

In order to enhance the process of phytoextraction to make the Pb bioavailable, a number of studies have been performed. These studies include either the addition of a chelator or microbes to mobilize Pb and make it bioavailable for the plants. Natural chelators commonly used are actually low molecular weight organic acids (LMWOA) which include oxalic, citric, fumaric and tartaric acid. Synthetic chelators have also been used in phytoextraction which are Ethylene diamine tetra acetic acid (EDTA), Diethylene triamine pentaacetic acid (DTPA) and Ethylene diamine disuccinic acid (EDDS), etc. (Shahid et al., 2012).

The use of Fungi, especially mycorrhizal fungi is also said to improve the availability and mobilization of Pb thereby enhancing the phytoextraction process (Bhargava et al., 2012). Plant Growth Promoting (PGP) Bacteria, have also been reported to be used in the phytoextraction of Pb. PGP bacteria are said to possess growth promoting traits that improve the growth of plants. They also make the metals available to plants by releasing siderophores and organic acids thereby converting them into soluble forms (Ullah et al., 2015).

2.6.1. Chelate Assisted Phytoextraction

Use of chelating agents especially EDTA has been extensively used for heavy metal phytoextraction. EDTA is considered and effective chelating agent because it improves the solubility of heavy metals (Shahid et al., 2014). Because of the high metal solubilizing ability, EDTA is normally used for Pb phytoextraction. Zaier et al. (2014) studied the effect of EDTA of the phytoextraction of Pb using *Sesuvium portulacastrum* in Tunis. It was found that 2265 µg Pb per plant was accumulated in the shoots after the application of EDTA to the soil contaminated with 800 mg kg^{-1} Pb^{2+}. Also, the plant biomass was improved considerably after the addition of EDTA as compared to control plants.

Ethylenediaminedisuccinic acid (EDDS) has also been used as a chelating agent for Pb phytoextraction by Attinti et al. (2017). In this study, vetiver grass was used which is the hyperaccumulator plant. The soil samples were taken from residential areas of Baltimore and San Antonio with Pb concentration from 1000 to 2400 mg kg^{-1}. The results revealed that Pb uptake by vetiver grass was increased after the addition of EDDS. The concentration of Pb after first application (10 mmol kg^{-1}) of EDDS was 105.3 mg kg^{-1} in roots and 22.5 mg kg^{-1} in shoots of vetiver grass. However, after the second application (30

mmol kg^{-1}), this concentration increased to 3.6 and 8.3 folds in shoots and roots of vetiver grass respectively. However, the use of chemical chelates pose threat to the ground water in the form of leaching. It has been found that the EDTA application caused the leaching of heavy metals in deeper soils and ground water. Also, the leaching of heavy metals depends upon the particle size and soil pH. Higher pH with greater particle size pose lesser risk of EDTA leaching (Lu et al., 2017). EDDS also caused leaching of Pb as it mobilized the metal and is also a risk to groundwater. To overcome this, optimum dosage must be selected which is balanced with the uptake by plant (Attinti et al., 2017).

Another solution to overcome leaching of heavy metals is to apply small amount of synthetic chelating agents but it may affect the enhancement of phytoextraction which is the sole purpose of chelators (Evangelou et al., 2007). As most of the synthetic chelating agents such as EDTA and EDDS cause leaching effect, hence they limit the phytoextraction process. Low molecular weight organic acids (LMWOA) such as citric acid may be used as an alternative of EDTA. Another advantage of citric acid is that it is highly biodegradable, limits the leaching of heavy metals and hence an environmental friendly approach (Shakoor et al., 2014).

Freitas et al. (2013) used citric acid with vetiver grass for Pb phytoextraction near the battery recycling site in Brazil. The average concentration of Pb in the area was found to be 1553 mg kg^{-1}. The removal efficiency of Pb also depended on the spacing of vetiver grass. The application of 40 mmol kg^{-1} citric acid caused an increase in Pb solubility from 136 to 314 mg L^{-1}. Pb accumulation in vetiver grass increased to 10, 7 and 7 times with different spacings (0.80, 0.65 and 0.50 m) compared to control in which 172 mg kg^{-1} Pb was accumulated. As citric acid is a low molecular weight organic acid, it increases the

mobility of Pb by decreasing soil pH through increased oxidation. Citric acid also forms metal organic complex which is taken up by plants. In addition to that, it not only improves photosynthetic characteristics but also the morphology of plants (Shakoor et al., 2014).

2.6.2. Microbe Assisted Phytoextraction

Recently, microbe assisted phytoextraction has emerged as a promising technique for the remediation of contaminated soil. It is an environmentally friendly as well as cost effective method. The incorporation of arbuscular mycorrhizal fungus and plant growth promoting rhizospheric bacteria in phytoextraction improves the process as the microbes form symbiotic association with plants. Not only the growth of plants is enhanced but bioavailability of contaminant is increased (Karimi et al., 2018). Also, the use of biological chelating agents is a greener solution for heavy metal contamination with lesser cost compared to chemical chelators (Munawar and Iram, 2013). The basic mechanism behind improved phytoextraction with the addition of microbes is their growth promoting traits and siderophores production. Plant growth promoting fungal and bacterial strains not only enhance metal accumulation but also the biomass of plants. Production of siderophores reduces IAA degradation and improvement of plant's resistance against heavy metals. Siderophores also bind to metals converting them into available forms by making metal-siderophores complex. Outer membrane receptor proteins on plants specific to siderophores allow their uptake and hence metal uptake in plants (Seneviratne and Vithanage, 2015; Ahmed and Holmstrom, 2014; Rajkumar et al., 2010).

2.6.2.1. Bacterial Assisted Phytoextraction

Bacterial assisted phytoextraction has been studied by Jing et al. (2013). In this study strains of *Klebsiella* and *Enterobacter* were isolated from rhizospheric soil and inoculated in *Brassica napus*. The study showed an increased Pb uptake in the roots (75%) and shoots

(49%) of the plant in the presence of *Klebsiella* strain. While, addition of *Enterobacter* sp. resulted in 65% increase in Pb uptake as compared to control in soil with up to 200 mg kg^{-1} of Pb. This confirmed that addition of *Klebsiella* and *Enterobacter* improves the metal uptake by plants (Jing et al., 2014).

Zea mays L. has also been used for the phytoextraction of Pb. Praburaman et al. (2016) combined the application of *Herbaspirillum sp.* with *Zea mays* and found that strain showed plant growth promoting traits. As a result, the metal uptake in roots of the plant was 25% while it was 77% in shoots of *Zea mays* as compared to control plants.

2.6.2.2. Fungal Assisted Phytoextraction

Phytoextraction of heavy metals with the help of fungi is considered an important technique for soil remediation. Fungi is found to have higher tolerance against heavy metals than bacteria because of high biomass and metal chelating properties. So, the addition of fungal strains also enhanced the process of phytoextraction (Hiroki, 1992; Deng and Cao, 2017).

MG isolate of *Trichoderma sp*. has been used for the phytoextraction of Pb contaminated soil along with *Helianthus annuus* in Tamil Nadu, India. *Trichoderma sp.* was segregated from decomposed wood and is said to improve the growth of plants. It was found that *Helianthus annuus* inoculated with *Trichoderma sp.* MG showed 59% and 48% Pb accumulation in shoots and roots respectively, in a soil contaminated with 250 mg kg^{-1} Pb. This can be due to the reason that MG isolate of *Trichoderma sp.* has growth promoting properties and may have reduced the phytotoxicity. This combination was found to be an effective solution for Pb phytoextraction (Govarthanan et al., 2018).

Deng et al. (2010) studied the effect of *Mucor sp.* on the phytoextraction of Pb. This strain was isolated from roots of rape plant from metal contaminated mining area in China.

Mucor sp. is known as a fungal endophyte and is resistant to heavy metals. The results revealed that *Mucor* sp. is able to accumulate more than one metals. Also, the strain was able to bioaccumulate high concentration of metal in a solution containing multiple metals. 74.4 mg g^{-1} Pb was bioaccumulated from a solution containing 2 mM Cd + 1 mM Pb. While, the biosorption capacity for Pb was 88.2 mg g^{-1}. The addition of active mycelia also increased the availability of Pb to plants by 77% (Deng et al., 2011).

2.6.3. Integrated Phytoextraction

Combined application of microbes and chelators have also been studied recently. It has been known that chelators enhance the mobility of metals while microbes enhance heavy metal uptake by plants. Therefore, to further improve the process of phytoextraction, combine application of chelators and bacteria or fungus may be useful in the remediation of contaminated soil (Kutrowska et al., 2016).

A similar study was performed by Jarrah et al. (2014) in which combined application of Arbuscular Mycorrhizal Fungus (AMF); *Glomus mosseae* and EDTA was done on Pb contaminated soil using Sunflower. It was found that applying AMF and EDTA together increased the concentration of Pb in Sunflower. Growth of plants in soil with 400 mg kg^{-1} of Pb showed higher uptake index when inoculated with *G. mosseae* and EDTA (Jarrah et al., 2014).

Chapter 3

MATERIALS AND METHODS

This section describes the framework of experimental procedure implemented during this study. The experimental work was performed in the Environmental Biotechnology Laboratory at IESE, NUST, Islamabad. In this study, fungal and bacterial strains along with citric acid as a chelator were added to the plants. The seedlings of *Pelargonium hortorum* were grown in two different concentrations of Pb contaminated soils. The selected amendments were added to one-month old seedlings and an exposure time of four months was given to them. After this, harvesting of plants was done for the analysis of heavy metals. Keeping in view the objectives, the methodology has been discussed in detail.

3.1. Treatments

3.1.1. Selection of bacterial strain

Initially, growth of four bacterial strains capable of solubilizing Pb was checked in the presence of five different chelators. Firstly, bacterial inoculum was prepared by dissolving bacterial strain in distilled water with the help of inoculation loop. The optical density of this inoculum was checked at 600 nm on UV/ visible spectrophotometer (Specord 200 plus Analytikae Jena, Germany) and was set to 0.5. Afterwards, 200 µL of each bacterial strain was added in 10 mL autoclaved nutrient broth (Oxoid). The broth contained predetermined concentrations of Pb as Pb $(NO_3)_2$ and amendments (ammonium nitrate: 10 mmol kg^{-1}, citric acid: 10 mmol kg^{-1}, compost: 10%, EDTA: 5 mmol kg^{-1} and nanoparticles: 100 mg kg^{-1}). As a control, liquid medium without the addition of metal and chelator was used

while another medium contained only Pb and no chelator. The inoculum was then placed in the shaking incubator (Lab Tech, Daihan Laboratories) as shown in figure 3.1 for 24 hours at 150 rpm and 30ºC temperature.

Fig. 3.1 Bacterial inoculum in the shaking

After 24 hours, the optical density of strain was checked again using UV/Visible Spectrophotometer (Specord 200 plus) as shown in figure 3.2. The growth of bacterial strains was determined by comparing the optical density to 0.5 (He et al., 2009). The experiment was performed in triplicates.

Fig. 3.2 Analyzing optical density with U.V. Visible Spectrophotometer

3.1.2. Selection of fungal strain

Three fungal strains with the ability of Pb solubilization were grown in autoclaved Potato Dextrose Broth (PDB) along with pre-determined concentrations of Pb as $(PbNO_3)_2$ and five chelators. Control was kept by taking PDB without addition of Pb or chelator. Another medium contained only Pb in it and no chelators. For the growth of fungal inoculum, 300 µL of it was added in 20 mL of PDB containing chelators and Pb. The optical density of fungal strain was set at 0.5 as that for bacteria. The strains were then placed in the Shaking Incubator (Lab Tech, Daihan Laboratories) at 150 rpm and 30ºC temperature. The optical density of fungal strain was checked after 48 hours at 580 nm wavelength using UV/Visible Spectrophotometer (Specord 200 plus). The whole experiment was performed in triplicates (He et al., 2009).

3.1.3. Selection of metal chelator

All the bacterial and fungal inocula were grown in the presence of five amendments with following concentrations:

- Ammonium Nitrate: 10 mmol kg^{-1}
- Citric Acid: 10 mmol kg^{-1}
- Compost: 10%
- EDTA: 5 mmol kg^{-1}
- Nanoparticle: 100 mg kg^{-1}

The above-mentioned concentrations of amendments were selected on the basis of previous study conducted by Gul et al. (2018). It was suggested that these concentrations of amendments showed better accumulation of metal in plants.

The optical density of strains was compared with the control to check the effect of each amendment on the growth of strains. The amendment which enhanced the growth of both bacterial and fungal strains was selected. The experiment was performed in triplicates.

3.1.4. Compatibility of selected bacterial and fungal strains

To check the growth compatibility of selected bacterial and fungal strains, disc diffusion method with slight modifications was performed. For this purpose, a 5mm disc of Whatman No. 1 filter paper was dipped in bacterial inoculum with optical density set at 0.5. The disc was placed in a petri plate of PDA. On the opposite side of the disc a plug of *Aspergillus flavus* was placed and the plate was sealed with para film. It was incubated for 4 days in an incubator (Memmert INO 200).

The growth of *Klebsiella quasipneumoniae subsp. similipneumoniae* with *Aspergillus flavus* confirmed that both strains can grow in the presence of each other (Mishra et al., 2011).

3.2. Soil heavy metal analysis

Soil sample of 0.5 g was taken and digested using 1:3 (v/v) HNO_3 and HCl. Samples were digested on a hot plate with the initial temperature of 50 °C and then raising the temperature gradually to 150 °C. The samples were digested until all soil disappeared and color changed to pale yellow or green. Distilled water was added in the sample up to 50 mL followed by its filtration using filter paper. The resulting filtrate was then stored in the plastic bottle in a refrigerator for further analysis. Atomic Absorption Spectrophotometer (A-7000 F Schimadzu) was used for Pb determination (Rashid et al., 2016) given in figure 3.4.

Fig. 3.4. Heavy metal analysis on atomic absorption Spectrophotometer

3.3. Site selection

To undertake the pilot scale experiment, a site was selected within NUST campus. The selected site was divided into three plots having the size of 1.69 m^2. The division is based on the different concentration of Pb in the soil. Following concentrations of Pb were found after soil analysis on the basis of which the study area was divided:

- T_0: 13 mg kg^{-1} Pb
- T_1: 1187 mg kg^{-1} Pb
- T_2: 2292 mg kg^{-1} Pb

Each plot contained approximately 300 kg of soil. The selected area has been shown in figure 3.5.

Fig. 3.5. Study area at IESE, NUST

3.4. Physicochemical Analysis of Soil

The parameters analyzed in experimental soil include pH, moisture content, water holding capacity, soil texture, nitrate-nitrogen, total organic carbon and total phosphorus.

3.4.1. Soil pH, texture and electrical conductivity

pH of the soil was measured to check whether the selected soil is appropriate for the growth of plant. Air dried soil (10 grams) with a particle size of less than 2 mm was taken in 50 mL conical flask followed by the addition of 50 mL distilled water using measuring cylinder. The mixture was placed at a mechanical shaker (figure 3.6) at 180 rpm for 30 minutes for proper mixing and left to stand for 1 hour.

Fig. 3.6. Soil samples in a mechanical shaker

The soil was then filtered with whatman no. 1 filter paper (McLean, 1982). Reading was taken with the help of pH and EC meter. However, soil texture was determined by saturation percentage method. In this method, equal amount of soil and water were taken (20 g each). Water was added slowly in the soil until a thin paste was formed (Malik et al., 1984). Using following formula texture was calculated:

Soil Texture (%) = Volume of water required / Weight of dry soil ×100

3.4.2. Soil moisture content

Air dried soil (10 grams) with a particle size < 2 mm was weighed using weighing balance and placed in a china dish. It was then dried in oven for 24 hours at 105 °C. The china dish was then removed from the oven carefully and was left for 30 minutes to cool. The soil was re-weighed (Estefan et al., 2013).

Following formula was used to calculate moisture content:

% moisture in soil= (Wet soil-dry soil) / (dry soil) ×100

3.4.3. Water Holding Capacity

Using weighing balance, 10 grams of air-dried soil was weighed. Whatman filter paper no. 42 was then placed in a funnel. Weighed soil was then placed on the filter paper. After this, 10 mL of distilled water was poured onto the soil using measuring cylinder. The filtrate was collected in a graduated cylinder. The final volume of filtrate was noted (Harding and Ross, 1964).

To calculate Water Holding Capacity, following formula was used:

WHC= (water added-water drained) / (water added) ×10

3.4.4. Nitrate-Nitrogen

For the determination of nitrate-nitrogen, 10g air dried soil was taken in an Erlenmeyer flask and 50 mL of 0.02 N $CuSO_4.5H_2O$ was added in it. The solution was placed in shaker for 15 minutes and then filtered using double whatman no. 42 filter paper. After filtration, 3 mL of filtrate was added in a 50 mL conical flask using pipette and placed in cold water for some time. After this, 1 mL of 0.1% chromotropic acid was added in the solution drop wise without mixing the solution. The solution was again placed in cold water. Preparation of 0.1% chromotropic acid took place by dissolving 0.368 g of this acid in 200 mL of concentrated H_2SO_4 and kept in dark bottle. Concentrated H_2SO_4 (6 mL) was added in the solution followed by swirling of the flask and cooling at room temperature. After 45 minutes, yellow color developed. For the preparation of stock solution, 3.6092 g oven dried KNO_3 was added in 500 mL of 0.02 N $CuSO_4.5H_2O$. This stock solution was diluted by taking 10mL of it and adding 200 mL of $CuSO_4.5H_2O$ in it. From this diluted stock solution, standard solutions of 1 to 7 mL were prepared. Standard curve was prepared using

these standards on UV/ Visible spectrophotometer (Specord 200 plus) at 430 nm wavelength and concentration of unknown samples was found from the curve (Estefan et al., 2013). Following formula was used to determine NO_3-N concentration:

NO_3-N (mg kg^{-1}) = ppm NO_3-N (from calibration curve) × V/ W_t × V_2/V_1

3.4.5. Total organic carbon

Walkley- Black method was followed for the analysis of total organic carbon in the soil. In a 500-mL beaker, 1 g of air dried soil was added followed by the addition of 10 mL 1N potassium dichromate solution and 20 mL concentrated H_2SO_4. After 30 minutes, 200 mL of DW water and 10 mL of H_3PO_4 was added in the soil and allowed to cool. Few drops of diphenylamine indicator were added in the mixture along with a magnetic bar and placed on the magnetic stirrer. The mixture was titrated against 0.5M ferrous ammonium sulfate solution till the color changed from violet blue to green (Estefan et al., 2013). Change in colors after titration is shown in figure 3.7.

Fig. 3.7. Color changed from violet blue to green in TOC analysis

Following formulas were used to calculate total organic carbon.

$$\mathbf{M = 10 / V_{blank}}$$

$$\mathbf{Oxidizeable\ Organic\ Carbon\ (\%) = (V_{blank} - V_{sample}) \times 0.3 \times M / Wt}$$

$$\mathbf{Total\ Organic\ Carbon = 1.334 \times Oxidizeable\ Organic\ Carbon}$$

3.4.6. Extractable phosphorus

Extractable phosphorus was performed using sodium bicarbonate method. In this procedure, 100 mL of 0.5 M $NaHCO_3$ was added in 5 g air dried soil. The soil was mixed at 200 rpm for 30 minutes and then filtered. From this this clear filtrate, 10 mL was taken in 50 mL flask which was diluted to 40 mL with Distilled water and 8 mL of reagent was added. This reagent was prepared by dissolving 1.056 g L-ascorbic acid in 200 mL of ammonium heptamolybdate-antimony potassium tartrate reagent. The standard stock solution was prepared by dissolving 2.197 g oven dried KH_2PO_4 in distilled water and brought to 1-L volume. This contained 500 ppm stock solution. Out of this stock solution, 50 mL was taken and diluted to 250 mL with distilled water. This contained 100 ppm diluted stock solution. Out of this diluted stock solution, 5, 10, 15, 20 and 25 mL was taken and further diluted with distilled water to prepare standard stock solutions.

These standards were then prepared the same way soil samples were prepared as shown in figure 3.8. They were run on UV/ Visible Spectrophotometer (Specord 200 plus) at 882 nm wavelength and calibration curve was obtained (Estefan et al., 2013).

Fig. 3.8. Standards for extractable phosphorus

Following formula was used to calculate extractable phosphorus:

Extractable P (mg kg^{-1}) = mg kg^{-1} P (from Calibration curve) × V / Wt × V_2 / V_1

3.5. Transplantation of seedlings

Seedlings of experimental plant geranium (*Pelargonium hortorum*) were purchased locally. Seedlings of identical size were selected and sown in each plot with 30 cm distance from each other. Transplantation of seedlings is shown in figure 3.9.

Fig. 3.9. Transplantation of seedlings

3.5.1. Application of amendments and plants harvesting

Selected amendments i.e. *Klebsiella quasipneumoniae subsp. Similipneumoniae, Aspergillus flavus* and citric acid were applied to one-month old seedlings grown in the pilot scale area. The inocula of fungus and bacteria were individually dissolved in 500 mL distilled water. The optical density was brought to 0.5 using U.V/ Visible spectrophotometer (Specord 200 plus). The volume of 10 mL was taken from each of the strains and then applied to plants in the soils with 1187 (T_1) & 2292 (T_2) mg kg^{-1} Pb. Citric acid was used as a metal chelator and 0.44 kg of citric acid was dissolved in 5 L of water. After this, 10 mL of citric acid was given to each plant in 1187 & 2292 mg kg^{-1} Pb soil. The plants were harvested after giving them 5 months exposure time of amendments. The application of amendments and harvesting of plants is given in figure 3.10a and b respectively.

Fig. 3.10 (a). Application of amendments

Fig. 3.10 (b). Harvesting of plants

3.6. Plant Analysis

Following analysis on plants were performed:

3.6.1. Plant Length measurement

After harvesting of *Pelargonium*, the plants were washed with water, and length was measured as given in figure 3.11.

Fig. 3.11. Plant length measurement

3.6.2. Plant biomass determination

Roots and shoots of *Pelargonium* were cut, and their fresh biomass was weighed one by one. For dry biomass, shoots and roots were placed in hot air oven (Lab Tech LDO-030 N) at 70°C for 48 hours as given in figure 3.12 (a) and the plant material was then weighed. Electric grinder was used for grinding the shoots and roots separately, depicted in figure 3.12 (b) and air tight sampling bags were used for the storage of plants until used for further analysis.

Fig. 3.12 (a). Hot air oven

Fig. 3.12 (b). Crushing of plants

3.6.3. Plant heavy metal analysis

For plant heavy metal analysis, 0.2 g grounded sample of plant was taken in 25 mL flask and digested using 3:1 ratio of nitric and perchloric acid. The sample was digested till the plant material disappeared and color changed to pale yellow/ green. The digested sample was then brought to volume adding distilled water and filtered using Whatman no. 1 filter paper. The sample was stored in plastic bottles in a refrigerator for further analysis (Rashid et al., 2016.

3.7. Statistical Analysis of Data

Statistical significance of the results was checked using the software “Statistics 8.1” by applying single factor ANOVA and LSD through all pair-wise comparison. Results were considered statistically significant when the probability was less than 0.05.

Chapter 4

RESULTS and DISCUSSION

4.1. Selection of treatments

4.1.1. Selection of bacterial strain

Figure 4.1 shows the growth of four bacterial strains in the presence of Pb and five chelators which are ammonium nitrate, citric acid, compost, EDTA and nano particles. The results showed that all of the bacterial strains had almost significant growth in the presence of compost when compared with control except *Microbacterium paraoxydans*. This is due to the reason that addition of compost significantly increased the microbial activity. It has been reported by Zhen et al. (2014) and colleagues that addition of compost helps in the increase of microbial biomass and their respiration rates. This in turn improves the development of microbes. Moreover, the growth of *Klebsiella quasipneumoniae subsp. similipneumoniae* in the presence of all five chelators was significantly higher compared to other three strains (*Pseudomonas beteli, Klebsiella variicola* and *Microbacterium paraoxydans*). In the presence of citric acid, 4% decrease was observed by *Klebsiella quasipneumoniae subsp. similipneumoniae* when compared with control. However, *Pseudomonas beteli* exhibited 26% decrease, *Klebsiella variicola* had a decrease of 25% while *Microbacterium paraoxydans* showed 60% decrease compared to control in the presence of citric acid.

This reveals that ammonium nitrate and citric acid had lesser negative impact on the growth of *Klebsiella quasipneumoniae subsp. similipneumoniae* when compared with other strains. Hence the strain was selected for the phytoextraction of Pb. *Klebsiella*

quasipneumoniae subsp. similipneumoniae has been reported for its high tolerance against heavy metals and can tolerate Pb levels of up to 1400 mg L^{-1} in liquid medium (Aslam et al., 2016; Sagar et al., 2017).

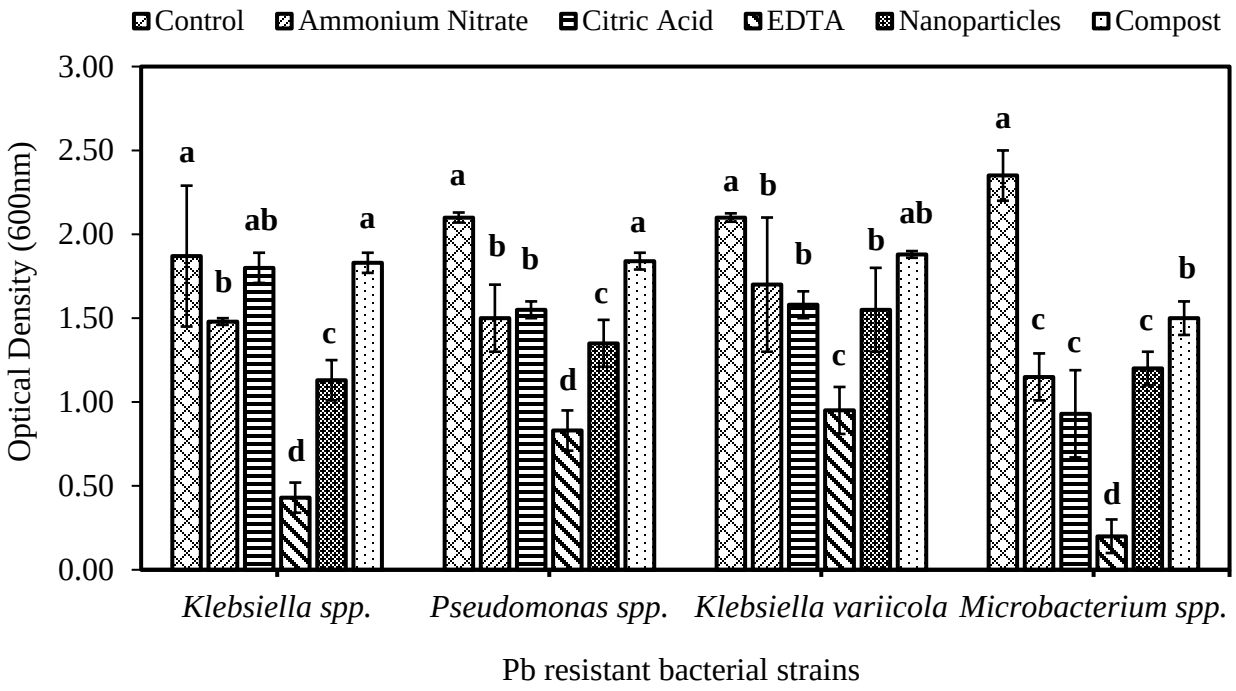

Fig. 4.1. Growth of bacterial strains in the presence of Pb and chelators. Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

4.1.2. Selection of fungal strain

Figure 4.2 shows the growth of three Pb solubilizing fungal strains; *Mucor* sp., *Aspergillus flavus* and *Aspergillus niger* in the presence of Pb and metal chelators. All the fungal strains showed significant decrease in the presence of chelators compared to control as significantly higher growth was observed in control. However, the overall results showed that after 48 hours, significantly lower growth was observed by all the fungal strains i.e. *Mucor* sp., *Aspergillus flavus* and *Aspergillus niger* in the presence of EDTA. Whereas,

growth of *Mucor* sp., showed no significant difference with ammonium nitrate and citric acid. Compared to control, a decrease of 54% was observed by *Mucor* sp., when grown with citric acid. Although *Mucor* sp., had better growth in the presence of citric acid but the strain produces chitosan which has bactericidal properties (Moussa et al., 2011). Hence, selection of *Mucor* sp. is not suitable to be integrated with bacterial strain. On the other hand, growth of *Aspergillus flavus* and *Aspergillus niger* had a significant difference when grown with ammonium nitrate and citric acid. Also, *Aspergillus flavu*s showed a decrease of 59% with citric acid whereas *Aspergillus niger* had a decrease of 70% when compared with control. According to Iskandar et al. (2011), *Aspergillus niger* is capable of growing at all levels of Pb whereas the highest biomass of this strain was observed at 250 mg Pb L^{-1}. Heavy metal tolerance of *Aspergillus flavus* has also been confirmed by Abdul Majeed et al. (2016) which reported 64.63% Pb removal efficiency of the strain. Oso et al. (2015) reported that *Aspergillus flavus* can tolerate and adsorb lead at as high as 1000 mg kg^{-1} concentration. Also, it has been reported by Kannahi and Senbagam (2014) that *Aspergillus flavus* can produce hydroxamate type siderophores which was confirmed by Czsaky method.

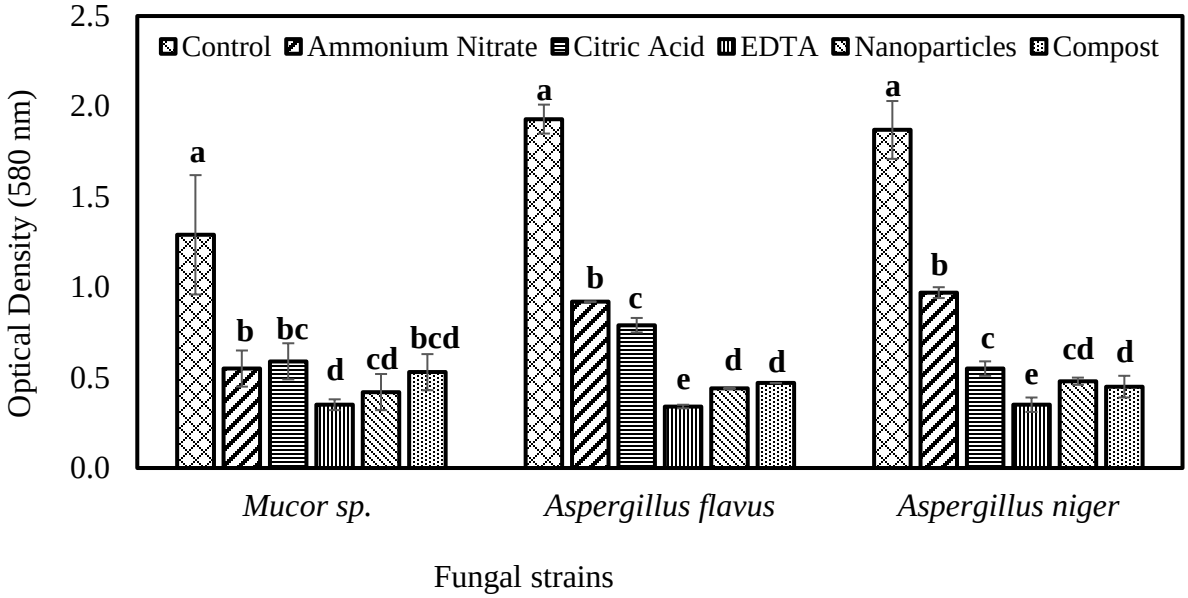

Fig. 4.2. Growth of fungal strains in the presence of Pb and chelators. Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

The selection of fungal and bacterial strain was further confirmed by growth compatibility test using disc diffusion method. The results shown in figure 4.3 clearly depict that *Klebsiella quasipneumoniae subsp. similipneumoniae* and *Aspergillus flavus* can grow in the presence of each other.

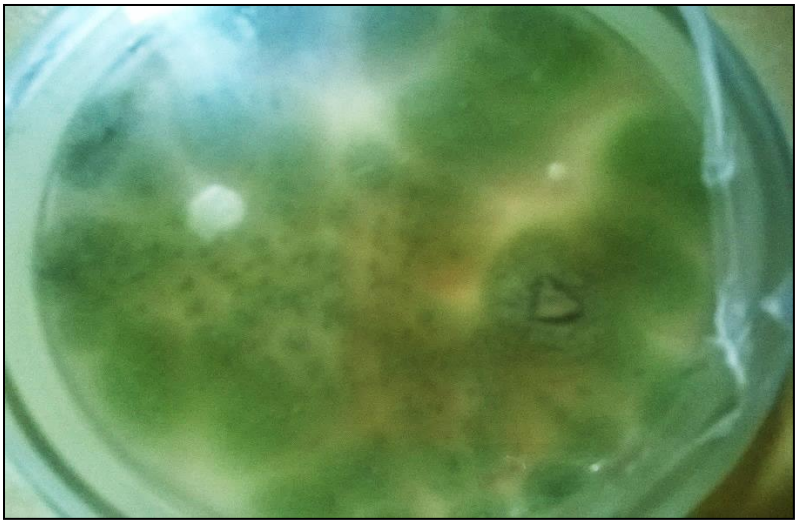

Fig. 4.3. Growth compatibility test of *A. flavus* and *Klebsiella quasipneumoniae subsp. similipneumoniae*

4.1.3. Selection of metal chelator

In order to select a metal chelator, growth of *Klebsiella quasipneumoniae subsp. similipneumoniae* and *Aspergillus flavus* in five chelators was compared. In case of bacteria, all the strains showed significantly higher growth in compost compared with other metal chelators. However, compost is not suitable to be used as a metal chelator because of its adsorptive properties. As more active functional groups are present in compost, they enhance the complexation process with heavy metals and adsorb them on the surface, thereby making them unavailable (Liu et al., 2018). Other than compost, growth of bacterial strains in the presence of citric acid had lesser negative impact compared to EDTA, nanoparticles and ammonium nitrate. Similarly, in case of fungal strains, 57%, 52% and 48% decrease were observed by *Mucor* sp., *Aspergillus flavus* and *Aspergillus niger*, respectively in the presence of ammonium nitrate. However, according to Karczewska and Milko (2010), ammonium nitrate has poor metal mobilizing capability when compared with citric acid and EDTA. It was also reported that 1.3 mg kg^{-1} Pb was extracted after

applying 5 mmol kg^{-1} while 0.2 mg kg^{-1} Pb was extracted by applying same concentration of ammonium nitrate.

On the basis of these results, citric acid was chosen as a metal chelator in combination with *Klebsiella quasipneumoniae subsp. similipneumoniae* and *Aspergillus flavus* because citric acid is bio-degradable in nature which reduces the leaching risk of metal-chelate complex in the soil. Also, due to economic feasibility citric acid is a better choice than other chelators (Gobran et al., 2000). Wuana et al. (2010) also studied the effect of citric acid on heavy metal removal. It was found that 0.05M citric acid after the batch experiment of 6 hours removed 31% of Pb from the soil contaminated with 292.5 mg kg^{-1} Pb. Moreover, at the application of 10 mmol kg^{-1} of citric acid, 3.5% increase in the plant dry biomass was observed.

4.2. Physicochemical analysis of soil

The physical and chemical analysis performed on experimental soil are pH, electrical conductivity (EC), moisture content, water holding capacity, soil texture, Nitrate-Nitrogen, extractable phosphorus and lead (Pb). The soil analysis showed that all treatments i.e. 13 mg kg^{-1} Pb (T_0), 1187 mg kg^{-1} Pb (T_1) and 2292 mg kg^{-1} Pb (T_2) had clay loam soil with the characteristics shown in table 4.2.

Table No. 4.2. Preliminary characteristics of experimental soil

Parameters	**13 mg kg kg^{-1}Pb (T_0)**	**11867 mg kg^{-1}Pb (T_1)**	**2292 mg kg^{-1}Pb (T_2)**
pH	7.7	7.5	7.48
Electrical Conductivity (dS m^{-1})	0.6	0.23	0.37
Moisture Content (%)	0.4	1	1.4
Water Holding Capacity (%)	6.9	8.2	7.3
Nitrate-Nitrogen (mg kg^{-1})	33.3	33.7	38.4
Total Organic Carbon (%)	0.18	0.23	0.32
Extractable Phosphorus (mg kg^{-1})	86	75	73.6

4.2.1. Soil Pb analysis

Pb analysis of pre and post harvested soil samples has been shown in figure 4.4. The results show that Pb levels in pre and post harvested soil is significantly higher ($p<0$) compared to 13 mg kg^{-1} Pb (T_0). However, in post harvested soil samples, after the addition of amendments and cultivation of plants, the Pb concentration all three study areas i.e. 13 mg kg^{-1} Pb (T_0), 1187 mg kg^{-1} Pb (T_1) and 2292 mg kg^{-1} Pb (T_2) was significantly decreased compared to samples in pre-harvested soil. Pb content in post harvested soil samples of 2292 mg kg^{-1} Pb (T_2) and 1187 mg kg^{-1} Pb (T_1) had 26% decrease as compared to the pre-harvested soil samples.

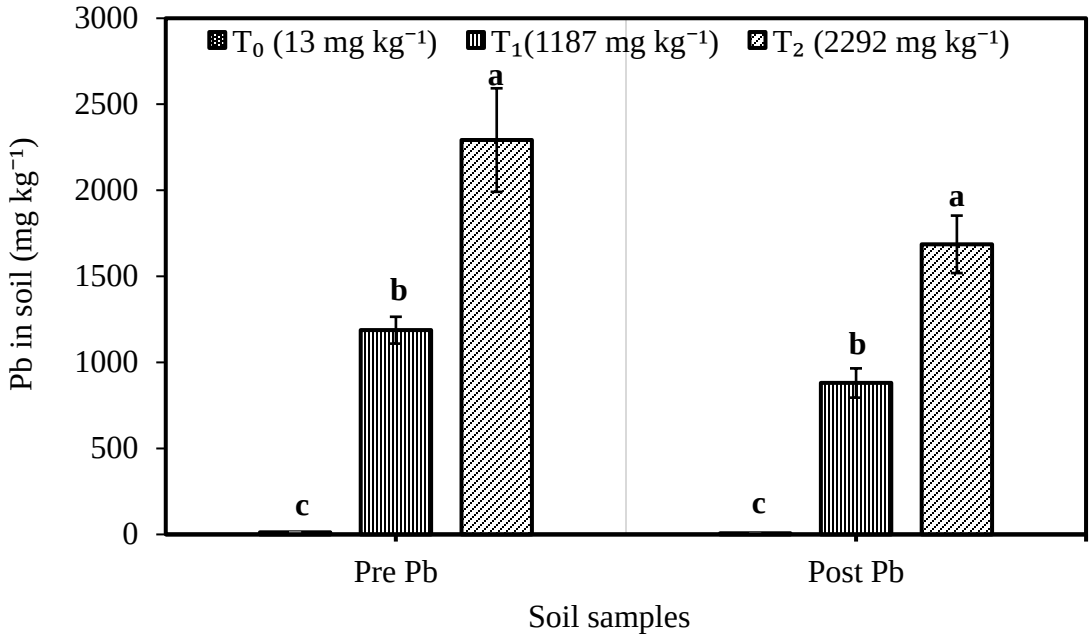

Fig. 4.4. Pb concentration in soil samples. Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

4.3. Plant growth parameters

4.3.1. Plant length

The different levels of Pb in soil and the addition of amendments (*Aspergillus flavus*, citric acid and *Klebsiella quasipneumoniae subsp. similipneumoniae*) showed different effects on the length of plants. Figure 4.5. shows the total length of plants grown in different levels of Pb. Plants grown in soil with 2292 mg kg^{-1} Pb (T_2) had significant effect ($p< 0.05$) on the total length of the plants. The average total length of plants in 13 mg kg^{-1} Pb (T0) was 33.2 cm while that of plants grown in 1187 mg kg^{-1} Pb (T_1) had a total length of 37 cm. The plants in 2292 mg kg^{-1} Pb (T_2) had an average total length of 45 cm hence plants grown in 2292 mg kg^{-1} Pb (T_2) had significantly higher total length when compared to the plants grown in 13 mg kg^{-1} Pb (T_0) and 1187 mg kg^{-1} Pb (T_1). According to Grobelak and Hiller

(2017), bacterial and fungal strains release siderophores that result in the growth promotion of plants which may be the reason of increased length in plants inoculated with *Aspergillus flavus* and *Klebsiella quasipneumoniae subsp. Similipneumoniae.* Ahemad and Khan (2011) reported that *Klebsiella spp.* produced significant amount of Indole Acetic Acid and siderophores which increases the growth rate of plants. Siderophores also play an important role in alleviating metal toxicity in plants by binding different metal ions and acting as a ligand (Costa et al., 2014).

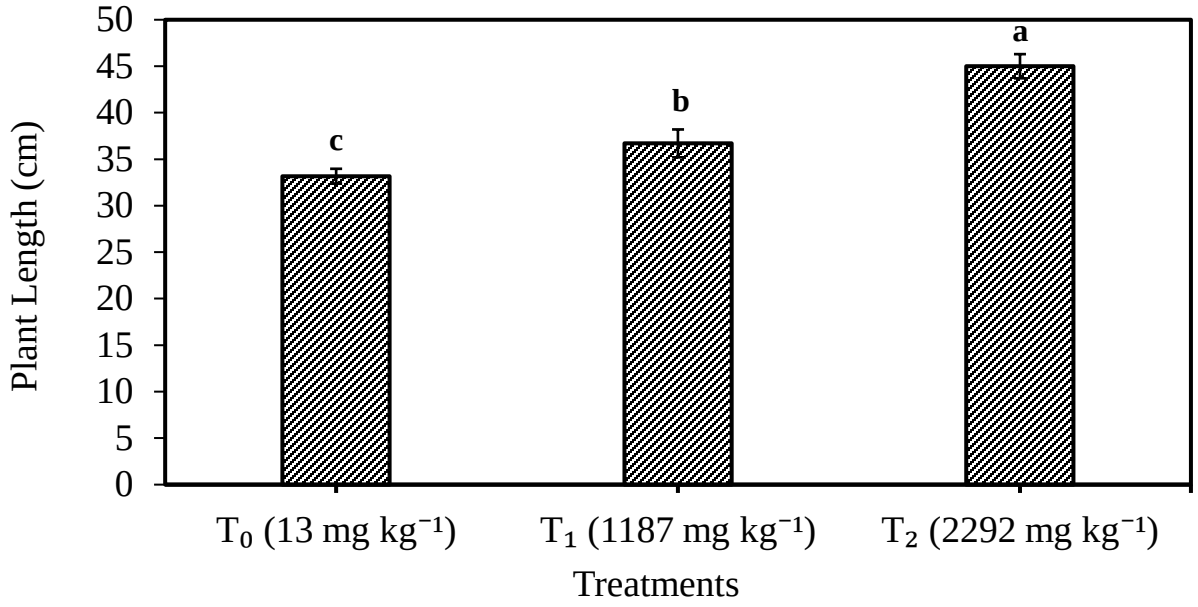

Fig. 4.5. Effect of amendments & different Pb levels on total length of *Pelargonium hortorum* Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

4.3.2. Root and shoot dry biomass

The different levels of Pb in soil and the addition of amendments (*Aspergillus flavus,* citric acid and *Klebsiella quasipneumoniae subsp. similipneumoniae*) showed different effects on the root and shoot dry biomass of plants. Figure 4.6 shows the root and shoot dry

biomass of plants grown in different levels of Pb. Plants grown in soil with 2292 mg kg^{-1} Pb (T_2) had significant effect ($p< 0.05$) on the root dry biomass. The average root dry biomass of plants in 13 mg kg^{-1} Pb (T_0) was 0.25 g while that of plants grown in 1187 mg kg^{-1} Pb (T_1) had an average of 0.4 g root dry biomass. The plants in 2292 mg kg^{-1} Pb (T_2) had an average root dry biomass of 0.5 g. The results showed a significantly higher increase in the root dry biomass of plants grown in soil with 2292 mg kg^{-1} Pb as compared to the plants grown in 13 mg kg^{-1} Pb (T_0) and 1187 mg kg^{-1} Pb (T_1). Higher root dry biomass may be associated with the addition of *Klebsiella* strain which is capable of producing Indole Acetic Acid because the hormone is helpful in the production of lateral roots in plants (Etesami et al., 2015). Liu et al. (2018) confirmed that *Klebsiella spp.* produces siderophores and its inoculation in seedlings of Soy bean improved the root length and volume by 52% and 43%, respectively.

In case of shoot dry biomass, plants grown in soil with 2292 mg kg^{-1} Pb (T_2) had significant effect ($p< 0.05$) on their dry biomass. The average shoot dry biomass of plants in 13 mg kg^{-1} Pb (T_0) was 2 g while that of plants grown in 1187 mg kg^{-1} Pb (T_1) had an average of 2.3 g root dry biomass. The plants in 2292 mg kg^{-1} Pb (T_2) had an average root dry biomass of 3 g. The results showed a significantly higher increase in the shoot dry biomass of plants grown in soil with 2292 mg kg^{-1} Pb (T_2) as compared to the plants grown in 13 mg kg^{-1} Pb (T_0) and 1187 mg kg^{-1} Pb (T_1). It has been reported by Rungin et al. (2012) that plant growth parameters such as length as well as root and shoot biomass were increased significantly when endophytic fungi was added because of its ability to produce siderophores. Similarly, Marques et al. (2010) reported that shoot biomass of *Zea mays* was increased by 100% when they were inoculated with plant growth promoting bacterial strains.

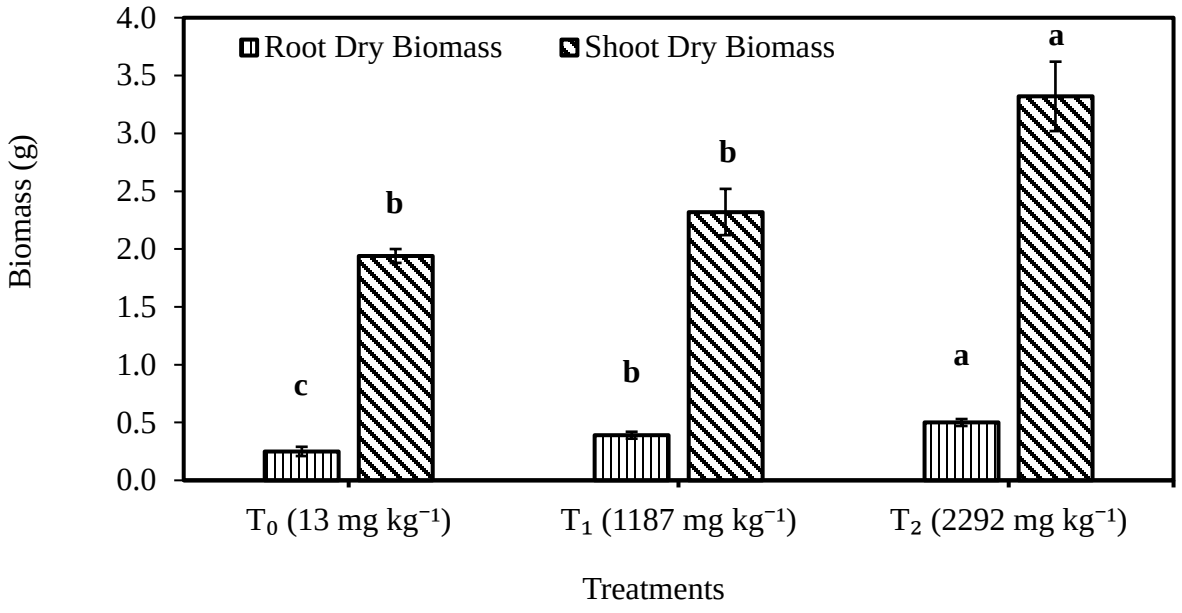

Fig.4.6. Effect of amendments and different Pb levels on root and shoot dry biomass of *Pelargonium hortorum* Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

4.3.3. Lead (Pb) concentration in roots and shoots

The concentration of Pb in the roots and shoots of *Pelargonium hortorum* grown in 2292 mg kg^{-1} Pb (T_2) and 1187 mg kg^{-1} Pb (T_1) has significantly increased ($p<0.05$) compared to the roots and shoots concentration of Pb of plants grown in 13 mg kg^{-1} Pb (T_0).

Roots and shoots concentrations of Pb in *Pelargonium hortorum* have been shown in figure 4.7. The results show that the Pb concentration is maximum in the shoots as compared to the roots of the plants. The plants grown in 2292 mg kg^{-1} Pb (T_2) and 1187 mg kg^{-1} Pb (T_1) had significantly higher concentration of Pb in the shoots compared to the plants in 13 mg kg^{-1} Pb. The average concentration of Pb in the shoots of plants in 13 mg kg^{-1} Pb (T_0) was 16.4 mg Pb kg^{-1} while that of shoots in 1187 mg kg^{-1} Pb (T_1) was 677 mg Pb kg^{-1}. The

highest concentration of 666 mg kg^{-1} Pb was observed within the shoots of plants grown in T_2 (2292 mg kg^{-1} Pb).

Similar trend was observed in the Pb concentration of roots where the plants grown in 2292 mg kg^{-1} Pb (T_2) and 1187 mg kg^{-1} Pb (T_1) was significantly higher to the compared to the roots of plants in 13 mg kg^{-1} Pb (T_0). The average Pb concentration by the roots of plants grown in 13 mg kg^{-1} Pb (T_0) was 11.3 mg Pb kg^{-1} while that of plants in 1187 mg kg^{-1} Pb (T_1) and 2292 mg kg^{-1} Pb (T_2) had 440 mg Pb kg^{-1} and 483 mg Pb kg^{-1} respectively. Similar results are presented by Mahdieh et al. (2013) in which highest concentration of Pb in roots and shoots of *Pelargonium hortorum* was observed in the culture solution contaminated with 2,500 mg L^{-1} of Pb $(NO_3)_2$. According to Ehsan et al. (2014) application of citric acid to *Brassica napus* L. not only improved gas exchange parameters of plants but also increased antioxidant enzyme activity. This could be the reason behind increased metal concentration in roots and shoots of plants.

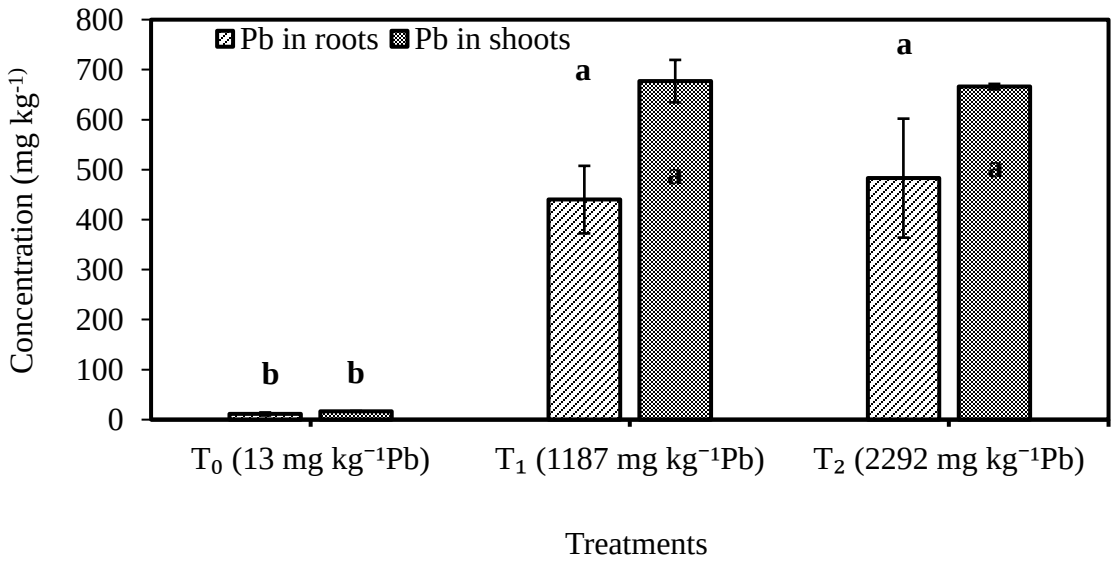

Fig. 4.7. Pb concentration in shoots and roots of *Pelargonium hortorum.* Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

4.3.4. Pb uptake by *Pelargonium hortorum*

Uptake of Pb by *Pelargonium hortorum* varied greatly in different concentrations of Pb amended soil along with the addition of *Aspergillus flavus*, citric acid and *Klebsiella quasipneumoniae subsps. similipneumoniae.* Pb uptake was calculated using the following formula:

$$\textbf{Pb uptake} = [(C_r \times B_r) + (C_s \times B_s)]$$

Where; C_r: Concentration of Pb in plant root

B_r: Dry Biomass of root

C_s: Concentration of Pb in plant shoot

B_s: Dry biomass of shoot

The results show that addition of *A. flavus*, citric acid and *Klebsiella quasipneumoniae subsps. similipneumoniae* in 2292 mg kg^{-1} Pb (T_2) and 1187 mg kg^{-1} Pb (T_1) has significant ($p<0.05$) impact on Pb uptake compared to soil with 13 mg kg^{-1} Pb (T_0).

Uptake of Pb by *Pelargonium hortorum* has been shown in figure 4.8. The results show that the Pb uptake was significantly higher in the plants grown in 2292 mg kg^{-1} Pb (T_2). The average uptake of Pb by each plant in 13 mg kg^{-1} Pb (T_0) was 0.03 mg Pb $plant^{-1}$ while that of plants grown in 1187 mg kg^{-1} Pb (T_1) was 1.75 mg Pb $plant^{-1}$. The highest Pb uptake was observed in the plants grown in 2292 mg kg^{-1}Pb (T_2) which was 2.4 mg Pb $plant^{-1}$. Higher uptake of Pb may be associated with the addition of *Klebsiella* strain because of its properties such as the production of siderophores, indole acetic acid and nitrogen fixation (Liu et al., 2016). Schalk et al. (2011) suggested that siderophores help in the mobilization of metal and in turn increases their uptake by plants. Dimkpa et al. (2008) proposed that siderophores production is improved in the presence of heavy metals as they act as a signal

for their production. The production of catecholate type siderophores by *Klebsiella pneumoniae* has been reported by Saha et al. (2016) which not only make plants resistant to metals but also increases their accumulation in plants (Rajkumar et al., 2010). Chen et al. (2014) also reported that plants inoculated with plant growth promoting bacteria accumulated higher concentration of heavy metals compared to uninoculated plants. The reason behind higher uptake was the production of siderophores by inoculated strain. Application of 5 mmol of citric acid resulted in 0.2, 0.25 and 0.8 mg of Cr accumulation in roots, stems and leaves respectively of each plant. The reason behind this is that citric acid reduces electrolyte leakage and malondialdehyde concentrations due to heavy metal stress as reported by Afshan et al. (2015).

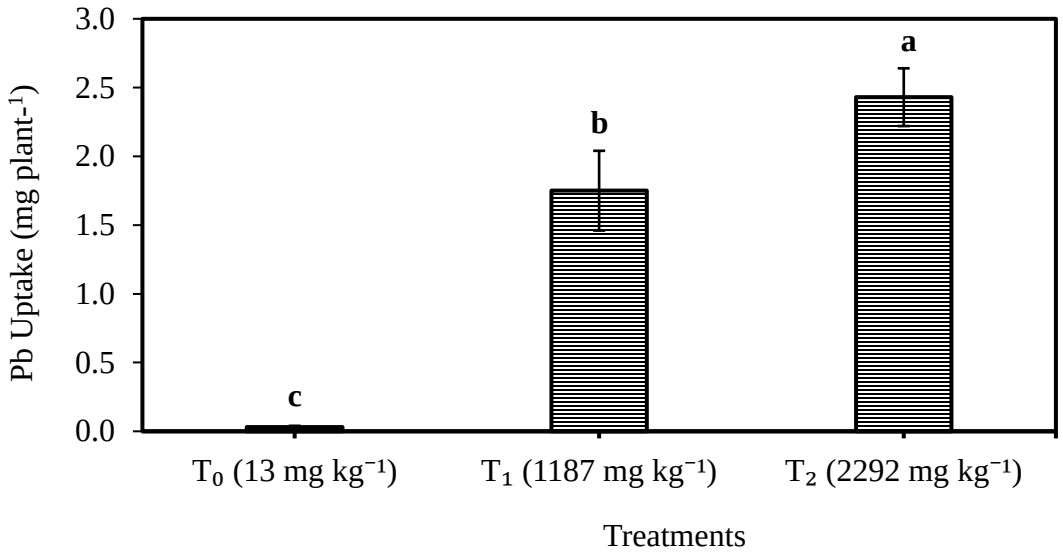

Fig. 4.8. Pb uptake *Pelargonium hortorum* Values shown in graph are mean (± SD) of three replicates. Same letters on the bars represent no significant difference between compared means.

Chapter5

CONCLUSIONS AND RECOMMENDATIONS

5.1. Conclusions

Based upon the current study, it can be concluded that the integration of Pb resistant fungal and bacterial strains i.e. *Aspergillus flavus* and *Klebsiella quasipneumoniae subsps. similipneumoniae* along with the addition of citric acid not only increased the overall growth of plants but also enhanced Pb uptake from soil contaminated as high as 2292 mg kg^{-1} Pb in the pilot scale. Both of these strains were compatible with each other and hence can grow together. Moreover, citric acid was better amendment because of its negligible impacts on microbial community as well as the environment. The combination of these three amendments is a good solution for Pb Phytoextraction at pilot scale. Furthermore, Pb uptake at the highest exposure i.e. 2292 mg kg^{-1} Pb (T_2) was maximum however, no flowering was observed at the highest exposure level probably due to increased vegetative growth.

5.2. Recommendations

From the current study, following recommendations can be made for the work to be done in future:

- Potential mechanisms of chelators, bacteria and fungi in soil should be explored in detail.
- Metal recovery, re-use and fate of harvested plant biomass must be considered.
- As the current study was on pilot scale, it is required to collaborate with industries to remediate Pb polluted soil at a larger scale.

- The combination of these three amendments is a good solution for Pb phytoextraction and need to be tested at field scale.

  Apart from mechanistic and applicability understandings, economic evaluation of phytoextraction is required. Biomass valorization is an important aspect to be studied in future.

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