Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L.

Thesis

SUBMITTED TO

BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY

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Dedicated

To Almighty

&

My Beloved

Parents

CERTIFICATE

This is to certify that the thesis entitled "**Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L.**" submitted by "**Mr. Shatrohan Lal**" is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university. The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the Doctor of Philosophy (PhD) regulations – 1999 as amended in 2010 and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

22/04/19

Date: 22/04/2019

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DECLARATION

I, Shatrohan Lal, hereby declare that the thesis work entitled "**Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L.**" is my own work carried out under the guidance of **Prof. Rajesh Kumar, Head, Department of Environmental Microbiology, Babasaheb Bhimrao Ambedkar University, (A Central University) Vidya Vihar, Raebareli Road, Lucknow.** The matter embodied in this thesis is written by me and has not been submitted to any other university for the fulfillment of the requirement of any other Degree or Diploma.

Place: - **Lucknow**
Date: 22/04/2019

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Shatrohan Lal

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ABBREVIATION

Al	:	Aluminium
ANOVA	:	Analysis of Variance
AR	:	Analytical Reagent
As	:	Arsenic
BCF	:	Bioconcentration factor
BLAST	:	Basic Local Alignment Search Tool
bp	:	Base Pair
BSA	:	Bovine Serum Albumin
Ca	:	Calcium
$CaCO_3$	:	Calcium Carbonate
CAS	:	Chrome Azurol S
Cd	:	Cadmium
Co	:	Cobalt
Cr	:	Chromium
Cu	:	Copper
DAS	:	Days After Sowing
DMRT	:	Duncan Multiple Range Test
dNTP	:	Deoxynucleotide Triphosphate
Dw	:	Dry weight
EC	:	Electrical conductivity
EDS	:	Energy Dispersive X-rays Spectroscopy
F	:	Fluoride
Fe	:	Iron

FTIR	:	Fourier-transform infrared spectroscopy
Fw	:	Fresh weight
g	:	Gram
$g\ L^{-1}$	:	Gram per Litre
h	:	Hours
HCN	:	Hydrogen Cyanide
$HgCl_2$	:	Mercuric Chloride
I	:	Iodine
ICP-OES	:	Inductively Coupled optical Emission Spectrometry
K	:	Potassium
Kg	:	Kilogram
MEGA	:	Molecular Evolutionary Genetics Analysis
mg	:	Mili gram
Mg	:	Magnesium
$MgCl_2$	:	Magnesium Chloride
mL	:	Millilitre
mM	:	Millimolar
Mn	:	Manganese
N	:	nitrogen
NaOH	:	Sodium Hydroxide
NCBI	:	National Center for Biotechnology Information
Ni	:	Nickel
Nm	:	Nanometer
^oC	:	Degree Celsius
OD	:	Optical Density

P	:	Phosphorus
Pb	:	Lead
PCA	:	Principle component analysis
pH	:	Potential of Hydrogen
rDNA	:	Ribosomal Deoxyribonucleic Acid
RMC	:	Root metal content
rpm	:	Revolutions Per Minute
rRNA	:	Ribosomonal Ribonucleic Acid
Se	:	Selenium
SEM	:	Scanning Electron Microscopy
Si	:	Silicon
SMC	:	Shoot metal content
TDB	:	Total dry biomass
TF	:	Translocation factor
TMA	:	Total metal accumulation
UV	:	Ultra-violet
v/v	:	Volume by volume
w/v	:	Weight by volume
Zn	:	Zinc
$\mu g\ L^{-1}$	:	microgram per liter
$\mu g\ mg^{-1}$	:	Microgram Per milligram
µM	:	Micro Molar

Chapter 1

Introduction

CHAPTER - 1

INTRODUCTION

Advent of industrialization, unplanned urbanization, deforestation, energy generation and modern agricultural practices, have resulted in countless incidents in the ecosystem on which the total quality of our environment depends (Spellman, 2017; Neely, 2018). More extensive and intensive exploitation of natural resources and energy production has created cumulative pressures on the quality of local, regional and global ecosystems. These exploitations produce toxic wastes and pose risks to human health, natural habitat, and all parts of our ecosystem. Amongst the most prevalent and toxic contaminants generated from anthropogenic activities, halogenated hydrocarbons, radionuclides, heavy metals, polycyclic aromatic hydrocarbons (PAHs), petroleum hydrocarbons (PHC), pesticides, solvents, salts etc. are included (Spellman, 2017). Plenty of literature well documents and indicates the serious consequences and hazards related to these toxic contaminants that are causing a serious impact on human and ecosystem health (Jia et al., 2018; Jaiswal et al., 2018; Ouabo et al., 2019).

Soil is a precious exhaustible natural resource, extremely important for the survival of plants, humans and terrestrial animal's growth and life support. Unfortunately, this resource is being contaminated by a numbers of pollutants including heavy metals day by day and we are lagging behind in our efforts to save the quality of this resource. Anthropogenic activities such as industrialization and urbanization are main cause for the overall deterioration of the soil health including assimilation of toxic metal compounds, which further degrades the overall quality of the soil. Heavy metal

contamination severely affects soils fertility and most important the crop yield (Marrugo-Negrete et al., 2017; Lwin et al., 2018).

1.1. Heavy metal toxicity in the Environment

Metals that have atomic density greater than 5 gm cm^{-3}, 5 times or more, greater than water and electronegative in nature are known as heavy metals (Lal et al., 2018). In other words, the term "heavy metals" refers to any metallic element that has a relatively high density and is toxic or poisonous even at low concentration. They are noteworthy environmental pollutant and their presence in the environment causes serious toxic effects in humans, animals and plants. The most common toxic heavy metals are cadmium (Cd), lead (Pb) chromium (Cr), nickel (Ni), arsenic (As), and mercury (Hg) (Ali et al., 2013; Moore et al., 2016). Their toxicity depends on the forms (i.e. metallic, ionic, complex or insoluble), accidental dose, exposure routes and duration of exposure, i.e. acute or chronic (Nordberg et al., 1978). All these may lead to various deleterious effects due to oxidative stress induced by free radicle generation and consequently leads to excessive damage. Apart from this, some heavy metals are essential for physiological functions of living cells and regulate many biochemical processes, but the higher concentration can lead to notorious health problems and severe poisoning (Oves et al., 2016). For instance, Zinc (Zn), Copper (Cu), Iron (Fe), and Selenium (Se) are essential elements for plants, humans and animals, but concentration, higher than permissible limit causes severe health problems.

Amongst all heavy metals, Cd and Pb have emerged as serious toxic environmental pollutants in the past few years because of their excessive use in manufacturing (electroplating, leather tannin, printed circuit board etc.) and agricultural (Chemical fertilizer, herbicides and pesticides) industries (Chen et al., 2016). Elevated concentrations of Cd and Pb in the soil enormously pollute the natural ecosystem as

well as alter or destruct the soil texture by reducing its fertility and nutrient availability (Shaheen et al., 2016). They affect plants, humans and animals,, more deleteriously after entering into the food chain by various routes (Machado et al., 2013). Cadmium and lead have relatively more solubility than other heavy metals due to their easy ionizing and bonding property to other compounds. These properties make them highly mobile in the water-soil-plant system (Machado et al., 2013). From contaminated soils, heavy metals enter into the plant through root cortical tissues and reach xylem via symplastic and/or apoplastic pathways (Song et al., 2016). Further, cadmium enters into root tissues via active transport or calcium transporter protein while lead enters through passive diffusion (Amari et al., 2017). Phytotoxic effects of cadmium and lead arise in two different ways (i) direct effects on plant cells or (ii) in-direct by affecting metabolic pathways of plants. Through direct toxicity, cadmium and lead bind to the cell wall or cell membrane and ultimately leads to significant changes in the properties of cell wall, affects enzymatic activity, antimitotic and genotoxic effects (Gastaldo et al., 2007), while, indirect toxicity leads to fall in availability of water interference with other micro-essential metal ions, induction of oxidative stress and disruption of photosynthesis (Amari et al., 2017). In addition, high accumulation of heavy metals generally leads to growth inhibition and finally death of the plant. Therefore, there is a need to develop methods for the remediation of heavy metal contaminated soils to protect human health and environment.

1.2. Soil contamination by heavy metals

Soil is a crucial component of our ecosystem receiving a large number of contaminants from various sources every day. Generally, it does not only acts as a sink for toxic pollutants, but also acts as a natural buffer by controlling the transportation of toxic elements and substances in the environment (Kabata-Pendias,

2010). In the soil, heavy metals are found either naturally or produced by anthropogenic activities (Wuana and Okieimen, 2011). Natural phenomena such as weathering and volcanic eruptions have been reported to contribute heavy metal pollution in the soil, whereas, human activities such as mining and smelting operations, coal burning in power plants, petroleum combustion, nuclear power stations and high tension lines, plastics, textiles, microelectronics, wood preservation, paper processing plants, domestic and agricultural use of metals and metal-containing compounds also contribute towards heavy metal pollution in soils (Wuana and Okieimen, 2011; Tchounwou et al, 2012; Begum and Huq 2016). Apart from this, soil contamination may also occur through atmospheric deposition, leaching of heavy metals, sediments re-suspension, metal corrosion, soil erosion of metal ions and metal evaporation from water resources to the soil and groundwater (Begum and Huq, 2016).

1.3. Extent of the problem

Over the past 50 years, more than 22,000 tons of Cadmium, 800,000 tons of Lead, 30,000 tons of Chromium, 939,000 tons of Copper and 1,350,000 tons of Zinc have been released into the environment globally by various anthropogenic activities and most of which have accumulated in the soil causing serious heavy metal pollution of the soil (Chen et al., 2016; Liu et al. 2018). Moreover, agricultural soil pollution by toxic heavy metals draws the attention of environmentalist not only because of the persistent nature of heavy metals in the soil but also due to their accumulation in food crops, where they contaminate the food chain and cause significant potential risk to human health (Haque et al., 2018; Salvo et al., 2018). Accumulation of heavy metals in food crops has remained as a serious concern due to the potential risks to human health in recent years, through bioaccumulation and biomagnification in food chain,

and their effects on the ecosystem. For instance, Chinese agriculture land was polluted by Cd, Hg, Cu, Ni, Zn and Pb and the highest pollution rate was by Cd 7.75%, followed by Hg, Cu, Ni and Zn, Pb. While, the rate of total contamination in Chinese agriculture field soil was 10.18%, mainly from Cd, Hg, Cu, Pb and Ni (Zhang et al., 2015). In addition, Indian environmentalists examined the quality of Indian agriculture land based on comprehensive environmental pollution index (CEPI) and revealed that the Indian agriculture land is also severely contaminated by toxic heavy metals like Arsenic, Lead, Chromium, Cadmium, and Zinc (Rajendiran et al., 2015). The researchers have recognised more than 43 critically heavy metal contaminated zones, which have more than 70 rating of CEPI allocated in the sixteen different states of India. The data presented by researchers based on CEPI have described that amongst all the forty-three sites, more than 50% sites are confined to only 4 states i.e. Uttar Pradesh, Maharashtra, Gujarat and Tamil Nadu of India (Rajendiran et al., 2015).

1.4. Technological approaches involved in the remediation of heavy metal contaminated soil

Numerous technological approaches such as physical (excavation, encapsulation, surface capping, land filling, and membrane filtration), chemical (soil washing, soil flushing, solidification, immobilization, coagulation, chemical precipitation, ion exchange, chemical oxidation or reduction and photocatalysis), thermal (vitrification) and electro-reclamation techniques are frequently used for the remediation of contaminated soil (Liu et al., 2018). But, most of them are having technological constraints, for example physical methods alter soil microflora, cause irreversible alterations in soil properties, are labour intensive and costly (Yao et al., 2012). In the same way, chemical processes are also very expensive, generate secondary pollutants,

and produce large quantities of sludge (Tangahu et al., 2011; Yao et al., 2012; Zubair et al., 2016; Khalid et al., 2017). All these approaches (physical, chemical, thermal and electro-reclamation) only change the form of the problem and fail to remediate/extract the pollutants thoroughly thereby minimize the soil pollution and improve the soil health (Gomes et al., 2016). Taking into consideration the shortcomings of the conventional methods, a plant based biological technique known as "Phytoremediation" has been identified, it is emerging, low-cost and eco-sustainable solution for the remediation of heavy metal contaminated soils.

Phytoremediation is a widely accepted technique in which plants are used as a remediator or accumulating agents for the removal of toxic heavy metals from contaminated soils (Ali et al., 2013; Ullah et al., 2015). Plants used in this technique, have an increased rate of heavy metal uptake, rapid translocation from root-to-shoot and excellent ability to detoxify and sequester heavy metals in aerial parts (Hrynkiewicz et al., 2018; Liu et al., 2018). Now it has been considered that phytoremediation is a best alternative approach for the removal of heavy metals from contaminated soil without affecting the biological activity, structure and fertility (Pinto et al., 2015; Sarwar et al., 2017; Hrynkiewicz et al., 2018; Liu et al., 2018). Depending on the process by which plants remove or reduce the toxic effects of heavy metals in the soil, phytoremediation technique can be categorised into sub-categories-

a. Phytoextraction
b. Phytostabilization
c. Phytotransformation
d. Phytostimulation

Phytoextraction: is the process in which heavy metals are sequestered or accumulated by plants from contaminated soils and concentrated them in harvestable parts.

Phytostabilization: is the process in which plants reduce the mobility of heavy metal from contaminated soil by preventing erosion, leaching, or runoff and to reduce bioavailability of metals in the environment, thereby preventing their migration to groundwater or their entry into the food chain.

Phytotransformation: is the process by which plants transform the toxic metals into non or less toxic forms and thereby are accumulated into plant tissues.

Phytostimulation: is the process in which plant releases exudates or enzymes into the root zone that stimulates the microbial activity thereby increasing solubilization and uptake of heavy metals in root.

Further, the success of phytoremediation depends on the potential of plant to yield high biomass and tolerate high concentration of metal stress. In this context, selection of plant is an important factor because most of the metal hyperaccumulator plants are slow-growing and usually produce limited amount of biomass. The plants that accumulate high concentration (>100 mg Kg^{-1} Cd, >1000mg Kg^{-1} of Ni, Pb and Cu or >10,000 mg Kg^{-1} of Zn and Mn dry weight) of metals into their aerial parts are frequently known as metal hyper-accumulator plants (Baker and Brooks, 1989). Approximately 101 families and more than 500 plants belonging to these families are reported as metal hyperaccumulator (Sarma, 2011). Some of the important families related to metal hyperaccumulator are *Asteraceae*, *Brassicaceae*, *Caryophyllaceae*, *Cyperaceae*, *Cunouniaceae*, *Fabaceae*, *Flacourtiaceae*, *Lamiaceae*, *Poaceae*, *Violaceae* and *Euphobiaceae* (Sarma, 2011).

Amongst all the plant species *Zea mays* L. has been reported many times for their excellent ability to accumulate high concentration of Cd and Pb in their biomass (Wang et al., 2016; Rizwan et al., 2017; Ahmad et al., 2018). It is capable of growing in diverse climates (Temperate and Tropical) and serves as a renewable source of energy as it is an important feedstock of bioethanol production in USA and Brazil (Schwietzke, 2009). Due to its luxuriant root system, high-biomass and adaptability, it is capable of continuous extraction of metals from contaminated soils by translocating them from roots to shoots (Nascimento and Xing, 2006) and is considered as a promising crop for phytoremediation of soils contaminated with multiple heavy metals.

Apart from this, *Canna indica* is another important plant frequently used in constructed wetlands for remediation of contaminated wastewater (Yadav et al., 2012). *C. indica* have good ability to accumulate considerable amount of hazardous metals like cadmium, lead, nickel and zinc etc. in various parts i.e. root, shoot and leaves (Subhashini et al., 2013). It is well adapted to various climatic conditions such as tropical, subtropical, humid and arid (Cheng et al., 2002). Due to their long flowering duration, easy growth, reproduction and adaptation to different climate, and beauty makes this plant suitable for phytoremediation as well as gardens/parks beautification. *C. indica* have the potential to grow in industrial sludge amended soil due to their fractionation and translocation of heavy metals like cadmium, lead, chromium, zinc, nickel, copper and manganese. Further their characteristic features like antioxidant and phytochelation are considerable for the tolerance to oxidative stress caused by several heavy metals (Bose et al., 2008).

Although phytoremediation is easily applicable and cost-effective technique, but it does have some inherent technical constraints like, it is restricted to the site with low

pollutant concentration; the higher concentration of contaminant may check the plant growth. Moreover, their slow growth and low biomass is also a hindrance in metal phytoremediation (Das and Kumar, 2016). Limited bioavailability of tightly bound fraction of metal ions from the soil is another demerit of phytoremediation technology. Slow transfer rate of metal from soil to root and root to shoot makes this technique inefficient. Phytoremediation technique may become efficient if fast-growing plants are inoculated with plant growth promoters along with significant metal chelators.

1.5. Plant growth promoting rhizobacteria (PGPR) assisted phytoremediation of heavy metals

PGPRs, whose role is still underestimated, play an important (or perhaps essential) role in enhancing plant growth as well as contribute in remediation of heavy metal contaminated soils (Ahmad et al., 2016; Hrynkiewicz et al., 2018). Among the microorganisms found in the soil, rhizospheric bacteria (PGPR) have the potential to tolerate metal toxicity and colonize in rhizospheric region in contaminated environment (Kamran et al., 2015). They are also capable of reducing heavy metal stress and promote phytoremediation process by inducing biomass production through various mechanisms such as fixation of atmospheric nitrogen, utilization of 1 aminocyclopropane-1-carboxylic acid (ACC) as a sole N source, production of siderophores and anti-pathogenic substances, production of plant growth regulators (phytohormones, such as auxins), and also through the transformation of nutrient elements like phosphorous and potassium (Marques et al., 2013; Etesami et al., 2018; Lal et al., 2019). Nevertheless, when heavy metal resistant rhizobacteria are used as a microbial inoculant either individually or as consortium, they efficiently reduce the toxicity of heavy metals by various mechanisms like - chelation of metal ions,

acidification, metal ions sequestration by releasing organic acids, exclusion and transformation of heavy metals from more toxic to less/non-toxic forms (Verma et al., 2017). Apart from these, metal binding proteins and peptides such as metallothioneins (MTs) and phytochelatins (PCs) are other tactics adopted by rhizobacteria to chelate and sequester metals and concurrently to defend against heavy metal stress (Basharat et al., 2018; Tiwari and Lata, 2018).

Plenty of research is being carried out for sustainable approaches to address the heavy metal toxicity in soil using plants but few of them are concentrated on rhizobacteria assisted phytoremediation technology. Hence, considering the threat of soils contamination, the role of PGPR in the effective and sustainable remediation of metal polluted soils, the present study was undertaken with the objectives (given in next section) to explore their interaction with two plants in cadmium and lead contaminated soil. In this study, heavy metal resistant rhizobacterial strains were chosen because they have the ability to thrive in metal stressed environment in a luxuriant way, support plant growth and also contribute to the remediation process.

1.6. Major Objective

To address the research gaps in knowledge regarding heavy metal resistant ability of rhizobacterial isolates and their plant growth promotory attributes and metabolites in the presence and absence of heavy metal and enhancement of *Canna indica* and *Zea mays* L. plant growth in Cd and Pb stressed environment was undertaken with the following objectives:

OBJECTIVES

1. Isolation and characterization of cadmium and lead resistant bacterial isolates from heavy metal contaminated sites of Uttar Pradesh.
2. Screening of isolates for plant growth promotory attributes such as siderophore, ACC deaminase, phosphate solubilization, Indole acetic acid, HCN, ammonia, and polysaccharides production.
3. Molecular characterization of bacterial isolates based on 16S rRNA gene sequencing.
4. Development of potential bacterial consortium and interaction studies of the consortium as well as of individual bacterial strains with *Zea may* L. and *Canna indica* for remediation of cadmium and lead under semi controlled conditions in the laboratory/glass house.

Chapter 2

Review of Literature

CHAPTER - 2

REVIEW OF LITERATURE

Rapid industrialization, which includes mining, smelting and leather processing during the last few decades, has dramatically led to elevated release of chemicals into the environment including heavy metals. The concentration of these heavy metals is increasing day by day causing several problems to plants and human health and needs immediate attention. A brief literature about heavy metals, problems and their possible biological solution are described as follows:

2.1. Definition of heavy metals and their source in environment

Heavy metal appears to include all metals of the periodic table with atomic numbers greater than 20, generally excluding the alkali metal and the alkali earth. Heavy metals are metallic, naturally occurring compounds that have a very high density ranging from 3.5- 7 g/cm^3 compared to other metals at least five times the density of water. Atomic weight of heavy metal ranges from 22.98 to < 40, and atomic number of heavy metals are >2 (Afal and Wiener, 2014; Emenike et al., 2018). Heavy metal contamination induces serious health and environmental hazards due to its toxic nature (Bouka et al., 2013; Wang et al., 2017). There are different sources for heavy metals in the environment. These sources can be either natural or anthropogenic origin. Both natural and anthropogenic inputs are correlated with the distribution of heavy metals in the soil.

2.1.1. Natural sources

The main natural sources of heavy metal pollutants in the soil are; 1) Weathering and abrasion of rocks, 2) Volcanic eruptions 3) Forest fires, 4) Aerosol formation over Seas. Soil erosion is also reported for heavy metal pollution in the soil. The two main

agents of soil erosion are wind and water (Bouka et al., 2013; Wang et al., 2017). During rainfall, sediment-bound heavy metals are distributed to the soil. Water containing agrochemicals with toxic metal concentration drop this sediment-bound metal in the soil even as it causes erosion. It is reported that volcanic eruptions produce hazardous impacts on the environment, climate, and health of exposed individuals (Pawar and Bhosale, 2018). Apart from the deterioration of social and chemical conditions and the gases released during eruptions, the activities from volcanoes are reported to be responsible for the release of heavy metals such as; As, Hg, Al, Rb, Pb, Mg, Cu, Zn and a host of others (Amarlal et al., 2006; Wuana and Okieimen, 2011; Pawar and Bhosale, 2018).

In addition, some aerosol (fine colloidal particles or water droplet in the air, in some cases they can be gas) particles may carry different kinds of the contaminant; like smoke cloud and heavy metals. These heavy metal containing aerosols usually accumulate on leaf surfaces in the form of fine particulates and can enter the leaves via stomata (Sardar et al., 2013; Akpor et al., 2014; Pawar and Bhosale, 2018).

2.1.2. Anthropogenic sources

Anthropogenic inputs like extensive use of agrochemicals (inorganic and organic) fertilizers, pesticides, waste water irrigation, sewage sludge supplementation, higher atmospheric depositions by industrial units and combustion of fossil fuels have led to elevated level of inorganic pollutants in the soils (Nicholson et al. 2003; Belon et al., 2012; Akpor et al., 2014; Sidhu, 2016). Fungicides, phosphate fertilizers and inorganic fertilizers have variable levels of Pb, Cd, Cr, Ni, Zn etc. depending upon their sources. Repeated use of phosphate fertilizers is enriching the agricultural soils with heavy metals (AlKhader, 2015; Sidhu, 2016).

The heavy metals essentially become contaminants in the soil environment because (i) their rates of generation via man-made cycles are more rapid, relative to natural ones, (ii) they get transferred from mines to random environmental locations where higher potentials of direct exposure occur, (iii) the concentrations of the metals in discarded products are relatively high compared to those in the receiving environment, and (iv) the chemical form (species) in which a metal is found in the receiving environmental system may render it more bioavailable (D'Amore et al., 2005; Wuana et al., 2011; Barbieri, 2016) . Heavy metals in the soil from anthropogenic sources tend to be more mobile, hence bioavailable than pedogenic, or lithogenic ones (Wuana et al., 2011; Barbieri, 2016).

2.2. Heavy metals toxicity in the soil

Heavy metal pollution in soil constitutes a highly complex disruption of ecological equilibrium. Soils naturally contain a broad diversity of metallic elements, and each metal may be present at variable concentrations and as different chemical species (Kayastha, 2014; Sodango et al., 2018). Heavy metals are one of the most persistent pollutants in soil and water. Heavy metals can be divided into two categories: essential and non-essential on the basis of their role in living systems. Essential heavy metals such as Mn, Fe, Ni, Zn are needed by living organisms for their growth, development and physiological functions, while non-essential heavy metals such as Cd, Pb, Hg and As are not needed by living organisms for any physiological function (Gohreet al., 2006; Pratush et al., 2018). As metals often occur in ionized forms in the soil, they react with negatively charged soil particles, meaning that both their concentrations and their bioavailabilities are relevant. The result of this situation is that soil biota must permanently regulate their activities in order to make essential metals available and take them up in the required concentrations, as well as to exclude

or detoxify detrimental forms or concentrations (Takáč et al., 2009; Kamal et al., 2010; Wuana et al., 2011; Oves et al., 2015;). Every 1000 kg of "normal soil" contains 200 g Chromium, 80 g Nickel, 16 g Lead, 0.5 g Mercury and 0.2 g Cadmium, theoretically (Suciu et al., 2008; Amouei et al., 2018).

The toxicity of heavy metals mainly depends upon its relative oxidation state, which is responsible for physiological bio-toxic effects. When these metals enter into the living organisms, they, combine with protein, enzyme, and DNA molecules, form highly stable bio-toxic compounds, thus altering their proper functioning and obstructing the bioreactions (Jan et al., 2015; Mishra et al., 2019). Arsenic (As), Cr, Cd and Pb are highly toxic metals that produce mutagenic, carcinogenic and genotoxic effects in plant and livings (Mansour et al., 2009; Ghosh, 2010). Among these, Cd and Pb are the most dangerous metals for human health (Sekara et al., 2005), the unnecessary quantity of these metals in edible things is linked with etiology of various disorders, particularly with cardiovascular, nervous, kidney as well as bone disorders (Sanchez-Castillo et al., 1998; Steenland and Boffetta, 2000). Out of many heavy metals entering the environment, two metals cadmium and lead have been focused in the present study.

2.3. Source of cadmium (Cd) contamination in soil and its effects on plants and human health

Cadmium is a heavy metal with high toxicity and has an elimination half-life of 10-30 years (Rahimzadeh et al., 2017). Cd is released in the biosphere from both natural and anthropogenic sources, while volcanoes and weathering of rocks are the major natural sources for mobilization of Cd from the earth's crust which contains about 0.2 mg/kg, and released to soil and aquatic systems (Gong et al., 1977; Casentini et al., 2010; Abbaslou et al., 2013). This process plays a significant role in the global Cd cycle, but

rarely results in elevated concentrations in any environmental compartment (Gong et al., 1977; Casentini et al., 2010; Abbaslou et al., 2013). A tremendous anthropogenic point source of Cd currently being recovered around the world is a by- product of Zn smelting and refining because Cd is closely associated with Zn in its similar ionic structures and electro negativities and both are strongly chalcophilic (Satarug et al., 2003).

As a result of wide-spread use, a very large amount of Cd is released into the environment. About 7,500-29,500 tons of Cd are directed to landfills per year and are deposited in the form of discarded products and production wastes (Järup, 2002; Lalor, 2008). The rest of the Cd is released through other various human activities. Cd waste from the industries, such as Zn production, phosphate ore implication and bio industrial manure are distributed into streams and mainly end up in soils. Another important source of Cd emission is the production of artificial phosphate fertilizers (Jailani et al., 2010; Roberts, 2014). Cadmium intoxication becomes detectable when it exceeds the threshold level of 50-70mg/ day (Strungaru et al., 2016). It enters the environment through its industrial and agricultural applications. Several compounds of cadmium are used in chemical industries and in the manufacture of pesticides and herbicides, used in agriculture (Tchounwou et al., 2012; Singh et al. 2017). When disseminated into soil, Cd can be detrimental to agro-ecosystems because it is relatively mobile and phytotoxic even at low concentrations. In response to Cd toxicity, plants have developed a protective cellular mechanism such as synthesis of phytochelatins and metallothioneins, metal compartmentalization in vacuoles, and the increased activity of antioxidant enzymes to neutralize Cd-induced toxicity (Choppala et al., 2014).

Cadmium induces water stress, the symptoms being, a decrease in stomatal conductance, transpiration rate, and leaf relative water content (Benešová et al., 2012;

Rucińska-Sobkowiak, 2016). This seems to be the result of physiological alterations in the plasma membrane properties, and these alterations could affect water uptake and balance (Javot and Maurel, 2002). Cadmium at phytotoxic concentrations could result in a decrease in the size and the number of xylem vessels, intracellular spaces, and chloroplasts (Sandalio et al., 2001) (Table 2.1). Once trace metals enter the cell, their toxicity in the cytoplasm or other important cell compartments can be decreased by sequestering metals into the vacuole (Viehweger, 2014).

International Agency for Research on Cancer (IARC, 1993; 2012) categorized cadmium as one of the contaminants by the US-EPA and acts as a human carcinogen. The classic evident of Cd toxicity in human is itai-itai disease discovered in Japan since 1912 (Bernhoft, 2013; Nishijo et al., 2017). The health implications of cadmium exposure are exacerbated by the relative exposures that cause severe respiratory irritation (Table 2.1). Occupational levels of cadmium exposure are a risk factor for chronic lung disease (through airborne exposure) and testicular degeneration and are still under investigation as a risk factor for prostate cancer (Jan et al., 2015; Otaibi et al., 2016; Mishra et al., 2019). Cadmium damages a specific structure of the functional unit of the kidney (the proximal tubules of each nephron) in a way that is first manifested by leakage of low molecular weight proteins and essential ions, such as calcium, into urine, with progression over time to frank kidney failure (Vaidya et al., 2008; Lopez-Giacoman and Madero, 2015).

2.4. Source of lead (Pb) contamination in soil and its effect on plants and human health

Lead occur naturally in the soil environment from pedogenesis processes of weathering of parent materials in trace levels (< 1000 mg/ kg) and is rarely toxic (Kabata-Pendias and Pendia, 2001; Pierzynski et al., 2000). Due to increase in

environmental levels of lead more than 1000-fold over the past years are mainly by anthropogenic activities (Pinho and Ladeiro, 2012). Major sources of lead pollution includes unwarranted use of lead containing substance in our daily life like lead based paints (contain lead chromate), lead glazed ceramics, lead-based solder (used to join copper pipe, brass and chrome plated brass faucets) (Rattner et al., 2008; Wuana et al., 2011). Moreover, extensive lead ore mining, tailings and smelting has caused high levels of soil environment contamination (Zhang et al., 2012; Singh and Li, 2014). High level of lead has been detected in plants and soils adjacent to smelting works. In addition, several pesticides containing substantial amount of Pb like lead arsenate are widely used in horticulture and agriculture (Wolz et al., 2003; Schooley et al., 2009; Wuana et al., 2011; McBride et al., 2015). Aerial emission of Pb from the combustion of petrol containing tetraethyl lead contributes substantially to the content of Pb in soils in urban areas (Wuana et al., 2011; Lenart-Boroń and Boroń, 2014; Sankhla et al., 2016).

Plants experience oxidative stress upon exposure to lead that leads to cellular damage and disturbance of cellular ionic homeostasis. It impairs plant growth, root elongation, seed germination, seedling development, cell division, transpiration, chlorophyll production, lamellar organization in the chloroplast (Pourrut et al., 2011; Flora et al., 2012). Besides that, it can accumulate in different parts of the plant and thereby enter the food chain ultimately affecting plant and human health (Table 2.1). Studies have proven that Pb have detrimental effects on soil microbial activity, especially microbial respiration (Hemida et al., 1997; Kushwaha et al., 2018). Pb can affect any organ in the body, but most sensitive parts are developing nervous system, hematological and cardiovascular system, reproductive system and kidney (Assi et al., 2016). Children are more vulnerable to Pb poisoning than adults. Its accumulation over time in human

bodies can cause serious illness which includes headache, short-term memory loss, mental confusion, sense of unreality, distorted perception, pain in muscles and joints, and gastro-intestinal upsets etc. (Marg, 2011; Assi et al., 2016; Kushwaha et al., 2018).

Table 2.1 Toxic effects of Cd and Pb on plant and human health above their permissible limit.

Heavy metals	WHO permissible limit for Plant	WHO permissible limit drinking water	Toxic effects of heavy metals on human	Toxic effects of heavy metals on plant	References
Cd	0.02	0.01	Carcinogenic, mutagenic, endocrine disruptor, lung damage and fragile bones, affects calcium regulation in biological systems	Inhibit the seed germination, stomatal opening, photosynthetic activity, causes severe chlorosis, alters the mineral uptake through plant, affect the plasma membrane permeability	Benavides, et al. (2005), Jan et al. (2015)
Pb	2	0.05	Excess exposure in children causes impaired development, reduced intelligence, short-term memory loss, disabilities in learning and coordination problems, a risk of cardiovascular disease	distortion of chloroplast ultrastructure, obstructed electron transport, inhibition of Calvin cycle enzymes, impaired uptake of essential elements, such as Mg and Fe, and induced deficiency of CO2 resulting from stomatal closure. inhibition of ATP production, lipid peroxidation, and DNA damage by over production of ROS	Pourrut et al. (2011), Kushwaha et al. (2018)

2.5. Strategies for mitigation of heavy metal stress in soil environment

Over the last few years, various remediation approaches have been developed to clean-up or restore heavy metal contaminated soils sites, such as soil capping, soil encapsulation soil washing, solidification, immobilization, coagulation, chemical precipitation, ion exchange, chemical oxidation or reduction, photocatalysis, electro-kinetic extraction, vitrification and phytoremediation (Liu et al., 2018). Further, these techniques can be divided into five major categories: physical, chemical, electrical, thermal, and biological remediation (Chowdhury et al., 2018). In general, these soil remediation methods employ different working mechanisms and demonstrate specific application advantages and limitations.

2.5.1. Physical approach

Physical approaches use high machinery tools and energy, are expensive and time consuming. Commonly used physical remediation techniques are as follows:-

2.5.1.1. Soil capping

In this method, contaminated soil site is simply covered by a layer of waterproof material to form a stable, protective surface cap. Further this capping serves as an impermeable barrier to surface water infiltration, preventing soil contaminants from further diffusing to surface water and groundwater. The capped soil, however loses its natural environmental functions especially in supporting plant growth (Liu et al., 2018).

2.5.1.2. Soil encapsulation

In this technique, contaminated soil sites are isolated and pollutants are enclosed, eliminating off-site dispersion of the contaminants and on-site bio-exposure to the

contaminants (Meuser, 2013). The low permeability caps, usually synthetic textile sheets or clay layers minimize surface water infiltration and thus prevent leaching of contaminants into the groundwater (Liu et al, 2018). The major drawback of this technique is to construct below ground vertical impermeable barriers at contamination sites.

2.5.1.3. Landfilling technique

In this technique, the metal contaminated soil is partially or completely removed by clean soil. This process is commonly called as "dig-and-haul" technique. The major drawback of this technique is that, it is only applicable for smaller area that might be severely contaminated and is very expensive (Yao et al., 2012).

2.5.2. Chemical approach

In this approach, soil is treated with various chemicals to remove heavy metal contaminants. Some of the chemical approaches are described below:-

2.5.2.1. Soil washing

It is a mixed physical and chemical process in which heavy metals are removed by soil washing through different chemicals. It relies on washing solutions to mobilize heavy metals by altering soil acidity, solution ionic strength, redox potential, or complexation (Peng et al., 2018). It includes two steps - the solubilization of metals and the removal of solubilized metals (Akcil et al. 2015). To enhance the soil washing performance, different additives are used to facilitate solubilization, dispersal, and desorption of metal contaminants from polluted sediments. Commonly used additives for washing purpose include inorganic acids (HCl, H_2SO_4, and HNO_3), organic acids (oxalic, citric, gluconic and ascorbic acids), chelators (EDDS, EDTA, and NTA), and surfactants (rhamnolipids and sophorolipids) (Akcil et al. 2015). The major drawback of this technique, the chemical additives used for soil washing are persistent in nature and may adversely affect the ecological environment (Peng et al., 2018).

2.5.2.2. Soil flushing

In this technique, the heavy metal contaminated soil is remediated by passing an extraction fluid through the soil. Further, this extraction fluid is then recovered, reused, and eventually treated and disposed off. The technique is useful to homogenous, coarse-textured soils with high permeability (Liu et al., 2018). Although this technique is operative in nature but it requires high cost to install solution collection wells or subsurface drains (Peng et al., 2018).

2.5.2.3. Soil chemical immobilization

Immobilization or sometimes called as solidification/stabilization, in which heavy metals are trapped or immobilized in the contaminated soil by introducing chemical agents into the original medium to solidify soil or convert the mobile pollutant fractions (i.e., soluble and exchangeable forms) into precipitates and/or strongly sorbed moiety (Tajudin et al., 2016). This technique does not remove or extract contaminants from the soil. Instead, the mobility/solubility of heavy metals and their concentrations in soil pore water are drastically decreased, minimizing their potential transport to plants, microorganisms, and water (Nejad et al., 2018). If the heavy metals are not removed, the chemical stabilization effect needs to be regularly monitored and evaluated. "Well-mixing" of stabilizing chemicals with contaminated soils is crucial to achieve satisfactory soil remediation effects, are the some drawbacks of this technique.

2.5.3. Thermal approach

Vitrification technique or sometimes also known as thermal approach, in which heat is used to transform contaminated soil into glasslike solids (Liu et al., 2018; Peng et al., 2018). In this technique, intensive energy is required to form a high temperature zone (>1500 °C) for melting contaminated soils, which further is converted into

molten "lava" and ultimately becomes glasslike material upon cooling (Meuser, 2013). By this technique, heavy metals are encapsulated in the glassy matrix while any organic contaminants are destroyed. After vitrification process, a vitrified product is obtained, which is strong, durable, chemically inert, and resistant to leaching. This process is not applicable to soils with high organic matter content (e.g., 7%) and high moisture content (e.g., 10%); nor it is applicable to soils, heavily contaminated by volatile or flammable organics. Overall, vitrification is a destructive process by which the processed soils no longer are able to support agricultural uses (Meuser, 2013; Liu et al., 2018).

2.5.4. Electrokinetic approach

In this technique, a low-intensity electric field is applied to remove heavy metals from contaminated soils in wet condition by electric adsorption through electrode (Akcil et al., 2015; Pedersen et al., 2015; Peng et al., 2018; Liu et al., 2018). The electric current causes the movement of small charged particles, ions between the electrodes. The charged metal particles concentrated at the polarized electrodes are subsequently removed by electroplating, precipitation, solution pumping, or ion exchange resin complexation processes (Peng et al., 2018; Liu et al., 2018). Depending on the overall velocity at which metal ions move in the soil, electrokinetic remediation may take a few days to several years, means it is a very slow and time taking process.

2.5.5. Biological approaches

These approaches involve the use of living organisms as a tool for the remediation of heavy metals in the soil. These are further categorised into two sub-categories – bioremediation and phytoremediation

2.5.5.1. Bioremediation

Microorganisms are widespread in nature and are well known to adapt the toxic metal environment. They have developed various strategies to evade the stress and toxicities associated with different heavy metals (Pan et al., 2018; Jin et al., 2018). Strategies used by microorganisms to resist heavy metal toxicity includes exclusion by using a permeability barrier, intracellular and extracellular sequestration, active transport efflux pumps, enzymatic detoxification, and reductions in cellular sensitivity to metal ions (Ahemad 2014; Pan et al., 2018; Jin et al., 2018). Microorganisms detoxify heavy metal in soil by several ways including transformation of oxidation states, (Cr(VI) to Cr (III), SeO^{2-}_4 to Se), biosorption (adsorb metal ions into their cell surface), bioaccumulation (sequester or accumulate heavy metals inside the cell), bio-assimilation (By producing low molecular weight proteins that assimilates or chelates metal ions), extracellular chemical precipitation (e.g., by S^{2-} from sulphur-reducing bacteria), and volatilization (e.g. dimethylselenide, trimethylarsine, and Hg vapor) Garbisu and Alkorta, 2003). Apart from this, several strains of bacteria produce biosurfactants such as surfactin, rhamnolipids, sophorolipids, aescin, and saponin to enhance the solubilization of metals in soils (Acikel, 2011; Lal et al., 2018). Moreover, biotransformation, biosorption, and bioaccumulation are the main three kinds of microbial remediation processes that affect heavy metal toxicity and transport, playing a critical role in microbial remediation of heavy metals. Microbes mediated remediation of heavy metals has low costs and is noninvasive; it can be done on-site and can be coupled with physical or chemical treatment technologies (Mani and Kumar 2014). Microbial remediation has been considered as a safe, easy, and effective technology (Verma et al., 2017). However, there are several limitations of bioremediation; it is time taking process and has limited practical approach; it can

be difficult to predict the bioremediation effect; and the related mechanisms are complicated and not always fully understood. For heavy metal contaminated soils, there are only two different strategies (biomobilization and bioimmobilization) which may be efficient to restore the heavy metal contaminated soils sites. Till date there is no heavy metal-contaminated soil remediation projects using microbial remediation alone have ever been reported.

2.5.5.2. Phytoremediation

It is an eco-friendly and holistic approach, in which plant is, used as a contaminant removal agent from metal polluted soils sites. The term phytoremediation is constructed by two words Greek prefix "phyto" (means plant) and Latin suffix "remedium" (means to clean or remove an evil). It is an efficient, cost-effective, environment friendly, *in situ* applicable and solar-driven remediation technology with a positive public perception (Ali et al., 2013; Mahar et al., 2016). Over the years, this technology has been intensively explored and rapidly developed. Now, it has been widely accepted and used technology to remove or reduce the heavy metal, radionuclide contamination from various environmental media, including wastewater, soil, and sediments. There are comprehensive articles and chapters on the principles and application feasibility of phytoremediation of metal contaminated soils (Ali et al., 2013; Sarwar et al., 2017; Peng et al., 2018; Jin et al., 2018, Chowdhury et al., 2018; Liu et al., 2018). Further, the key success of this technology depends on the availability of the metal of concern in a plant-available form in the soil. Plants are only able to uptake soluble fractions of metals, hence, metal fractions that are strongly bound to soil particles are unavailable for plant uptake (Yadav et al., 2018). Moreover, phytoremediation technology comprises different techniques for the amelioration of heavy metal contamination in the soil and sediments using different

mechanisms depending on their applications. However, all the mechanisms cannot be applied for remediation of all the pollutants.

2.5.6. Types of phytoremediation

Depending upon the toxicity of contaminant and available strategy, phytoremediation technique have been categorised into four major types - phytoextraction, phytostabilization, phytovolatilization and phytofilteration.

2.5.6.1. Phytoextraction process

Phytoextraction (sometimes also known as phytosequestration, phytoaccumulation, or phytoabsorption) is a critical biochemical process to remove heavy metals from contaminated soils. Among the phytoremediation technology, phytoextraction is a major process for efficient removal of heavy metal from contaminated soils (Ali et al. 2013; Dixit et al. 2015). The phytoextraction process further includes three steps – i) cultivation of suitable plant species at the polluted site; ii) harvest metal-enriched biomass from the site; iii) postharvest treatment to produce market value (e.g., energy recovery from thermal treatment or used as a bio-ore) (Sarma 2011). Further successful phytoextraction depends on several factors including bioavailability of heavy metals, soil properties, heavy metal speciation, and the plant species (Ali et al. 2013). For selecting ideal plant for phytoextraction process, there are several requirements such as high growth rate, significant aboveground biomass, a widely distributed and highly branched root system, ability to accumulate the target heavy metals from soil, ability to translocate the accumulated HMs from roots to shoots, ability to tolerate the toxicity of target HMs, ability to adapt to prevailing environmental and climatic conditions, resistance to pathogens and pests, easy cultivation and harvest, and herbivore repulsion to avoid food chain contamination (Ali et al., 2013; Peng et al., 2018). The effective phytoextraction of heavy metals and

their accumulation in the extractable plant parts have been achieved by; 1) the expression of metal ligands/transporters, changes in enzyme kinetics involved with sulphur metabolism, 2) alterations in redox states resulting in the formation of heavy metals, and 3) the formation of intermediate moieties as products of primary metabolism (Fasani et al., 2018). Metal translocation to plant shoot results in effective phytoextraction after harvesting of root biomass (Ali et al., 2013; Tangahu et al., 2011; Mahar et al., 2016). Approximately 400 plants have a high affinity for metal absorption such as Ni, Zn, and Cu, removing them via phytoextraction process. Recent phytoextraction studies include many different plant species, such as *Cichorium intybus* L. and *Ricinus communis* L. (Fuentes et al., 2018), *Pteris vittata*, *Ricinus communis* (Yang et al., 2017a, b), *Puccinellia frigida* (Rámila et al., 2016)), *Helianthus annuus* (Farid et al., 2017), *Pisum sativum* (Tariq and Ashraf 2016), *Brassica napus* (Dhiman et al., 2016)), *Jatropha curcas* (Marrugo-Negrete et al., 2015), *Brassica juncea* (Kathal et al., 2016) and *Stanleya pinnata* (Bañuelos et al., 2015).

2.5.6.2. Phytostabilization process

In this process, the plant roots and microbial interactions reduces the mobility of contaminants through stabilization or immobilization of toxic compounds in soil thereby reducing their bioavailability in the environment (Ali et al., 2013; Mahar et al., 2016; Khalid et al., 2017; Yadav et al., 2018). Immobilization of heavy metals in soils using plants can be achieved through precipitation, complexation or metal valence change/reduction in the rhizosphere (Ali et al., 2013). Different chemical forms of metals have different valences thus conferring different degrees of toxicity. One way to alleviate this problem is by planting optimal species that are efficient in the hazardous conversion of toxic metals into less poisonous form. For example,

highly toxic chromium (VI) can be reduced by some deep-rooted plants to the less soluble and bioavailable compound, chromium (III) (Ali et al., 2013). Phytostabilisation has proved useful for the treatment of Pb, As, Cd, Cr, Cu, and Zn contaminated soil and has also been successful in addressing the removal of metals and other inorganic contaminants in sediments. There are certain plant species such as *Agrostis* spp. and *Festuca* spp. that are most commonly used in the phytostabilization of Cu, Zn and Pb polluted soils in European countries (Mahar et al., 2016). Metal-tolerant species like *Epilobium dodonaei* (Randelović et al., 2016), *Iris sibirica* (Ma et al., 2017), Rose plant (Ramana et al., 2013), *Lupinus luteus* (Dary et al., 2010), *Brassica juncea* (Shiyab et al., 2009; Banuelos et al., 2005), *Hordeum vulgare*, *Vicia villosa* (Katoh et al., 2017), *Typha domingensis* and *Phragmites australis* (Bonanno, 2013) also promote metal stabilization and soil conservation as a result of their excluder behaviour. Phytostabilization process also facilitates some additional positive resolutions: 1) waste stabilisation and reduction in subsequent complications, 2) minimizing erosion by water/wind where soil exposure is halted, 3) hydraulic control (Mahar et al., 2016; Ma et al., 2017; Yadav et al., 2018). The ideal characteristics of plant species used for phytostabilization should be: 1) able to tolerate elevated levels of heavy metals, 2) possess large production of root biomass, 3) greater ability to immobilize contaminants, and 4) stagnated and increased toxin retention in the roots.

2.5.6.3. Phytovolatilization process

In this process, plants are used to extract certain metals from soils and then their release into atmosphere by volatilization process (Sarma, 2011). Arsenic (As), mercury (Hg) and selenium (Se) are present in gaseous form in the environment but can be absorbed back into the ground due to weather events. Plants such as *Arabidopsis thaliana*, *Brassica juncea* and *Chara canescens* can uptake heavy metals

and convert them to gaseous species through the partitioning of the contaminants into air spaces within a plant with subsequent release into the atmosphere (Khalid et al., 2017). For instance, arsenic transformed into arsenite and arsenate can be effectively volatilized in the fronds of *Pteris vittata* (Sakakibara et al., 2010). Phytovolatilization efficiency of *Brassica juncea* is up to 40 g Se/ha which is very promising for Se decontamination from soil (Mahar et al., 2016). For successful phytovolatilization plant root systems should include: 1) a lower water table, 2) advection with gas fluxes due to fluctuations in diel water tables, 3) increase in soil permeability and 4) hydraulic chemical redistribution (Limmer and Burken, 2016).

2.5.6.4. Phytofiltration process

Phytofiltration is the process in which plants roots (rhizofiltration) or seedlings (blastofiltration) are used to absorb or adsorb toxic metal pollutant, mainly from water or from the soil (Sarma, 2011). The ideal plant for phytofiltration should: 1) produce a substantial quantity of root biomass or surface area, 2) be able to accumulate and tolerate significant amounts of target metals, 3) involve easy handling, 4) have a low maintenance cost, 5) ability to grow under submerged conditions, and 6) have a minimum of secondary waste that further requires disposal (Yadav et al., 2018). Phytofiltration remediates metals such as Pb, Cd, Ni, Cu, Cr, V and radionuclides (uranium (U), caesium (Cs), and strontium (Sr). Phytofiltration provides a cost-effective and eco-friendly solution for the purification of wastewaters (Rezania et al., 2016). Presently, the use of native plants for phytofiltration of problematic heavy metals is under investigation to increase phytoremediation efficiency. The plant species studied include *Pistia stratiotes* for Cu, Zn and Pb (Galal et al., 2017a, b), *Limnocharis flava* for Cd (Marrugo-Negrete et al., 2017), *Salix matsudana* for Pb (Tang et al., 2017) and *Typha domingensis* for Cd, Cr and Hg (Vymazal, 2016). *Micranthemum umbrosum* and *Warnstorfia fluitans* also have great potential for

phytoremediation. *M. umbrosum* works efficiently in hydroponic nutrient solutions for removal of As and Cd without any phytotoxic effects, while *W. fluitans* works efficiently in arsenite/arsenate contaminated water bodies for the absorption of As from contaminated water without displaying any toxic effects of the metalloid (Sandhi et al., 2018). Another study regarding metal uptake by phytofiltration has demonstrated that a mixed culture of *Phragmites australis* and *Typha latifolia* provide a highly efficient removal of Cu, Cd, Cr, Ni, Fe, Pb and Zn within 14 days of the initial pollution of water bodies (Kumari and Tripathi, 2015).

2.5.7. Hyperaccumulator plants

The success of phytoremediation of heavy metal depends on the proper selection of the plants. Hyperaccumulator plants (that accumulate particular metal or metalloids in their living tissues to levels that may be hundreds or thousands of times greater than is normal for most plants) can accumulate heavy metals into their aboveground biomass (e.g., shoots and leaves) at concentrations 100 to 1000-fold higher than the plants belonging to the non-hyperaccumulator group (Peer et al., 2005; Reeves et al., 2018a; Reeves et al., 2018b). Some other factors like Bioconcentration factor (BCF) and translocation factor (TF) also play determining roles for heavy metal hyperaccumulation inside plant tissues. Hyperaccumulator plants are able to rapidly translocate metals from root to shoot via xylem. It has been observed that hyperaccumulator plants such as *Thlaspi caerulescens* and *Sedum alfredi* can translocate metals from root to shoot within 48 - 72 h (Baker and Brooks, 1989).

2.5.7.1. Maize (*Zea mays* L.)

Maize (*Zea mays* L.), is an annual cereal crop spread worldwide with high growing capacity. It produces high extensive fibrous root system with high biomass and a number of seed in adverse conditions. This is a heavy metal tolerant and metal

accumulating plant with moderate bioaccumulation factor. It is capable of growing in diverse climates (Temperate and Tropical) and serves as a renewable source of energy as is an important feedstock for bioethanol production in the USA and Brazil (Schwietzke 2009). Due to its luxuriant root system, high-biomass and adaptability, it is capable of continuous phytoextraction of metals from contaminated soils by translocating them from roots to shoots (Nascimento and Xing 2006) and considered as a promising crop for phytoremediation of soils contaminated with multiple heavy metals (Wuana and Okieimen 2010). Recently, Awokunmi et al., 2018 reported that *Zea mays* L. accumulated 101, 110, and 104 mg Kg^{-1} Cd, Pb and Cr, respectively in their harvestable parts growing in waste dumping sites.

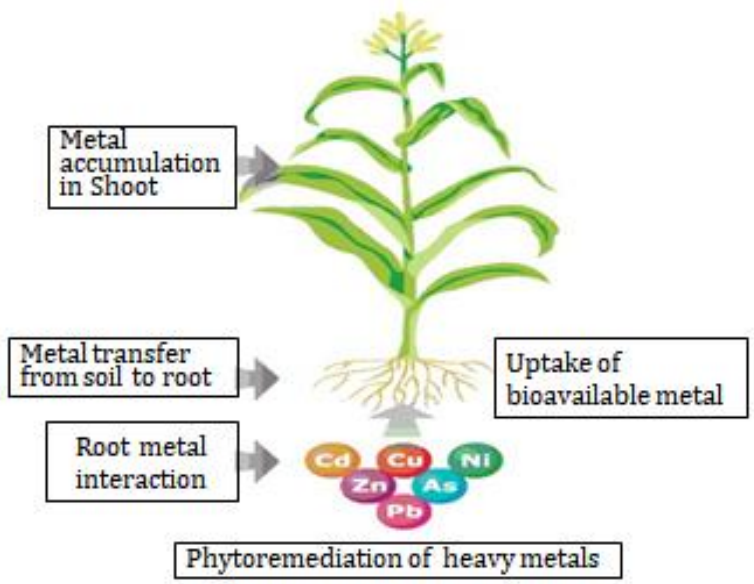

Figure 2.1 Mechanisms of heavy metal phytoremediation by hyperaccumulator plant

2.5.7.2. *Canna indica* L.

Canna indica is also important plant and frequently used for phytoremediation of heavy metals as well as organic pollutant in terrestrial and aquatic systems (Yadav et al. 2012). *C. indica* is having good ability to accumulate considerable amount of

hazardous metals like Cadmium, Lead, Nickel and Zinc etc. in their various parts i.e. root, shoot and leaves (Subhashini et al. 2013). It is well known for growing in various climatic conditions such as tropical, subtropical, humid and arid (Cheng et al. 2002). For their long flowering duration, easy growth, reproduction and adaptation to different climate, and beauty makes this plant suitable for phytoremediation as well as gardens/parks beautification. *C. indica* is having the potential to grow in industrial sludge amended soil due to their fractionation and translocation characteristics of heavy metals like cadmium, lead, chromium, zinc, nickel, copper and manganese. Further their characteristic features like antioxidant and phytochelation are considerable for the tolerance to oxidative stress caused by several heavy metals (Bose et al. 2008). In a study, Cheng et al., (2002) reported that *Canna indica* grown in hydroponic condition in the presence of Cd metal ions tolerates up to 400 ppm cadmium concentration and concluded that *Canna indica* is potential specie for phytoremediation of cadmium with some limitations only at higher concentrations. Another study carried by Boss et al., (2008), reported about *Canna indica* grown in different concentration of industrial sludge containing Cr, Fe, Cd, Cu, Ni, Zn, Mn and Pb heavy metals. The metal accumulation in various parts of *C. indica* after 90 days of experiment, was in the order of Fe > Cr > Mn > Zn > Ni > Cu > Cd > Pb and the metal translocation was found lesser in shoot. With the increasing percentage of sludge amendments in soil, the metal concentrations increased in different parts of *Canna indica*.

2.5.8. Limitations of Phytoremediation

Although phytoremediation is a promising technology but it has several limitations that require further intensive research on plants and site-specific soil conditions. The major drawback of this technology is that it is a time-taking process, compared with

other conventional physical and chemical remediation processes that might take weeks to months to clean up a heavy metal-contaminated site, phytoremediation may need several years to achieve the same result (Clemens, 2001; Tong et al., 2004; LeDuc and Terry, 2005; Karami and Shamsuddin, 2010; Mukhopadhyay and Maiti, 2010; Ali et al., 2011; Ramamurthy and Memarian, 2012). Plants with low biomass yields and reduced root systems do not support efficient phytoremediation and most likely do not prevent the leaching of contaminants into aquatic system. Another limitation of this technology is that the depth of the root system dictates the extent of remediation possible, which makes this technology effective only for sites with shallow contamination. It is hard to mobilization of more tightly bound fraction of metal ions from soil i.e., limited bioavailability of the contaminants in the soil. It is applicable to sites with low to moderate levels of metal contamination because plant growth is not sustained in heavily polluted soils. Another important drawback of this technology is that a risk of food chain contamination in case of mismanagement and lack of proper care.

Table 2.2 Various Technique for remediation of heavy metal contaminated soils and their advantages and disadvantages (adopted from Yadav et al., 2018)

Techniques	Advantages	Disadvantages	References
Physical methods			
Coagulation	Low cost, simple to use, well proven, readily available chemicals	Sludge generation, unpleasant water taste, residual aluminium, coagulation and flocculation cannot treat wastewater	Fu and Wang (2011), Bundschuh et al. (2010)
Adsorption	Effectiveness of some adsorbents are efficient at wide pH range, easy operating conditions, commercially available sorbents, regeneration ability of used adsorbents, comparatively low cost	Low selectivity, production of waste products, some adsorbents are pH sensitive, considerable adsorbent regeneration time, competing ions reduce efficiency	Jadhav et al. (2015), Babel and Kurniawan (2003), Aklil et al. (2004) and Yadav et al. (2017a,b),
Membrane filtration	Small space requirement,	High initial and	Jadhav et al. (2015)

Techniques	Advantages	Disadvantages	References
	low pressure, highly efficient, no chemicals required, ability to purify water completely	operational cost, high water rejection, brine disposal problem, need for posttreatment remineralisation	
Biosorption	High efficiency even with low concentration of biosorbent, eco-friendly, cost-effective, no generation of secondary compounds, short operation time, ability to handle multiple heavy metals, high surface area to volume ratio, rapid kinetics of adsorption and desorption	High temperatures, biomass characteristics and concentration, acidity, metal affinity to biosorbent affects biosorption	Sharma et al. (2016a,b) and Abbas et al. (2014
Chemical methods			
Chemical precipitation	Low capital cost, simple operation, particular components removed, high degree of selectivity	High acid content, production of a large quantity of toxic sludge, extra operational cost for sludge disposal problem	Kurniawan et al. (2006)
Ion exchange	Sensitive to binding the particles, specific ability to swap cationic resins, high treatment capacity, high removal efficiency, fast kinetics	Ion exchange is nonselective and highly sensitive to pH of solution, expensive, regeneration causes secondary pollution	Mohammadi et al. (2005); Volesky (2001); Kang et al. (2004)
Electrochemical removal	High separation selectivity, rapid and well-controlled process, provides good reduction yields, produce less sludge	Corrosive, high energy consumption, high operational cost	Fu and Wang (2011)
Chemical oxidation or reduction	Specific chemicals required for specific metals decontamination (not universal), biological system is having slow rate of chemical reaction in soil due to slow rate of released chemical from roots	Mineralisation, steam stripping, air stripping, more research still needed, climate-sensitive, produces toxic byproducts	Volesky (2001); Bundschuh et al. (2010)
Photocatalysis	Efficient treatment, low-cost operation, high stability, removal of both metal and organics, less harmful byproducts	Long process time, limited applications	Barakat (2011)

Techniques	Advantages	Disadvantages	References
Biological methods (Bioremediation)			
Micro-organism based	Cost effective, minimal site disruption, eco-friendly, high efficiency, performed on site, eliminates waste permanently, reduces long-term liability, greater public acceptance, regulatory encouragement, use of mobile bioreactors, remediation by own consortia of previously selected and adapted zymogenous microorganisms	Time-consuming, depends on environmental parameters, highly specific, process is sensitive to level of toxicity, release toxic metabolites, tailored to site-specific conditions	Girma (2015); Verma et al., 2017
Plant-based (Phytoremediation)			
Phytoextraction	Abundant biomass in short period of time, limits dust generation, less waste to dispose off, cost-effective, applicable for laboratories, pilot and field study	Slower process, the concentration of the contaminant is important, depends on the depth of contamination, toxic metals leaches into groundwater, phytomass must be disposed properly	Meyerholt (2013)
Phytovolatilisation	Economically efficient, contaminant transformed into a less toxic substance, applicable for soil, sediments, sludges, accelerates degradation processe	Re-deposition of pollutant back into ecosystem by precipitation (elemental Hg)	USEPA (2000)
Phytofiltration	Environmental friendly, inexpensive procedure, applicable for groundwater and surface water, after harvesting crop may be converted to biofuel briquette	Long-term maintenance depends on type of contaminant and depth, hinders plant growth, highly species specific	Singh et al. (2015)
Phytostabilisation	Disposal of hazardous material/biomass is not required, low cost and less disruptive, highly efficient, plant reduces soil erosion, applicable in field and mining area, tolerant of high levels of	Soil cannot (only with extensive efforts, time and money) be made suitable for plant growth (soil structure, high salinity, toxic substances other than metals), conflicting	USEPA (2000); Kumpiene et al. (2007

Techniques	Advantages	Disadvantages	References
	contaminants, enhances soil fertility, achieving ecosystem restoration	results between plant growth and metal leaching, metal concentrations in vegetables not sufficiently reduced	
Phytodegradation	Enzymatic breakdown of pollutants, applicable for soil, sediment sludges, groundwater and surface water	Depends on factors such as the concentration and composition, plant species, and soil conditions	Subrahmanyam and Prasad (2011)
Rhizosphere bioremediation	Increased microbial activity, release of organic acid, rhizosphere enhances biodegradation, applicable for soil, sediment sludges, groundwater, cost-effective, environmental friendly, self-sustaining method of contaminant removal.	Continous adjustment of pH in influent solution to obtain optimum metals uptake, slow-growing root systems, laboratory and greenhouse studies might not be feasible in field	Rungwa et al. (2013); Subrahmanyam and Prasad (2011)

2.5.9. Plant growth promoting rhizobacteria

Every plant has its specific microflora in their rhizosphere and this microflora plays an important role in plant growth promotion as well as heavy metal detoxification/removal in contaminated soils. Plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978) are a heterogeneous group of bacterial communities naturally existing in the rhizosphere, plays important role in plant growth promotion (Zubair et al., 2016). PGPRs are not only significant from agricultural point of view, but also play an important role in soil remediation strategies, not only by enhancing growth and successful establishment of plants in polluted soils, but also by increasing the availability of contaminants. Their density in the rhizosphere is much higher than the rest of soil because of the availability of high levels of nutrients, especially small molecules such as amino acids, sugars and organic acids that are exuded from the roots of most plants. These exudates support large and

active beneficial bacterial populations existing in the rhizosphere (Lynch and Brown, 1997). PGPR in metal contaminated soils helps plants by several ways like they help in mineral uptake, contribute in essential vitamins, stomatal regulation, osmotic modification, and adaptation of root morphology (Bauer et al., 2013; Vacheron et al., 2013).). Further, heavy metals contaminated soils affect nutrient uptake and retards plant growth (Ouzounidou et al., 2006). Under such nutrient limiting conditions, PGP bacteria help in providing essential nutrients to plants. For example, *Rhizobium leguminosarum* bv. trifolii can fix nitrogen in the presence of heavy metals and support plant growth (Nonnoi et al., 2012). Chromium resistant *Cellulosimicrobium funkei* isolated from *Phaseolus vulgaris* rhizosphere solubilize PO_4, produce IAA, EPS, ammonia, catalase, biosurfactant, protease, amylase, and lipase in the presence of chromium. The root elongation assay with *C. funkei* significantly increased root length in the presence of chromium (Karthik and Arulselvi, 2017). Bacteria that assist in the remediation/detoxification of heavy metals can contribute to this process directly or indirectly. Direct mechanisms involves production of EPS, siderophore, metallothioneins, biosurfactants, organic acid, and phytochelatins, increases the bioavailability, solubility and accumulation of metals and indirectly by the improvement of plant growth and protection against pathogens that further facilitate the accumulation of heavy metals (Ullah et al., 2015a, b; Shi et al., 2016; Gupta and Kumar, 2017). A large number of PGPRs have been identified and evaluate for plant growth promotion and heavy metal detoxification including the genera: *Pseudomonas*, *Azospirillum*, *Burkholderia*, *Bacillus*, *Enterobacter*, *Pantoea*, *Rhizobium*, *Erwinia*, *Serratia*, *Alcaligenes*, *Arthrobacter*, *Acinetobacter*, and *Flavobacteria* (Verma et al., 2017).

2.5.9.1. Mechanisms of plant growth promotion and heavy metal detoxification by PGPR

Plant growth promoting rhizobacteria promotes plant growth by two different ways either directly or indirectly. Usually in heavy metal contaminated soil PGPR promotes plant growth by direct means by fixing atmospheric nitrogen, production of siderophore (Chelate or sequester iron from the soil), production of phytohormones (such as Auxins and Cytokinins), solubilize mineral phosphorous that readily available to plants, produce ACC deaminase enzyme (modulate the level of ethylene within the plant during abiotic stress).

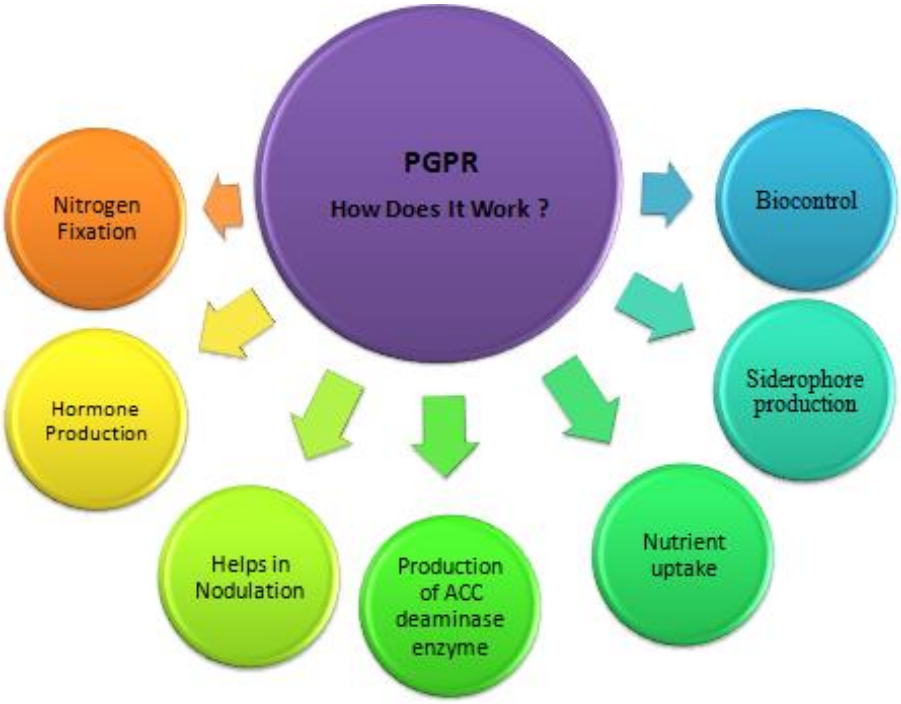

Figure 2.2 Diverse mechanisms of plant growth promotion by PGPR in contaminated soils

Apart from direct mechanisms PGPR promote plant growth in contaminated soils by indirect means including: antibiotic production, depletion of iron from the rhizosphere, induction of systemic resistance, synthesis of antifungal metabolites,

production of fungal-cell wall lysing enzymes, competition for inhabitants on the roots, and HCN production (Glick, 2010; Rajkumar et al., 2012). Further PGPR also have the potential to detoxify heavy metals by several ways including they alter metal bioavailability in soil through acidification, chelation, complexation, precipitation, and redox reactions by producing several metabolites (Mishra et al., 2017).

2.5.10. PGPR assisted Phytoremediation of Heavy metal contaminated soil

Heavy metal resistant plant growth promoting rhizobacteria not only contribute in the growth promotion of host plant but also accelerates heavy metal remediation from contaminated soil through diverse mechanisms (Rajkumar et al., 2012). Through direct mechanisms they are involved in the production of extracellular polymeric substances (EPS), siderophore, metallothioneins, biosurfactants, organic acid, and phytochelatins that increases the bioavailability, solubility and accumulation of metals and indirectly by the improvement of plant growth and protection against pathogens that further facilitate the accumulation of heavy metals (Ullah et al., 2015a, b; Shi et al., 2016; Gupta and Kumar, 2017). Plant-associated rhizobacteria can also reduce heavy metal stress by the synthesis of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase which reduces the high levels of ethylene produced in abiotic stress (heavy metal stress) by consuming its immediate precursor, the ACC.

2.5.10.1. Phosphate solubilizing bacteria (PSB) assisted phytoremediation of heavy metal contaminated soil

In most of the heavy metal contaminated soils, metals are strongly bounded by soil particles; therefore, they are not easily available for uptake by hyperaccumulator plants (Gamalero and Glick 2012; Rajkumar et al., 2012). In this context, PSB plays promising role, since they have the capability to solubilize the insoluble and

biologically unavailable metals such as phosphorous and Zn (Verma et al. 2011), by secreting low molecular weight organic acids; thus, they facilitate metal bioavailability for plant uptake (Ahemad, 2015). A variety of organic acids production has been reported by PSB such as oxalic acids, citric acids, 2-ketogluconic acid, valeric acid, tartaric acid, succinic acid, glycolic acid, lactic acid, formic acid, malonic acid, piscidic acid and malic acid which have solubilize or chelate the heavy metal ions in contaminated soils (Panhwar et al. 2013). For example Patel et al., (2010) reported the role of organic acids oxalic acid, malic acid in the solubilization of phytate in the presence of heavy metals. Their results demonstrated that application of phytate solubilizing bacteria probably enhanced the accumulation of heavy metals in *Cajanus cajan*. Similarly, Han et al., (2006) reported the role of organic acid, acetic acid and malic acid in inducing Cd uptake by maize roots and reported that the organic acids with lower molecular weight was able to increase large amount of Cd accumulation in maize. Another study by Panfili et al. (2009) reported a similar effect on the Cd uptake and distribution among durum wheat roots in the presence of citric acid. These results suggested that metal-organic acid complexes indirectly contribute to the metal uptake through metal-complexes dissociation within the diffusion layer and/or at the root surface thus increasing the concentration of free metal ions (Han et al., 2006; Panfili et al., 2009). Although root associated rhizobacteria have the ability to produce organic acids and these acids are capable to mobilize toxic and essential metal ions (Uroz et al., 2009) but an important question that has yet to be adequately resolved is whether PGPR act as sources or sinks of organic acids in the soil contaminated by heavy metals. Ashraf et al., (2017) reviewed several laboratory studies with soil microbes have explained to some extent, in which the level of organic acid influx is directly regulated by the external concentration (Ashraf et al.,

2017). A detailed characterization of the factors those control the fate and behaviour of organic acids in soil (e.g., heavy metal/nutrient-mobilization efficiency, concentrations required for metal mobilization, sorption to the soil's solid phase and biodegradation), are key to identify the precise mechanisms of microbial organic acids in the metal contaminated rhizosphere soils.

2.5.10.2. Rhizobacterial siderophore mediated phytoremediation of heavy metal contaminated soil

Plant growth promoting rhizobacteria are well reported to produce iron chelating compounds known as siderophores in response to low iron availability in the rhizosphere (Sinha and Mukherjee 2008). Siderophore are low molecular weight (400- 1,000 Da) organic compounds with high affinity to chelate and transport iron (Fe^{3+}) (Dimka et al., 2008). Apart from iron chelation siderophores also capable to chelates toxic metal ions, such as Cd, Pb, Zn, Ni, As, Al, Cu, In and Ga (Sinha and Mukherjee 2008; Rajkumar et al. 2010; Braud 2010; Schalk et al. 2011). Furthermore, siderophores having diverse functional groups which are based on their overall structure that coordinates the iron. In their metal binding sites, siderophores have either hydroxycarboxylic acid, catechol or hydroxamic acid moieties sites and thus can be classified as hydroxycarboxylate-, catecholate- or hydroxamate-type siderophores. The acquisition mechanisms of iron by siderophore describes that it is an energy-dependent phenomenon that can occur via four different ways: (1) shuttle transport mediated by penetration of the iron-siderophore complex and cleavage of these two compounds by an intracellular reductase; (2) taxicab transport mediated by the production of a siderophore that chelates iron and remains outside the membrane, while iron is transferred through ligand exchange to an internal siderophore pool (Renshaw et al. 2002; Comensoli et al., 2017); (3) hydraulic acquisition mediated by

the penetration of the iron-siderophore complex into the cell and subsequent cleavage of the complex by intracellular reductive and degradative reactions; and (4) reductive acquisition mediated by the production of a siderophore that chelates iron and remains outside the cell, followed by iron reduction near the membrane and uptake of Fe^{2+}(Renshaw et al., 2002; Comensoli et al., 2017). Several studies has been carried out in the favour to explore the siderophore mediated enhanced uptake of heavy metals by plants, in this context Sinha and Mukherjee, (2008) reported Cd-induced siderophore production by *Pseudomonas aeuroginosa* isolated from rhizospheric region of plant, showed Cd-induced siderophore production maximally at 1.75 mM of Cd concentration under culture condition. It stimulates the growth of mustard and pumpkin plants in Cd-added soil through its establishment in rhizosphere (Sinha and Mukherjee, 2008). Similarly, Dimkpa et al. (2009a) found that the siderophores produced by *Streptomyces tendae* F4 significantly enhanced uptake of Cd by sunflower plant. Another recent study carried by Hesse et al., (2018), reported that siderophore production was highest in the presence of copper by wild type *Pseudomonas aeuroginosa* isolated from rhizosphere region.

2.5.10.3. Exopolysaccharides mediated phytoremediation of heavy metals

Exopolysaccharides (EPS) are a complex blend of high molecular weight biopolymeric metabolite secreted by bacteria, fungi, few plants and microalgae for protection against environmental stress. They not only protect cell against dewatering or toxic substances but serves as a carbon and energy source too (Gadd, 2004; Gupta and Diwan, 2017). EPS produced by rhizobacteria mainly consists of polysaccharides, proteins, humic substances, uronic acid, nucleic acid, lipids and glycoproteins surrounding the cells which bind metals (Das et al., 2009; Sheng et al., 2010). Different investigators have reported about a variety of rhizobacterial species and a

diverse range of EPS (Table 2). Rasulov et al. (2013) reported the remediation efficiency of EPS produced by *Azotobacter chroococcum* strain XU1 up to 33.5 mg g^{-1} for Pb, and 38.9 mg g^{-1} for Hg respectively. The metal absorption behaviour of alginate (EPS) produced by *Azotobacter* in soil helps in the remediation of toxic metals by creating micro-environment of essential metal ions to maintain soil ecology and accelerate the growth of the plant (Rasulov et al., 2013). In another report on biosorption of Cu^{2+} and A^{g+} by exopolysaccharide produced by four marine bacterial strains, the maximum remediation were 400 mg g^{-1} EPS (6.29 mM g^{-1}) and 333 mg g^{-1} EPS (3.09 mmol g−1) for Cu^{2+} and Ag^{3+}, respectively (Deschatre et al., 2013). Another recent study was carried by Li et al. (2017a, b) in which they showed the role of EPS in Ni^{2+} biosorption onto aerobic/anaerobic granular sludge. The maximum biosorption was achieved 65.77 mg g^{-1} for aerobic sludge and 54.18 mg g^{-1} for anaerobic sludge respectively. Moreover EPS producing rhizobacteria increases root and shoot growth of wheat under drought stress (Hussain et al., 2014). EPS produced by rhizobia helps in the synthesis of biofilm where they get protection from environmental anomalies and may help the plants by extracting more water and nutrients (Vanderlinde et al., 2010). EPS also plays a significant role in metal complexation thereby reducing their bio-accessibility and bioavailability by infiltration of heavy metals (Wei et al., 2011; Gupta and Diwan, 2017). Joshi and Juwarkar (2009) reported that the immobilization of Cd and Cr after inoculation of EPS-producing *Azotobacter* spp. was 15.2 mg g^{-1} of Cd and 21.9 mg g^{-1} of Cr. The tactics for achieving a significant amount of toxic heavy metal removal through bacterial EPS must be focused on utilizing the non-neutral, negatively charged EPS (EPS packed with abundant anionic functional groups) to be incorporated as a suitable biosorbents. Some of the reported commercial bacterial EPS with the required

anionicity are alginate (*P. aeruginosa*, *Azotobacter vinelandii*), gellan (*Sphingomonas paucimobilis*), *hyaluronan* (*P. aeruginosa*, *Pasteurella multocida*, *Streptococci attenuated* strains), xanthan (*Xanthomonas campestris*), *galactopol* (*Pseudomonas oleovorans*), *fucopol* (*Enterobacter* A47) (Freitas et al., 2011; Öner, 2013). EPS with different chemical compositions were tested for their ability to sorbed mercury, and it was observed that the EPS containing hexosamines was most effective in removing mercury from the solution whereas EPS consisting neutral sugars removed the least amount of mercury from the solution (Cruz, 2014). It was revealed that the EPS produced by Ni-resistant *Cupriavidus pauculus* bacteria isolated from serpentine soil was a homopolymer of rhamnose containing uronic acid, protein, and nucleic acid (Pal and Paul, 2013). Unimpaired whole microbial cells and additionally cell bound EPS, have discovered broad application for metal remediation in industrial as well as environmental wastewater sources (Kumar, 2016).

Table 2.3 Exopolysaccharide producing bacteria from contaminated soil and rhizosphere

Types of EPS	Source/Origin	Bacterial species	Heavy metal	Removal efficiency	Reference
alginate	Soil	*Bacillus sp. CIK-516 and Stenotrophomonas sp. CIK-517Y*	Nickel	Maximum accumulation of Ni 609 mg kg^{-1}dry weight	Akhtar et al. (2018)
	Mangrove rhizosphere	*Kocuria flava AB402 and Bacillus vietnamensis AB403*	Arsenic	tolerate 35mM and 20mM of arsenite respectively	Mallick et al. (2018)
	Soil	*Fluorescent Pseudomonas strain Psd*	Zinc		Upadhyay et al. (2017)
	Gram negative microbial consortia		Zinc, lead, Chromium, Nickel, Copper, Cadmium Cobalt	75 to 78% reduction in metal load	Gawali et al. (2014)
		Azotobacter chroococcum	Lead, Mercury	40.48% Pb^{2+}(33.5 mg Pb^{2+}/g of EPS); 47.87% Hg^{2+} (38.9 mg of Hg/g EPS)	Rasulov et al. (2013)

Types of EPS	Source/Origin	Bacterial species	Heavy metal	Removal efficiency	Reference
		Lactobacillus plantarum	Lead	276.44 mg Pb^{2+}/g EPS, at 1000 ppm initial metal load	Feng et al. (2012)
		Ensifer meliloti	Lead, Nickel, Zinc	89% Pb^{2+}, 85% Ni^{2+}, 66% Zn^{2+} reduction from 50 ppm initial load	Lakzian et al. (2008)
Homogenous consortial EPS	Soil isolates	*Bacillus firmus*	Lead, Copper, Zinc	1103 mg Pb^{2+}/g EPS (98.3 %,), 860 mg Cu^{2+}/g EPS (74.9%), 722 mg Zn^{2+}/g EPS (61.8%)	Salehizadeh, and Shojaosadati, (2003)
	GRAS status	*Paenibacillus jamilae*	Lead, Cadmium	200 - 300 mg Pb^{2+}/g EPS, 21 mg Cd^{2+}/g of EPS	Morillo et al. (2006)
	PGPR consortia	*Gordonia alkanivorans strains SMV185.1, SMV185.5, SMV207.37, Macrococcos caseolysticus and Lysinibacillus macrolides*	Arsenic, Mercury	85% for As^{5+}, As^{3+}, 45% for Hg^{2+}	Franchi et al. (2017)
	PGPR consotia	*Cellulosimicrobium funkei AR8, Cellulosimicrobium funkei AR6,*	Chromium	EPS to 14.79 and 5.89% in 50 lg/mL of Cr^{6+} treated AR6 and AR8 strains, respectively.	Karthik et al. (2016)
	EPS mediated synthesized CdS nanoparticle	Pseudomonas aeruginosa JP-11	Cadmium	88.66% form aqueous solution	Raj et al. (2016)
	Agar Beads immobilized	*Paenibacillus polymyxa*	Lead	111.11 mg Pb^{2+}/g EPS	Mokaddem et al. (2014)
	Hydrocarbon contaminated water microbial consortium		Zinc, Copper, Cadmium	87.12% Cd^{2+}; 19.82% of Zn^{2+}; 37.64% of Cu^{2+} reduction from 1 ppm initial metal load	Martins et al. (2008)
		Paenibacillus polymyxa	Copper	1602 mg Cu^{2+}/g EPS	Acosta et al. (2005).
	Microbial mats and Deep sea hydrothermal vents	*Paracoccus sp., Alteromonas sp., Vibrio sp., Vibrio diabolicus, Pseudoalteromonas*	Mercury	Uptake capacities ranged from 0.005 to 0.454mM	Cruz et al. (2017)

Types of EPS	Source/Origin	Bacterial species	Heavy metal	Removal efficiency	Reference
		sp., Alteromonas sp.		Hg/g for the different EPS(sorbed upto 82%)	
	Marine	Paracoccus sp., Alteromonas sp, Vibrio sp	Copper and Silver	400mg g^{-1} EPS (6.29 mM g^{-1}) for Cu^{2+} and 333 mg g^{-1} EPS (3.09 mM g^{-1}) for Ag^{2+}	Deschatre et al. (2013)
Heterogenus consortial EPS	Activated sludge mixed consortia		Zinc, Copper, Chromium Cadmium	85 to 95% reduction from initial metal load of 10-100 ppm	Liu et al. (2001)
Dead biomass EPS		*Bacillus cereus, Bacillus pumilus, Pantoea agglomerans*	Chromium	89.87%, 89.23%, 85.5% reduction from initial metal load of 50 ppm	Sultan et al., (2012)
Dead biomass EPS	Activated sludge isolate	*Ochrobactrum anthropi*	Chromium, Cadmium, Copper	57.8 mg Cr^{6+}/g EPS at initial metal load of 280 ppm, 26 mg Cu^{2+}/g EPS at initial metal load of 91.6 ppm 29.5 mg Cd^{2+}/g EPS at 100.6 ppm initial metal load	Ozdemir et al. (2003)
Modified EPS		*Pseudomonas putida, Rhizobium alamii*	Cadmium	N.A.	Xu et al. (2012), Wei et al. (2011), Schue et al. (2011).
	Phosphorylated bacterial EPS (cellulose)	*Acetobacter*	Lead, Copper, Manganese, Zinc, Cobalt	90% reduction from initial metal load of 0.1mM/dm^3 (Fe^{3+} > Cu^{2+}> Mn^{2+} >Zn^{2+}; Co^{2+})	Oshima et al. (2008)
Immobilized EPS	Alginate bead immobilized	*Chryseomonas luteola*	Cadmium, Cobalt, Copper, Nickel	64.10mg Cd^{2+}/g EPS 55.25mg Co^{2+}/g of EPS 1.989mM Cu^{2+}/g EPS 1.224mM Ni^{2+}/g EPS	Ozdemir et al. (2005a) Ozdemir et al. (2005b)

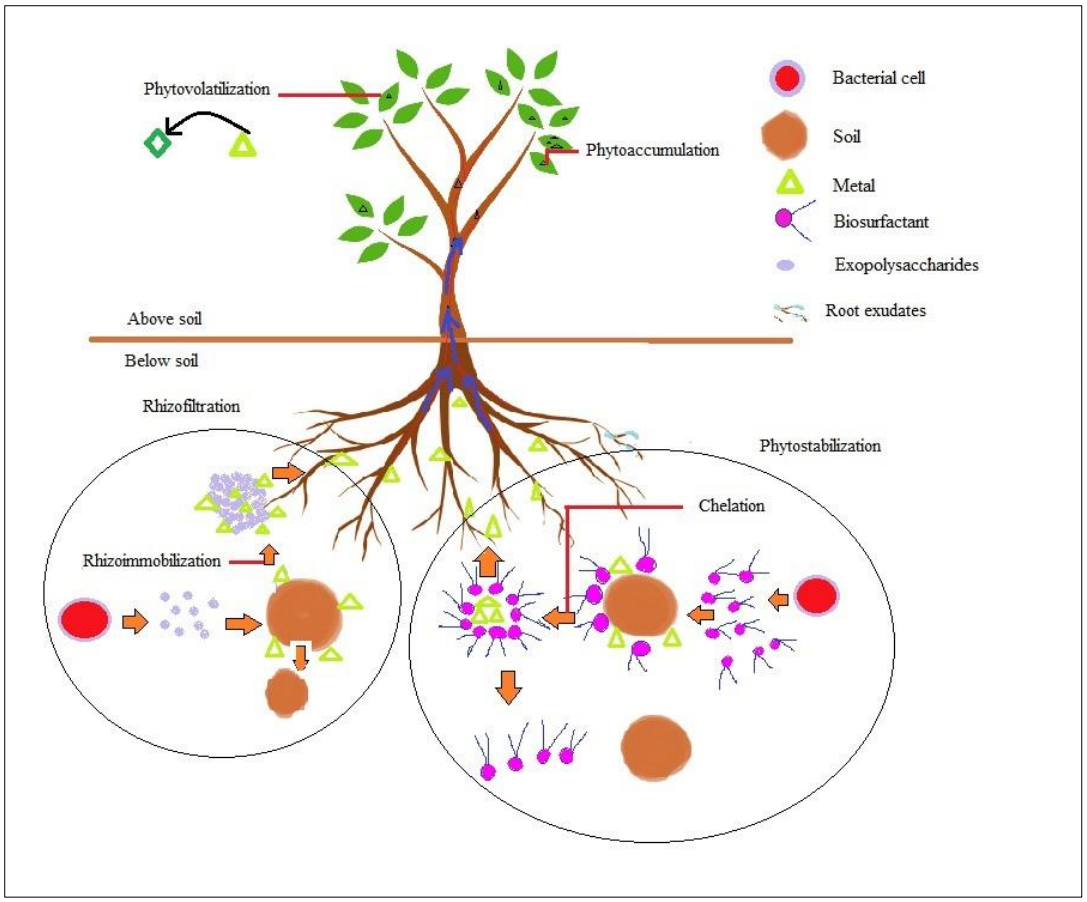

Figure 2.3 Diverse mechanisms by PGPR in rhizobacteria assisted phytoremediation of heavy metals (adopted from Lal et al., 2018)

2.5.10.3.1. Interaction mechanism between exopolysaccharide and heavy metal ions

The interaction between EPS and heavy metal appears very complex in the form of electrostatic attraction in which surface complex formation and chemical interaction between heavy metal ions and the functional groups of EPS occurs (Dobrowolski et al., 2017). It has been reported that due to the presence of acyl group, EPS shows anionic property, which increases the interaction with other cationic heavy metals (Cd^{2+}, Pb^{2+}, Co^{2+} and Ni^{2+}) and forms EPS-metal complex (Kaushal and Wani, 2016). Generally, EPS produced by rhizobacteria depicts a strong binding capacity to heavy metals and entrap precipitated metal sulphides and oxides, leading to the development

of EPS-metal complexes and subsequently enhancing the heavy metal remediation (Joshi and Juwarkar, 2009; Xu et al., 2012). In a study carried by Xu et al. (2012), EPS produced by *Pseudomonas putida* transformed the bioavailability of Cd^{2+} into organic species by means of complexation. Carboxyl and phosphate groups are mainly responsible for Cd^{2+} binding ability of EPS produced by *P. putida* (Wei et al., 2011). Moreover, electrostatic interaction seems to be the major mechanism through which EPS helps in the remediation of heavy metals. This interaction is mainly attributed to competition between divalent and trivalent cations; trivalent cations directly competed with divalent cations for EPS binding sites. Trivalent cations were more competitive than divalent cations for binding because they formed more strong bonds with EPS (Yan et al., 2017). The strength of interactions between the particular surface groups (mainly hydroxyl, acetamido or amino groups) and the metal ions depends on the type and activity of adsorption centre and the ion properties (Dobrowolski et al., 2017). Recently a thermodynamic study carried out on the interaction between EPS and heavy metal ions showed that the binding between heavy metals and EPS was spontaneous and driven mainly by enthalpy change. Environmental factors have also significant impact on the adsorption performance (Yan et al., 2017). Another study on the interaction of EPS and Ni^{2+} showed a stable operation of the granular sludge-based system, influencing the microbial activity and surface characteristics of sludge (Li et al., 2017a, b). Moreover, it is well reported that EPS have a high binding ability for heavy metal due to their abundant functional groups (e.g., carboxyl and hydroxyl groups).

As evidenced from above literature of review the research done till date, researcher worldwide are still working on the mechanisms behind plant-microbe-metal interactions as no concrete findings or sustainable technology has emerged till date

due to some or other reasons but interestingly, plant growth promoting rhizobacteria have emerged as an effective candidate to be applied in the remediation of heavy metal contaminated soils with plants like *Zea mays* L. and *Canna indica,* so the present study was taken to address the issues not taken up till now.

Chapter 3

Materials and Methods

CHAPTER - 3

MATERIALS AND METHODS

This chapter embodies the standard methods and protocols that were used to accomplish the objectives of the present study. All the ingredients and the media used in the experiments were purchased from Hi-media, SRL, Bangalore Genei, Merck, India and Sigma chemicals USA. The composition of various media is provided in Appendix, while some of the important reagents and media compositions are mentioned in the text. All the reagents used were analytical reagent (AR) grade with 99.99% purity. Analytical instruments, their make and model are suitably mentioned in the text. Further chapter also describes about the sampling sites from which the samples were taken for the isolation of the heavy metals (Cd and Pb) resistant rhizobacteria (Bacterial isolates), their maintenance and characterization. Details of pot experiments with *Canna indica* and *Zea mays* L. plant to study the interaction of heavy metal resistant rhizobacteria for phytoremediation of Cd and Pb contaminated soil is also included in this chapter. This chapter has been divided into sections and sub-sections wherever necessary for the ease of understanding.

3.1. Description of sampling sites and collection of sample

The rhizospheric samples (soil and roots) were collected from industrially contaminated sites of Lucknow, Kanpur nagar and Kanpur dehat region of Uttar Pradesh, India. The first sampling site was Charbagh locomotive work shop and its adjoining areas of Lucknow. The site was selected because, metal forging and smelting process occurs at high level and a large quantity of wastewater and sludge discharged from the site. The second site was Sarojini nagar industrial area of

Lucknow, at this site electroplating, metal smelting and metal forging industries running which discharged a large quantity of wastewater and sludge around the area. The third selected site was Telco industrial area Lucknow. At this site, a large unit of TATA motors is running which discharge large quantity of sludge and wastewater. Fourth selected site was Eveready battery industry Aishbagh Lucknow, at this site lead acid battery manufacturing unit is running which discharge a high quantity of wastewater and sludge. The fifth sampling site was Panki power plant Kanpur, a coal based power plant generating high amount of fly ash. The sixth sampling site was Vijay nagar industrial area Kanpur, the site was selected because a huge numbers of metal smelting, metal forging, electroplating, paint and chemical fertilizer production units are running that generates high quantity of wastewater and sludge. The seventh sampling site was Jainpur industrial area Kanpur dehat, here a numbers of paint and electroplating production units running which generates high quantity of sludge and wastewater.

Further, samples were collected by digging soil up-to 6 cm depth after removing the upper organic layer of soil using a sterile grub hook from each site in a sterile sample collection bag and the samples were immediately transported to the laboratory. Further, the descriptions of sampling sites were given in table 3.1 and Figure 3.1.

3.2. Physico-chemical characteristics of soil samples

For physico-chemical analysis, pulverized soil samples were mixed and homogenised. After that, they were ground, air dried and passed through a 2.0 mm sieve.

Table 3.1 A brief description of sampling sites

S. No.	Sampling Sites	No. of Samples collected	Samples code
	Lucknow		
1	Charbagh locomotive work shop and Its adjoining Areas (Coordinate 26°49'51"N 80°55'31"E)	3	CH
2	Sarojini Nagar Industrial Area (Coordinate 26°44'40^{0} N, 80°52'12"E)	3	SA
3	Telco Industrial Area (Coordinate 26°55'8"N 81°3'16"E)	3	TA
4	Eveready battery industry Aishbagh (Coordinate 26°50'27"N 80°53'0"E)	1	EF
	Kanpur Nagar		
5	Panki Power Plant (Coordinate 26°28'31"N 80°14'35"E)	2	PC
6	Vijay Nagar Industrial Area (Coordinate 26°28'12"N 80°15'49"E)	2	VA
	Kanpur Dehat		
7	Jainpur Industrial Area (Coordinate 26°21'33"N 79°59'21"E)	2	JA

3.2.1. Soil pH

For measurement of soil pH, soil sample was brought to saturation stage with Milli Q (MQ) water in the ratio of 1:2.5 (10 g soil: 25 mL water). Contents were stirred for 30 minutes and then allowed to settle down and pH measured with the help of glass electrode pH meter as described by Jackson (1973). pH meter (Eutech Instruments, USA) was calibrated with standard buffers (pH 4, 7 and 10) and the value was noted.

3.2.2. Soil Electrical Conductivity (EC)

10g dried soil sample was taken in a beaker and added with 25 mL of MQ water. The above solution was stirred for 30 minutes in order to allow bulk of the soil to settle. Conductivity meter was calibrated with standard potassium chloride (0.01N) solution and conductivity was measured.

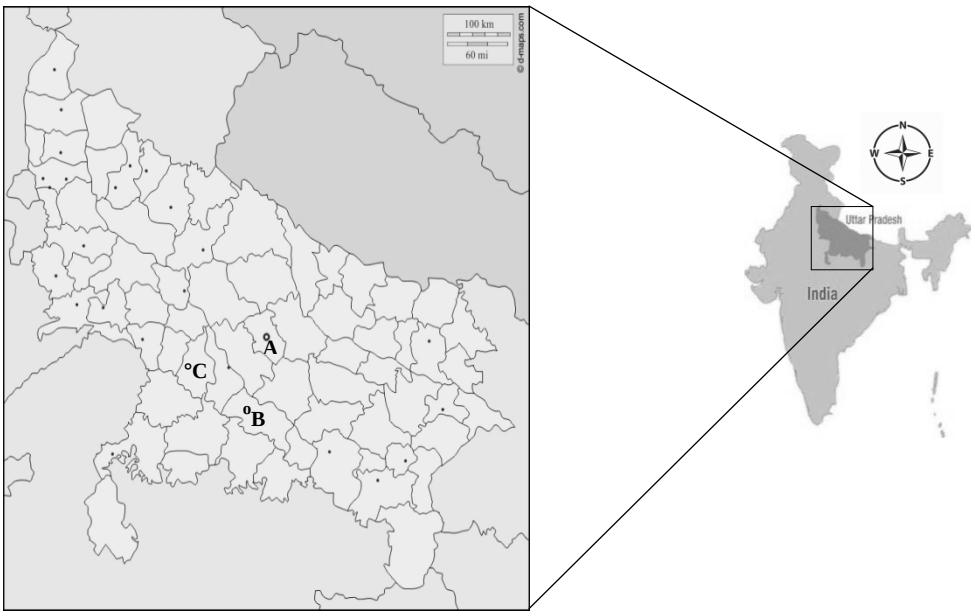

Figure 3.1 Location of sampling sites; A – Lucknow, B – Kanpur nagar, C – Kanpur dehat.

3.2.3. Soil Organic matter

Reagents required:

a) 1N potassium dichromate ($K_2Cr_2O_7$) - 49.04g $K_2Cr_2O_7$ was added in distilled water and volume was made to 1 litre.

b) 1N ferrous ammonium sulphate - 19.6 g FAS was added in distilled water and volume was made to 100 mL.

c) Diphenyl amine indicator - 0.5 g of Diphenyl amine indicator (DPA) was dissolved in a mixture of 20 mL water and 100 mL concentrated H_2SO_4.

d) H_2SO_4 - 96% with distilled water

0.5g of sieved soil sample was taken in 500 mL conical flask and 10 mL of 1N $K_2Cr_2O_7$ was added. The flask was swirled for mixing the soil and reagent. 20 mL of H_2SO_4 was added and the flask was allowed to stand undisturbed for 30 minutes. Afterwards 200 mL of distilled water was added before adding 1 mL of diphenylamine indicator. The content was titrated with freshly prepared 1N ferrous

ammonium sulphate. A blank without soil sample was also run following the similar steps. Organic matter (OM) was calculated after the estimation of organic carbon; the formula is as follows (Walkley and Black, 1934).

$$\text{Organic carbon } \% = \frac{10 \ \text{B} - \text{T} \ \times 0.003 \times 100}{\text{B} \times \text{weight of soil (g)}}$$

Where, B is volume of ferrous ammonium sulphate solution for blank titration; T is volume of ferrous ammonium sulphate solution for soil sample.

Organic matter (OM %) = Organic carbon (%) × 17.24 (van bemmelen factor).

3.2.4. Soil Kjeldahl Nitrogen:

Reagent required:

Digestion reagent –

Solution (A) was prepared by dissolving 134g K_2SO_4 in mixture of 650 mL MQ water added with 200 mL concentrated H_2SO_4.

Solution (B) was prepared by dissolving 2 g HgO in 25 ml $6(NH_4)_2SO_4$.

Solution (C) was prepared by adding 25 mL of solution (B) into 200 mL of solution (A) by continuous stirring and final makeup of the solution was 1 litter. This solution was stored at a temperature approximate 14 °C to prevent crystallization.

Sodium hydroxide - Sodium thiosulphate reagent - Prepared by dissolving 500g NaOH and 25g sodium thiosulphate and diluted to 1 litter with MQ water.

Mixed indicator - Prepared by dissolving 200mg methyl red in 100 mL 95% ethyl alcohol. This solution was mixed with the solution of 100 mg methylene blue in 50 mL 95% ethyl alcohol.

Boric acid - Prepared by dissolving 20g H_3BO_3 in MQ water and 10 mL mixed indicator. Contents were diluted with 1 litter MQ water in volumetric flask.

Sodium hydroxide (6N) solution - Prepared by dissolving 240 g NaOH in 1 litter MQ water.

Phenolphthalein indicator - Prepared by dissolving 0.5 g powder phenolphthalein in 500 mL 95% ethyl alcohol + 50 mL MQ water. Drop wise 0.02 N NaOH was added till faint pink colour developed.

Sulphuric acid (0.02 N) - Prepared by diluting 0.72 mL concentrated sulphuric acid to 1 litter of MQ water.

Total nitrogen was estimation by following three steps i.e. 1) digestion 2) neutralization and 3) titration. For this, 50 mL of the sample was taken in Kjeldahl flask with 50 mL digestion reagent. Digestion was continued till the sample appeared light green to ensure complete decomposition/destruction of organic matter. Contents were cooled and diluted with distilled water to 150 or 300 mL depending on the capacity of the flask. The Kjeldahl flask was placed properly in distillation apparatus and heated. Phenolphthalein (0.5 mL) was added followed by sodium hydroxide - sodium thiosulphate reagent till pH raised just above 8.2. Distillation was continued to collect 100 or 200 mL distillate in 50 mL boric acid. The tip of the condenser was extended well below the level of boric acid solution. After completion of distillation, the concentration of ammonia was measured by titrating the distillate with 0.02 N H_2SO_4 till the indicator turned pale lavender colour. Blank was prepared in the same way using MQ water. Calculation of total nitrogen was done using the following formula:

$$\text{TKN} = \frac{\text{a} - \text{b mL} \times \text{Normality of sulpuric acid (0.02N)} \times 14 \times 1000}{\text{Volume of sample (mL)}}$$

Where, a = mL 0.2 N H_2SO_4 required for sample; b = mL 0.2 N H_2SO_4 required for blank.

3.2.5. Soil available phosphorus

The available phosphorus was determined by following the method Olsen (Olsen et al., 1954).

Reagent required:

Extracting solution - 42 g of $NaHCO_3$ was added in MQ water and volume was made up to 1 litre. The pH was adjusted to 8.5 with NaOH (1M).

Reagent A - 5.6g ammonium molybdate was mixed with 250 mL distilled water, and 0.13 g antimony potassium tartarate in 100 mL distilled water was added to 70 mL of conc. H_2SO_4, mixed thoroughly with continuous stirring and volume was made to 1 litre with MQ water.

Reagent B - 0.529g of ascorbic acid was added in 100 mL of reagent A and mixed properly.

Darco-G60 (activated Charcoal)

1g soil was taken with 20 mL of extracting solution added with 2 pinch of Darco-G 60. Kept in a shaker for 30 minutes and was filtered through Whatman Filter paper no.42. 10 mL aliquot of filtrate was transferred to a 100 mL beaker. 10 mL reagent B (freshly prepared) was added and final volume made with 50 mL MQ water. After 10 minutes of incubation at room temperature the intensity of the colour was measured at 660 nm against blank. Standard curve was prepared by standard solution of KH_2PO_4.

3.2.6. Soil K^+, Ca^{++} and Mg^{++} estimation

Reagent required:

Extracting solution- 58 mL of glacial acetic acid was added into 600 mL distilled water. Then, 70 mL of conc. ammonia (sp. gravity 0.90) was added into the solution. Solution was diluted to one litre, and the pH of solution was adjusted to 7.0 with the help of ammonia or acetic acid.

5g of soil samples was taken with 25 mL of extracting solution. This mixture was shaken for 5 minute on incubator shaker and filtered through the whatman No.1 filter paper. The sample was measured by flame photometer after calibration. The amount of potassium, calcium and magnesium were calculated using standard curve.

3.2.7. Soil Elemental analysis

Reagent required:

a. **Nitric acid (HNO_3 concentrated)**

b. **Perchloric acid ($HClO_4$ concentrated)**

1g of dried soil samples were taken into a conical flask and digested with HNO_3 and $HClO_4$ in a ratio of 3:1. The flask was heated gently on the digestion unit till clear solution appeared. The digested samples were diluted with double distilled water and volume made to 100 mL. The suspensions were filtered through Whatman filter paper grade 1 (pore size 11 µm). The digested samples were analysed for heavy metals (Zn, Cu, Cd, Ni, Pb and Cr) through Inductive Coupled Plasma Optical Emission Spectroscopy (ICP-OES) Perkin-Elmer (Analyst Model Optima 5300 V). The metal content was expressed in ppm dry weight (dw) of soil sample.

3.3. Isolation of Rhizobacteria

Isolation of rhizobacteria was done by using serial dilution and plating techniques on Luria-Bertani (LB) agar medium in triplicate. Roots of the plant from contaminated sites were soaked in sterile MQ water for 20 min and washed to remove the adherent soil. The samples were kept in shaking incubator for 1 h at 30 °C (150 rpm) in phosphate buffer (PB). Subsequently, serial dilutions were prepared from this initial suspension and 1 mL was used to prepare different dilutions of 10^{-3}, 10^{-4} and 10^{-5} by serial dilution technique. Finally, 0.1 mL of suspension from each dilutions was

spread on the solid surface of Luria-Bertani (LB) agar medium containing 100 mg L^{-1} of Cd and Pb, added as $Cd(NO_3)_2$ and $Pb(CH_3COO)_2$ individually or in combination on separate Petri dishes and kept for incubation at 30±2 °C. The growth was observed periodically. After incubation, bacterial colonies were selected and streaked on to nutrient agar plates. The isolated strains were purified and maintained on slant (NA slants) at 4 °C, as well as 20% (v/v) glycerol stock at -20 °C.

3.4. Screening of rhizobacterial isolates for maximum heavy metal tolerance

The intrinsic tolerance capacity of rhizobacterial strains against heavy metal stress was evaluated by agar plate dilution method (Holt et al., 1994). Stock solution of Cadmium nitrate [$Cd(NO_3)_2.7H_2O$] and Lead acetate [$Pb(C_2H_3O_2)_2$] salts concentration 10^6 mg L^{-1} was used for preparing different concentration (Cd 0-1200 and Pb 0-1500 ppm) of agar media plates. All these concentrations were maintained in LB agar media plates in gradually increasing order (in multiple of 100). The selected rhizobacterial isolates were streaked on these heavy metals (Cd and Pb) amended plates and incubated at 30±2 °C for 4 - 5 days (Sarathambal et al., 2017). The maximum concentration at which the rhizobacterial isolates were growing easily recorded as maximum tolerance concentration (MTC) and beyond this concentration, there was no growth.

3.5. Evaluation of Plant growth promoting traits of selected isolates

Various plant growth promoting traits *viz.* ACC deaminase, phosphate solubilization, IAA, and siderophore production of selected strains were checked qualitatively and quantitatively. All respective media were prepared either alone (control) or supplemented with 500 ppm Cd and 500 ppm Pb.

3.5.1. 1-Aminocyclopropane-1-carboxylic acid (ACC) deaminase activity

ACC deaminase activity of potential rhizobacterial isolates were analysed by the utilization of 1-aminocyclopropane-1-carboxylic acid (ACC) as nitrogen source supplements during cell growth using DF salts minimal medium (Dworkin and Foster, 1958). Further, for quantification, an assay was performed by following the method of Honma and Shimomura (1978), later modified by Penrose and Glick (2003), which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The number of mM of α-ketobutyrate produced by this reaction was determined by comparing the absorbance at 540 nm of a sample to the standard curve of α-ketobutyrate ranging between 0.1 and 1.0 mM.

3.5.2. Siderophore production activity

For detection of siderophore, universal CAS assay was followed (Schwyn and Neilands, 1987) but the method for media preparation was adopted by Pérez-Miranda et al. (2007). In brief, a deferrated medium (1%, v/v), was prepared and overlaid onto the fresh culture plate (supplemented with 500 ppm Cd and 500 ppm Pb) of the bacterial isolates. After a maximum period of 15 min, development of blue to orange colour surrounding cultured colony confirms the siderophore production by the bacterial isolates (as described in the traditional CAS assay for siderophore). Further, quantitative estimation of siderophore production was conducted according to the method performed by Shin et al. (2001). Each test was performed thrice with three replicates.

3.5.3. Indole-3 acetic acid (IAA) production activity

Indole-3 acetic acid (IAA) production experiment was done by following the method described by Bric et al. (1991) with slight modification. The TSB medium containing

different concentration of tryptophan (100, 300, and 500 µg mL^{-1}) supplemented with 0 and 500 ppm Cd and Pb in triplicates and were allowed to grow in shaking incubator for five days at 30±2 °C. Bacterial cultures were then separated by centrifugation at 12,000 rpm for 15 min. Finally, 2 mL of Salkowsky's reagent was added to the supernatant and further incubated for 25 min in dark at 30 °C (Patten and Glick, 2002). IAA production was observed as development of a pink-red colour. The absorbance of the pink coloured complex was read at 530 nm in a UV–Vis Spectrophotometer (Model WAT 207000, Singapore). The calibration plot was constructed using dilutions of a standard IAA (Hi-Media, India) solution and the uninoculated medium with the reagent as a control. The quantity of IAA produced in the culture broth was expressed as µg mg^{-1} protein.

3.5.4. Phosphate solubilization activity

Phosphate solubilization activity was evaluated on NBRIP media containing 0.5%, TCP (Tricalcium phosphate) as a source of inorganic phosphate and Bromophenol Blue (BPB) dye as an indicator (Nautiyal, 1999). The selected rhizobacterial isolates were streaked (point streaking) on solid surface of NBRIP media and incubated at 30±2 °C for 7 seven days. The development of red colour from blue indicates the positive results for isolates. Further, quantitative estimation in culture supernatant was checked by the Molybdate Blue Method (Fiske and Subbarow, 1925) on 7th day of incubation, recording absorbance at 660 nm using spectrophotometer. All tests were performed in triplicates for reducing the experimental error.

3.5.5 HCN and Ammonia production activity

The selected rhizobacterial isolates were screened for HCN production by following the method of Lorck (1948). Media was prepared by amending the 4% glycine L^{-1}.

Sterilized Whatman filter paper soaked in 2% sodium carbonate and 0.5% picric acid solution was placed in the lid of plate after inoculation of rhizobacterial isolates. The plates were wrapped properly with parafilm and incubated at 30±2 °C for 72 h. Development of orange/dark brown colour from yellow confirmed the positive results of isolates.

In order to test the ammonia production, selected bacterial isolates were grown in peptone water broth at 30±2 °C for 96 h. After incubation period, few drops of Nessler's reagents were added in culture supernatant. Development of brown colour (+++), Faint yellow colour (++) and light yellow (+) in test tubes confirmed the production of ammonia (Cappucino and Sherman, 1992).

3.5.6. Extracellular polysaccharide (EPS) production activity

Bacterial isolates were screened for EPS production by following the method of Subudhi et al. (2016). The isolates were inoculated in liquid LB media prepared by amending the 1% glycerol and incubated at 110 rpm shaking incubator at 30±2 °C for 4 days. Further, the supernatant was mixed with three volume of ethanol slowly adding along the sidewall of the flask and allowed to stand at 4 °C overnight to precipitate EPS. The precipitated EPS was filtered and measured after drying at 80 °C. Further, extracted EPS was characterized with the help of FTIR spectroscopy technique.

3.6. Morphological characterization of selected isolates

Rhizobacterial strains were characterized morphologically with the help of light microscopy followed by Gram's staining and Scanning electron microscope (SEM). The morphological characteristics of rhizobacterial colonies were analysed after growing them on their respective media.

3.6.1. Gram's reaction of rhizobacterial isolates

Sir Christian Hans Gram (1884) developed an empirical method to differentiate the bacteria into two groups named as gram positive and gram negative. In this method a heat fix smear of bacterial cell firstly treated with Crystal violet and after 1 minute Gram's iodine solution poured over the smear. After that a decolourizer is added over the smear followed by distilled water rinsing. Finally, a counter stain safranin added followed by distilled water rinsing. Dry slides covered with coverslips were observed under microscope. The principle behind this test is, if bacteria retains primary stain (crystal violet) regarded as Gram positive whereas, if the bacterial cell loses the primary stain colour and is stained by counter stain safranin it is referred as Gram negative.

3.6.2. Scanning electron microscopic characterization of rhizobacterial cells

To observe the morphology of rhizobacteria under scanning electron microscope, overnight lag phase culture was fixed with 5% glutaraldehyde solution first in eppendorf tube and kept into refrigerator at 4 °C. The fixed cells were centrifuged at 4500 rpm for 10 min at 4 °C for removal of glutaraldehyde. Buffered washed cells were further processed for dehydration with the help of ethanol or acetone of different concentrations. Further, dehydrated cells were mounted on stubs coated with carbon tape followed by Palladium/Gold coating. Finally samples were observed under scanning electron microscope.

3.6.3. Biochemical characterization of selected isolates

Various biochemical tests were performed on respective media to confirm the identity of isolates as per the standard method describes by Aneja (2003) and Cappuccino and Sherman (1992).

3.6.3.1. Indole production test

The rhizobacterial isolates were inoculated in tryptone water and incubated for 48 h at 30±2 °C. Indole production was detected by adding the Kovac's reagent (dimethyl aminobenzaldehyde) into supernatant. The development of cherry red reagent layer indicates the positive results.

3.6.3.2. Methyl Red-Vogues and Proskauer test (MR-VP)

The rhizobacterial isolates were inoculated into sterilised MR-VP broth and incubated at 30±2 °C for 24 h. Further the culture broth of each test tube was transferred equally to other sterilized set of test tubes. Further, MR reagent (Methyl Red indicator) was added in one set and VP reagent (Barritt's reagent A & B) to another set of test tubes. Development of yellow and red colour indicates the positive result for MR and VP test respectively (Cappucino and Sherman 2002).

3.6.3.3. Motility test

Bacterial isolates were checked for motility test, for this sterilized semisolid SIM agar was poured in a test tube. A stab streaking with the help of needle loop making a single stab down to the center of the tube about half of the depth of medium was done. After inoculation, each set of test tube was incubated at 30±2 °C for 24 hrs. A hazy and diffused growth that spread throughout the medium renders the positive results of the test.

3.6.3.4. Hydrogen sulphide production activity

Bacterial isolates having the ability to reduce sulphur compounds into hydrogen sulphide in their metabolic process duly acknowledge for H_2S producing bacteria and a test measure for their identification is known as hydrogen sulphide test. Rhizobacterial isolates were tested for H_2S production by inoculating them into SIM

(sulphide-Indole-Motility) agar through stab inoculation technique. The inoculated tubes incubated at 37±2 °C for 24-48 h. Formation of black precipitate in the tubes is an indication of positive results of the test.

3.6.3.5. Citrate utilization test

Bacteria that utilize citric acid as sole source of carbon are duly rendered as citrate utilizing bacteria. The rhizobacterial isolates were tested for citrate utilization test by inoculating them onto Simmon's citrate agar medium and incubated at 30±2 °C for 48 h. Change in colour of medium from blue to green is indicative of positive results for the test.

3.6.3.6. Oxidase test

Bacteria having the cytochrome oxidase systems catalyses the transport of electron between donor bacteria and redox dye (tetramethyl-p-phenylene-diamine) are duly rendered as oxidase positive bacteria. Selected isolates were tested for oxidase test by inoculating into nutrient broth and incubated at 30±2 °C for 48 h. After incubation 2-4 drop 1% α-naphthol and then after add 4-6 drop1% p-aminodimethylaniline oxalate. Shake vigorously for proper oxygenation of the culture. Development of a deep purple/blue colour indicates oxidase production.

3.6.3.7. Urease production Test

Urea is a diamide of carbonic acid, several bacterial genera produce urease that hydrolyse urea into ammonia and carbon dioxide. The selected isolates were tested for urease production by inoculating them onto urea supplemented agar slant, incubated at 30±2 °C in ambient air for 18 to 24 h. Bacteria that produce urease hydrolyse urea rapidly and change the colour of slant from light orange to magenta colour.

3.6.3.8. Lipase production test

Bacterial isolates were tested for lipase production by inoculating them on Tributyrin agar (TBA) media and incubate at 30±2 °C for 24-48 h. A cleared zone appears around the culture shows positive results.

3.6.3.9. Catalase production test

Catalase production test was performed to detect for the presence or absence of catalase enzyme. Most of the aerobes and facultative anaerobes have characteristic catalase activity. Aerobic microbes usually utilize oxygen and produce hydrogen peroxide, which are toxic to nucleic acid and many cellular proteins. Hence, this test is used to detect for the presence of catalase enzyme in bacteria. A few drops of 0.3% H_2O_2 solution was poured on bacterial colony. Presence of bubbles is an indication of the catalase production.

3.6.4. Molecular characterization of the selected isolates by 16S rRNA gene sequencing

The selected rhizobacterial isolates were characterized through 16S rRNA gene sequencing analysis. The facility was jointly extended from Aakaar Biotechnologies Private Limited Lucknow, India and Genei Laboratories Private Limited Bengaluru India. The genotypic identification was carried out by amplification of partial nucleotide sequences of 16S rRNA. The 16S rRNA gene was amplified through PCR (Applied Biosystem Model #9902) using the conserved eubacterial primers pA (5′AGAGTTTGATCCTGGCTCAG; *Escherichia coli* bases 8-27) and pC5B (5′ TACCTTGTTACGACTT; *E. coli* bases 1507-1492). The PCR products were sequenced and homologies of both the strains were identified using BLASTn of National Centre for Biotechnology Information database. The aligned sequences were submitted to gene bank NCBI and accession number obtained. Moreover, the

evolutionary analysis was conducted using MEGA7 by neighbour-joining (NJ) method.

3.7. Antibiotic sensitivity test

The bacterial strains were tested for antibiotic sensitivity by following the Kirby – Bauer disc diffusion method (Buaer, 1966). The bacterial cultures of $1X10^8$ colony forming units (CFU) mL^{-1} were spread on Mueller Hinton Agar (MHA) plates and then antibiotic discs were placed and incubated for 18 h at 30±2 °C. The antibiotics used in the current study were - Amikacin (30 mcg), Piperacillin (10 mcg), Gentamycin (10 mcg), Chloramphenicol (30 mcg), Ceftriaxone (30 mcg), Ceftazidime (30 mcg), Cefoxitin (30 mcg), Ampicillin (10 mcg). After incubation, inhibition zone by antibiotics was recorded.

3.8. Bacterial compatibility test

The bacterial strains were tested for compatibility test through agar diffusion method before developing the consortium. The well plate technique was used on nutrient agar media. Wells were prepared with the help of sterilized microtips in solid nutrient agar media plates and bottom of the wells sealed with 1% soft agar and then filled with different bacterial isolates to be tested for compatibility against one isolates while another was streaked with the help of swab. The plates were incubated for 48 h at 30±2 °C. No inhibition between the test isolates were considered as compatible and selected for further study.

3.9. Seed germination test

The seed germination assay with *Canna indica* and *Zea mays* L. plants with selected rhizobacterial strains as well as developed consortium were performed according to Belimov et al. (2005) with slight modifications. The bacterial cultures were grown in LB medium with amended heavy metals (Cd and Pb) separately overnight at 30 °C

and 110 rpm. Further, the cultures were centrifuged at 7000 rpm and washed with phosphate buffer twice. All cultures were then re-suspended in deionized-sterilized water so as to maintain a population of 10^8 cells mL^{-1}. Seeds of *Canna indica* and *Zea mays* L. were surface sterilised with 0.5% (v/v) sodium hypochlorite for 10 min and rinsed several times with deionized-sterile water and then mix 5mL of bacterial suspension in the NB tubes. 5 mL of 500ppm heavy metal solution (Cd & Pb separately) was added to sterile petri dishes. Suitable controls (without any heavy metals) were also taken (Sterile water). Sterile Whatman filter paper no. 4 was soaked in each of petri plates taking care that solution was sufficient enough only to moisten the filter paper. After that seeds with bacterial suspension was transferred over the moist filter paper in the petri plates and distributed evenly throughout the plate with the help of sterile forceps. Daily monitoring of seed germination was recorded and the emergence of the radicle was taken as a criterion of germination. All the experiments were carried out in triplicate and results were averaged.

Seed germination percentage and seed vigour index (SVI) treated with rhizobacterial consortium and individual strains under different metal concentrations were calculated by following formula:

$$\text{Germination Percentage} = \frac{\text{Seeds germinated at each day}}{\text{total seeds sown}} \times 100$$

Seed germination % was recorded at 3, 5 and 7 days after sowing (DAS).

Seed vigour index (SVI) was calculated by multiplying germination (%) and seedling length. Higher seed vigour index (SVI) is indication of a more vigorous seed. SVI was calculated by following formula:

$$\text{SVI} = \text{Mean Root Length} + \text{Mean shoot length} \times \%\text{seed germination}$$

3.10. Pot Experiment

To study the effect of rhizobacterial consortium as well as each individual isolates on plant (*Canna indica* L. and *Zea mays* L.) growth and accumulation of heavy metal under different concentration of Cd and Pb stress soil, a mesocosm study was accomplished in April-July (for *Zea mays* L.) and July-November (for *Canna indica*). Experiments were conducted in Net house at Environmental Sciences research station of Babasaheb Bhimrao Ambedkar University, Lucknow, India.

3.10.1. Preparation of heavy metal (Cd and Pb) treatment in soil

Top soil (0-20 cm) free from contamination was collected from Horticulture research farm of Babasaheb Bhimrao Ambedkar University Lucknow, India. The soil was air dried homogenized and sieved through 2 mm sized sieve. Before amendment of heavy metal the soil was checked for physico-chemical properties by standard method as described in section 3.1.1. The dried soil was artificially contaminated by stock solutions of Cd and Pb heavy metals. The six concentrations i.e. 0, 100, 200, 300, 400, and 500 ppm of Cd and Pb were maintained separately in the soil.

3.10.2. Inoculum Preparation

Selected heavy metal resistant rhizobacterial isolates were grown in LB broth amended with Cd and Pb heavy metals at 30±2 °C for 24 h with continuous shaking at 110 rpm. The fresh grown young bacterial cultures were centrifuged at 10,000 rpm for 15 min and pellets were washed with distilled water. The obtained pellets were diluted with distilled water to yield a final concentration of 10^8 CFU/mL. Further, these heavy metal resistant strains PC1, PC3, SA were designated as T1, T2, T3 and their consortium (T4) were used to treat targeted plant seed under natural environmental conditions in a pot assay.

3.10.3. *Canna indica* and *Zea mays* L. seeds and their sterilization

The breeder seeds of *Zea mays* L. (Var. UMC-10) were obtained from Narendra Dev University of Agriculture and Technology Kumarganj, Faizabad, Uttar Pradesh, India, while *Canna indica* seeds were gifted by Dr. Siddhartha Gautam (Supra Organix private Ltd) Dehradun, India. Both plant seeds were surface sterilized by 0.5% (v/v) sodium hypochlorite solution for 10 min and rinsed several times with deionized-sterile water. In a pot assay, all the four bacterial treatments T1 T2 T3 & T4 were tested for their remediation efficacy.

3.10.4. Experimental design

Experimental design of treatment is given in Table 3.2. Complete randomized block design (CRBD) was used. Sterilized pots (wiping by 95% ethanol solution) about 30 cm (diameter) x 28 cm (height) filled with 10 Kg soil, were used in this study. In each pot, 12 surface sterilized seeds of *Canna indica* and *Zea mays* L., were sown and pots were monitored for emergence of seedlings. After germination, excess plants were removed to maintain a population of 10 plants per pot and the pots were irrigated with sterile distilled water throughout the experiments.

Table 3.2 Tabular presentation of pot experimental design

	***Zea mays* L.**				
Treatments	**Control**	**Bacterial isolate T1**	**Bacterial isolate T2**	**Bacterial isolate T3**	**Bacterial Consortium (T1+T2+T3)=T4**
HM(Cd & Pb) Concentration 0 ppm	0 ppm+No culture	0 ppm+T1	0 ppm+T2	0 ppm+T3	0 ppm+T4
100 ppm	100 ppm+No culture	100ppm+T1	100 ppm+T2	100 ppm+T3	100 ppm+T4
200 ppm	200 ppm+No culture	200ppm+T1	200 ppm+T2	200 ppm+T3	200 ppm+T4
300 ppm	300 ppm+No culture	300ppm+T1	300 ppm+T2	300 ppm+T3	300 ppm+T4
400 ppm	400 ppm+No culture	400ppm+T1	400 ppm+T2	400 ppm+T3	400 ppm+T4
500 ppm	500 ppm+No culture	500ppm+T1	500 ppm+T2	500 ppm+T3	500 ppm+T4
	Canna indica				
Treatments	**Control**	**Bacterial isolate T1**	**Bacterial isolate T2**	**Bacterial isolate T3**	**Bacterial Consortium (T1+T2+T3)=T4**
HM(Cd & Pb) Concentration 0 ppm	0 ppm+No culture	0 ppm+T1	0 ppm+T2	0 ppm+T3	0 ppm+T4
100 ppm	100 ppm+No culture	100ppm+T1	100 ppm+T2	100 ppm+T3	100 ppm+T4
200 ppm	200 ppm+No culture	200ppm+T1	200 ppm+T2	200 ppm+T3	200 ppm+T4
300 ppm	300 ppm+No culture	300ppm+T1	300 ppm+T2	300 ppm+T3	300 ppm+T4
400 ppm	400 ppm+No culture	400ppm+T1	400 ppm+T2	400 ppm+T3	400 ppm+T4
500 ppm	500 ppm+No culture	500ppm+T1	500 ppm+T2	500 ppm+T3	500 ppm+T4

HM – Heavy metal, C – Control, T1 – Strain PC1, T2 - Strain PC3, T3 - Strain SA and T4 – PC1+PC3+SA

3.11. Sampling procedure for plants and soil

Plant sampling was done at 30, 60 and 90 DAS to study the physiological, biochemical and metal accumulation by plants grown under different concentration of cadmium and lead. For physiological parameters and metal accumulation analysis,

three plants from each set of pots corresponding to the sampling time were uprooted. Soils samples (0-10 cm depth of pot) from each pot of each treatment and their corresponding control were collected at each sampling and analysed for heavy metal contents.

3.12. Analysis of plants for physiological growth and development

3.12.1. Shoot and root length analysis

The plants from each treatment were uprooted at the sampling time. The shoot length of both the test plants were measured by meter scale from the base of primary leaf to base of hypocotyl and mean shoot length was expressed in centimetres (cm). The uprooted plant roots were thoroughly washed with distilled water and was measured using meter scale from the tip of primary root to base of hypocotyl and mean root length was expressed in centimetre (cm).

3.12.2. Total dry biomass of plant

Different parts of each plant were separated into leaf, stem and root for measurement. Plant parts were wrapped separately in a weighed labelled aluminium foils. For dry weight determination, the samples after fresh weight measurement is kept in an oven maintained at 80 °C till a constant weight achieved. The samples were transferred to desiccators, allowed to cool, weighed and expressed in grams (g).

3.13. Biochemical characteristics of plants

3.13.1. Photosynthetic pigments and carotenoid content

Reagent required - 80% acetone

To determine the photosynthetic pigments (chlorophyll ‘a’, ‘b’, total chlorophyll) and carotenoid content of plants, 0.5 g of leaf materials were homogenized with 10 mL of chilled 80% acetone. The extract was centrifuged at 5000x g for 10 min. Using small

quantities of acetone, extract was centrifuged repeatedly till the supernatant became colourless. The clear supernatant was taken and volume made 10 mL with 80 % acetone. The extract was kept in dark till the optical density was measured. The optical density of the extract was read at 480, 510, 645 and 663 nm using spectrophotometer against the 80% acetone. The chlorophylls and carotenoid contents were calculated using the formula described by Goodwin (1976);

$$\text{Chlorophyll a (mg g}^{-1}\text{ fw)} = 12.7\ (OD\ 663) - 2.69\ (OD\ 645)$$

$$\text{Chlorophyll b (mg g}^{-1}\text{ fw)} = 22.9\ (OD\ 645) - 4.68\ (OD\ 663)$$

$$\text{Total Chlorophyll (a+b) (mg g}^{-1}\text{ fw)} = 20.2\ (OD\ 645) + 8.02\ (OD\ 663)$$

$$\text{Total Carotenoids (mg g}^{-1}\text{ fw)} = 7.6\ (OD\ 480) - 1.49\ (OD\ 510)$$

Where, OD =optical density and fw = fresh weight of leaf.

3.13.2. Proline estimation

Proline was estimated using the method described by Bates et al. (1973).

Reagent required:

A) **Acid Ninhydrin reagent**- 1.25 g ninhydrin was added in 30 mL glacial acetic acid and 20 mL ortho phosphoric acid, with agitation till dissolved and stored at 4° C, The reagent was used within 24 h of preparation.

B) **3% Aqueous Sulpho-salicyclic Acid**

C) **Glacial Acetic Acid**

D) **Toluene**

E) **Purified proline:** from Sigma-Aldrich

Proline purification

0.5 g of leaf material was homogenized in 10 mL 3% sulphosalicylic acid and centrifuged. After centrifugation, 2 mL of the aliquot was added to 2 mL of acid ninhydrin reagent and 2 mL of glacial acetic acid. The tubes were caped and the

reaction mixture was allowed to boil in water bath at 100 °C for 1 h. Reaction was terminated by placing the tube in ice bath. Afterward, 4 mL toluene was added to the reaction mixture and stirred for 20-30 sec. Toluene layer of the mixture was separated and allowed to come to room temperature. Red colour intensity of the separated layer was measured at 520 nm. A series of pure proline standard was also prepared in a similar way. Concentration of proline in the test sample was calculated from the standard curve and expressed as mg proline g^{-1} fresh weight of leaf.

3.14. Elemental analysis of cadmium and lead in plant parts

1 gm of dried and fine powdered test material of plant parts (root and shoot) were digested in a tri-acid mixture of HNO_3:$HClO_4$:H_2SO_4 in the ratio 10:4:1 (15 ml mixture) for the estimation of heavy metal contents (Cd and Pb). The digestion was performed in 50 mL conical flasks and to facilitate complete digestion, the samples in tri-acid mixture were kept overnight at room temperature. These flasks containing samples and tri-acid mixture were heated on a hot plate at 180 °C until the clear solution was obtained. This solution was then allowed to reduce in volume on digestion unit. This was followed by a slow but complete evaporation of acids. Then, the volume of the digested samples was made to 100 mL with distilled water. Samples were filtered and analysed by Inductive coupled plasma optical emission spectroscopy (ICP-OES) Perkin-Elmer (Analyst Model Optima 5300 V) at specific wavelength of specific metal (Table 3.3). The concentrations of elements in plant parts were calculated using formula:

$$\text{Element} \quad \frac{\text{mg}}{\text{g}} = \frac{(\text{C} - \text{B})}{\text{W}} \times \text{V}$$

Where, C=metal concentration in sample; B=metal concentration in blank; W=weight of sample and V=volume of sample taken.

Table 3.3 Standard wavelength of ICP-OES for elemental analysis

Elements	Wavelength (nm)
Cadmium	228.80
Lead	220.2
Chromium	267.71
Cobalt	230.786
Copper	324.75
Iron	238.20
Magnesium	279.071
Manganese	257.61
Arsenic	193.696
Nickel	213.60
Calcium	315.880
Phosphorus	214.912
Potassium	766.455
Zinc	206.2

3.15. Data processing and statistical analysis.

All the experimental data was analysed in three replication using SPSS program (IBM version 17.0). Analysis of variance (ANOVA) was performed on morphological and biochemical activity. Growth parameter and heavy metals treatment with rhizobacterial inoculation were considered as independent variables, whereas one-way analysis of variants has been implemented on the heavy metal concentration in plant tissues. Significant differences in means were separated by the Duncan multiple range tests (DMRT) at $p<0.05$. The principle component analysis (PCA) was performed based on the correlation data of total dry weight (TDB), total metal accumulation (TMC), root metal content (RMC), shoot metal content (SMC), bioconcentration factor (BCF) and translocation factor (TF). PCA was used to determine the homogenous characteristics of both the experimental plants and the correlation amongst each variable studied under heavy metals treatment with and without

rhizobacterial inoculation at different growth stage (DAS). Standardized PC1 and PC2 scores were plotted using Past3 software (2015).

The metal bioconcentration factor (BCF) was obtained following the formula: BCF= concentration of metals in root or plant tissues (C_{root}) / concentration of metals in soil (C_{soil}). Translocation factor (TF) is obtained through the formula: TF= concentration of metal in seed or root/ concentration of metal in root or soil. All the results obtained were then summarized and presented in next chapter.

Chapter 4

Results

CHAPTER - 4

RESULTS

The present investigation was carried out to isolate bacteria from heavy metal contaminated soil, screening for cadmium and lead-tolerance, PGPR properties and their efficacy for remediation of heavy metals using the hyperaccumulator plants *Zea mays* L. (Var. UMC - 10) *and Canna indica.* This association of plant and metal tolerant microbe is aimed at improving the efficiency of phytoremediation of heavy metal polluted soils (Belimov et al., 2005).

This chapter embodies the results obtained for the different experiments performed as listed in the previous chapter. The results are described under sections and sub-sections as in previous chapter.

4.1 Physico-chemical characteristics of soils sample collected from metal(s) contaminated site

Soil samples collected from different sites of Lucknow and Kanpur (Lucknow-Charbagh locomotive workshop and its adjoining areas, Sarojini nagar Industrial area, Telco industrial area, Eveready Industry-Aishbagh; Kanpur-Panki power plant,Vijay nagar industrial area, Jainpur industrial area) varied slightly in the physico-chemical parameters tested. pH of the soil samples ranged from 6.89 to 7.02 indicating slightly acidic nature. Amongst all, soil samples from Sarojini nagar industrial area Lucknow showed the lowest pH (6.89), while the highest pH was recorded for soil samples of Charbagh locomotive workshop and its adjoining areas (7.02). Similarly, electrical conductivity (EC) of soil samples was recorded between 1.18 to 3.14 dS m^{-1}, which represents more free metal ions in the soil samples. Among the total samples collected from different sites, soil sample from Jainpur industrial area showed the maximum

EC (3.14 dS m^{-1}) value, however, the minimum EC was recorded for the soil samples of Vijay nagar industrial area (1.18 dS m^{-1}). The total carbon and organic carbon content of the different soil samples ranged from 2.11 to 3.22 and 0.5 to 1.97 respectively; the lowest organic carbon content (0.5) was recorded for the soil from Sarojini nagar industrial area, while the soil samples from Telco industrial area possessed highest organic carbon percentage (1.97). The total Kjeldahl nitrogen (TN), total phosphorus (TP) and total potassium (TK) were measured in percent g^{-1} soil. The TN, TP and TK content of the different soil samples ranged from 0.15 to 0.32, 0.85 to 0.47 and 2.01 to 1.02 respectively. Furthermore, the total available nitrogen in the soil was highest in the samples of Panki power plant (0.32%) followed by Telco industrial area (0.27%), Eveready battery industry Aishbagh (0.24%), Jainpur industrial area (0.22%), Vijay nagar (0.21%) and Sarojini nagar industrial area (0.5%). Moreover, the available TP and TK content were showing highest value in the samples of Panki power plant (TP - 0.85 and TK-2.01%) followed by soil samples of Telco industrial area (TP - 0.66%, TK - 1.87%), Charbagh and Vijay nagar industrial area (0.65% and 1.76%), Eveready battery industry and Jainpur area (0.61% and1.62%), Jainpur area and Sarojini nagar (0.59% and 1.32%), Sarojini nagar and Charbagh (0.52% and 1.11%), Vijay nagar industrial area and Eveready battery industry (0.47% and 1.02%) soil samples.

The heavy metal contents in soil samples were estimated and it was recorded that the range of Cadmium (Cd) in all the samples was 1.03 to 5.03 ppm; while, in case of Lead (Pb), the range was 25.78 to 98.21 ppm. Moreover, soil sample from Sarojini nagar industrial area Lucknow contains maximum Cadmium concentration (5.03 ppm) followed by Panki power plant (4.73), Jainpur industrial area (2.72 ppm), Telco industrial area (1.97 ppm), Eveready battery industry (1.71 ppm) and Vijay Nagar

area (1.22 ppm). The lowest Cadmium content was recorded for soil from Charbagh area (1.03 ppm). However, in case of Pb heavy metal, maximum concentration was recorded for sample from Jainpur industrial area (98.21 ppm) followed by Sarojini nagar area (78.67 ppm), Eveready battery industry (76.67 ppm), Panki power plant (71.78 ppm), Vijay nagar area (49.31 ppm), Telco industrial area (45.89 ppm); while, minimum Pb concentration was recorded for Charbagh area samples (25.78 ppm). The detailed descriptions of sampling sites and summarized physico-chemicals results of the samples are given in table (Table 4.1).

4.2. Cadmium and lead resistant bacterial load in soil samples collected from metals contaminated site

In this experiment total thirty morphologically distinct bacterial isolates were isolated initially from sixteen different samples based on primary screening which involved the notable growth in Cd and Pb supplemented media at 100 ppm. Furthermore, out of thirty, four isolates (two for Cd and two for Pb) were isolated from Charbagh area, five isolates (four for Cd and one for Pb) from Sarojini nagar samples, five (two for Cd and three for Pb) from Telco industrial area samples, four (two for Cd and two for Pb) from Eveready battery industry samples, five (two for Cd and three for Pb) from Panki power plant samples, three (two for Cd and one for Pb) from Vijay Nagar area samples and four (one for Cd and three for Pb) from Jainpur area samples as shown in Fig 4.1. Isolates on LB media are depicted in Plate 4.1.

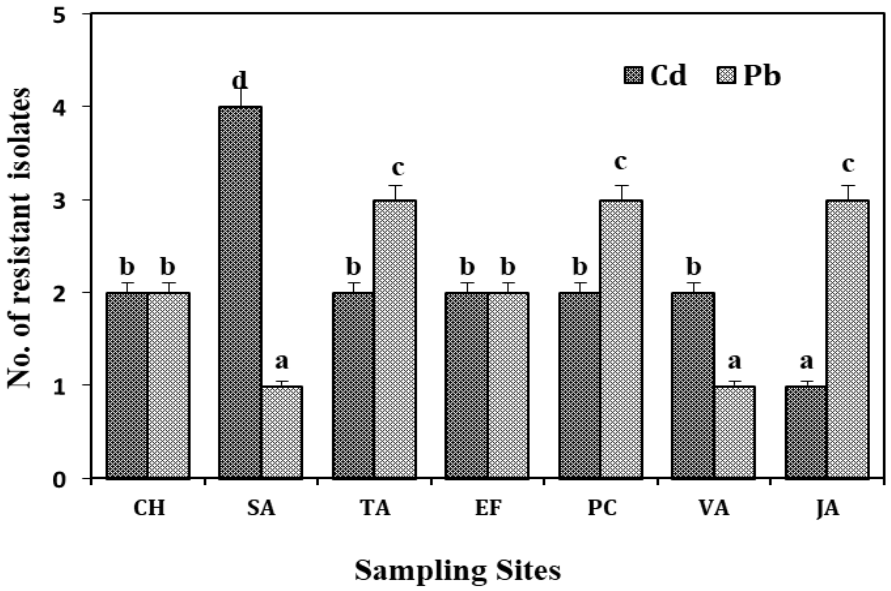

Figure 4.1 Number of heavy metals (Cd and Pb) resistant bacteria from different sampling sites.

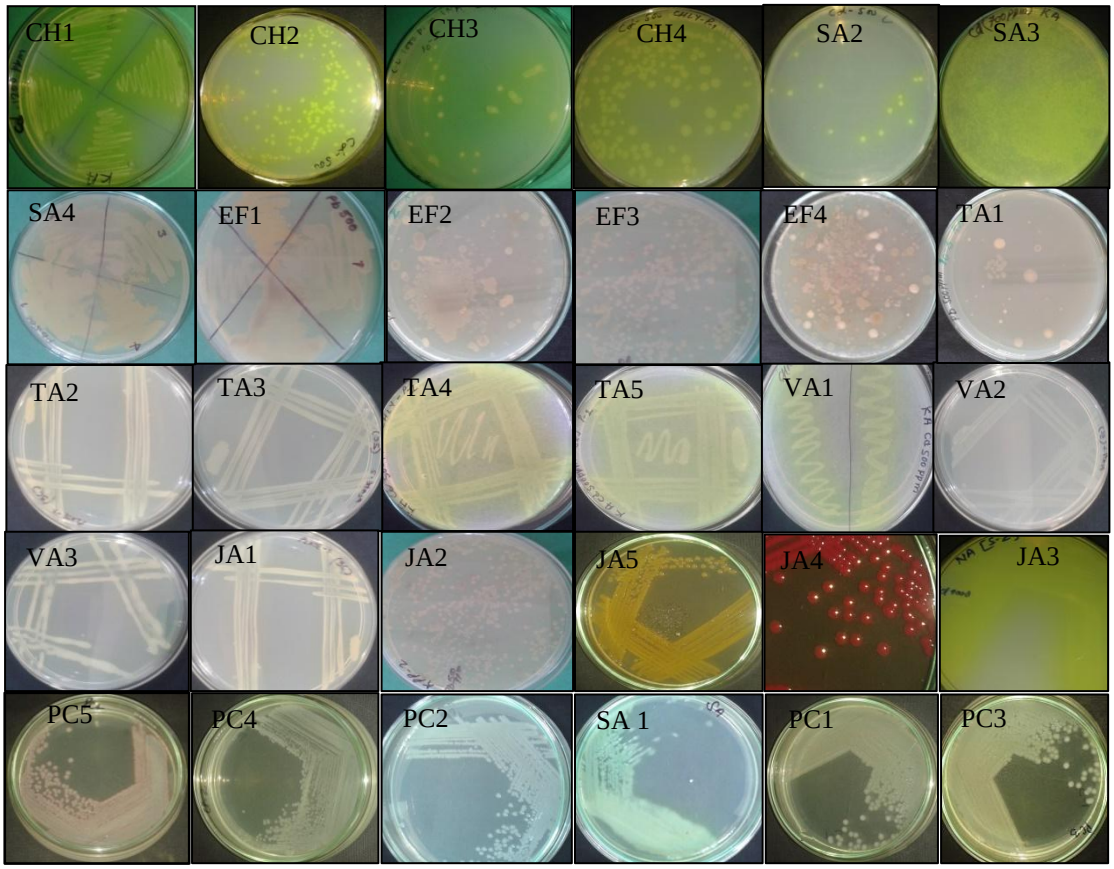

Plate 4.1 Representative culture plates of bacterial isolates from different sampling sites

Table 4.1 Physico-chemical and elemental characteristics of collected soil samples. Different small letters in each parameters indicate statistically significant differences between sampling sites according to DMRT (0<0.05).

Parameters	Sampling sites							Standard
	SA	**CH**	**JA**	**PC**	**TA**	**EF**	**VA**	
pH	6.89 ± 0.2^{a}	7.02 ± 0.04^{b}	6.98 ± 0.03^{b}	6.90 ± 0.01^{b}	6.91 ± 0.05^{b}	7.01 ± 0.04^{b}	6.94 ± 0.01^{b}	$6.5\text{-}7^{A}$
EC (dS m^{-1})	0.82 ± 0.02^{c}	0.63 ± 0.05^{b}	1.04 ± 0.01^{d}	0.83 ± 0.01^{c}	0.77 ± 0.03^{b}	1.03 ± 0.05^{d}	0.28 ± 0.01^{a}	1.0^{B}
OC (%)	0.5 ± 0.02^{a}	1.67 ± 0.05^{b}	1.76 ± 0.04^{b}	1.73 ± 0.02^{b}	1.97 ± 0.05^{c}	1.41 ± 0.05^{b}	1.37 ± 0.04^{b}	-
OM (%)	2.13 ± 0.05^{a}	3.21 ± 0.04^{c}	2.31 ± 0.02^{b}	2.92 ± 0.05^{b}	3.22 ± 0.05^{c}	2.11 ± 0.04^{a}	2.19 ± 0.05^{a}	-
N ppm	15 ± 0.02^{a}	25 ± 0.001^{c}	22 ± 0.01^{b}	32 ± 0.001^{d}	27 ± 0.01^{c}	24 ± 0.001^{c}	21 ± 0.01^{b}	$15\text{-}30^{C}$
P ppm	12 ± 0.06^{a}	25 ± 0.001^{c}	19 ± 0.002^{b}	32 ± 0.001^{d}	25 ± 0.01^{c}	21 ± 0.01^{b}	14 ± 0.02^{a}	$7\text{-}8^{D}$
K ppm	232 ± 0.05^{b}	111 ± 0.05^{a}	262 ± 0.06^{c}	301 ± 0.05^{d}	287 ± 0.05^{c}	202 ± 0.05^{b}	276 ± 0.05^{c}	$50\text{-}120^{E}$
Fe (ppm)	561.64 ± 2.8^{c}	151.64 ± 1.3^{a}	189.48 ± 1.4^{a}	671.64 ± 2.1^{c}	331.64 ± 3.2^{b}	153.64 ± 1.2^{a}	359.13 ± 3.4^{b}	150^{E}
Mn (ppm)	38.86 ± 0.70^{b}	16.86 ± 1.4^{a}	58.66 ± 1.3^{d}	53.86 ± 0.3^{d}	47.63 ± 0.5^{c}	10.26 ± 0.4^{a}	65.16 ± 1.5^{e}	437^{E}
Zn (ppm)	354.04 ± 4.2^{d}	22.81 ± 1.2^{a}	72.55 ± 5.6^{b}	552.81 ± 3.7^{e}	37.48 ± 0.5^{a}	60.81 ± 0.8^{b}	122.55 ± 3.1^{c}	$50\text{-}100^{E}$
Cu (ppm)	10.21 ± 0.28^{c}	8.21 ± 0.16^{a}	12.44 ± 1.4^{d}	10.47 ± 0.12^{c}	9.34 ± 0.17^{b}	8.13 ± 0.16^{a}	8.44 ± 0.6^{a}	$6\text{-}60^{D}$
Cd (ppm)	5.03 ± 0.08^{d}	1.03 ± 0.02^{a}	2.72 ± 0.07^{b}	4.73 ± 0.05^{c}	1.97 ± 0.05^{ab}	1.71 ± 0.05^{ab}	1.22 ± 0.05^{a}	$0.07\text{-}1.1^{D}$
Ni (ppm)	12.44 ± 0.21^{e}	4.39 ± 0.21^{a}	11.85 ± 0.25^{d}	11.21 ± 0.12^{d}	11.21 ± 0.13^{d}	10.39 ± 0.21^{c}	8.98 ± 0.05^{b}	$75\text{-}150^{C}$
Pb (ppm)	78.67 ± 1.4^{cd}	25.78 ± 1.2^{a}	98.21 ± 1.8^{d}	71.78 ± 1.7^{c}	45.89 ± 1.2^{b}	76.67 ± 1.2^{c}	49.31 ± 1.3^{b}	$10\text{-}70^{D}$
Cr (ppm)	1.54 ± 0.03^{b}	0.72 ± 0.001^{a}	11.54 ± 1.2^{c}	279.54 ± 24^{d}	0.2 ± 0.001^{a}	1.2 ± 0.05^{b}	189.14 ± 2.1^{d}	65^{E}

A-WHO (2002); B- Muhr et al. (1965); C- Indian standard by Awashthi (2000) and European Union, (2000); D- Codex Alimentarious Commission joint with FAO/WHO (1996); E- WHO (2000).

SA – Sarojini Nagar Industrial area Lucknow, **CH** – Charbagh Locomotive workshop and adjoining areas Lucknow, **TA** - Telco Industrial area Lucknow, **EF** - Eveready battery Industry Lucknow, **JA** – Jainpur Industrial area Kanpur Dehat, **PC** – Panki Power Plant Kanpur, **VA** -Vijay Nagar Industrial area Kanpur

4.3. Heavy metal (Cadmium and lead) tolerance test of the isolates

Results from present study showed that, out of the thirty isolates, 94% isolates were growing at 100 to 200 ppm of Cd concentration, and 4% were growing at 200 to 600ppm Cd concentration; while only 1% isolates were able to grow upto 1200 ppm of Cd concentration. In case of lead, only 1% isolates were able to grow in the range of 100 to 1500 ppm and 2% isolates in the range of 100 to 750 ppm while of Pb concentration, rest isolates were growing at ≤ 500 ppm of Pb concentration (Fig 4.2). Three isolates *viz.* SA, PC1 and PC3 were growing at higher concentration of 1200 ppm in case of cadmium and 1500 ppm of lead. On the basis of maximum tolerance, these three isolates SA, PC1 and PC3 were selected for further study.

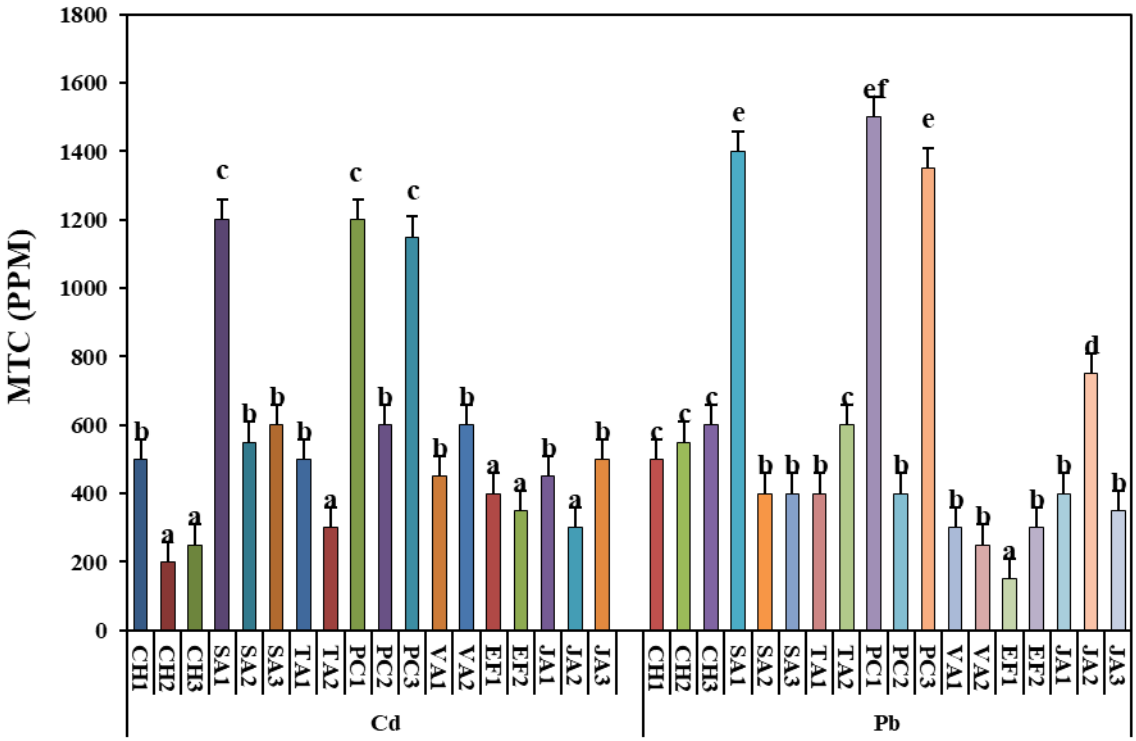

Figure. 4.2 Maximum tolerance concentration for heavy metals (Cd and PB) of isolated bactetrial strains.

4.4. Evaluation of plant growth promoting traits of the selected bacterial isolates under cadmium and lead stress condition

Selected rhizobacterial strains were tested for their ability to produce plant growth promotory (PGP) attributes *viz.* ACC deaminase activity, IAA production, phosphate solubilization, siderophore, extracellular polysaccharide production, ammonia and HCN production. Results revealed that the selected bacterial isolates (SA, PC1 and PC3) were showing multiple plant growth promoting activities in the presence and absence of both (Cd and Pb) metal ions up to 500 ppm concentration. Beyond this concentration, there was a decline in the expression of plant growth promotory properties, hence 500 ppm concentration of both (Cd and Pb) metal ions were selected for further experiments. There was a mixed response in the expression of PGP traits; in some cases there was a decline in expression of a particular property in presence of metal ion while in some cases there was an increase (Table 4.2).

4.4.1 ACC deaminase activity

Results from this experiment showed that all the three strains (PC1, PC3 and SA) were showing the capability to cleave ACC by producing ACC deaminase enzyme which is essential to balance the ethylene level in cells. The qualitative assay was performed by streaking the strains on DF salt media. Results revealed that all the strains were growing easily in DF salt medium using ACC as a sole source of nitrogen (Plate 4.2).

Further quantitative assessment of ACC-deaminase activity was determined by monitoring the amount of α-ketobutyrate (KB) produced from the cleavage of ACC. It was observed that strain SA produced 22.56±0.01, strain PC1 produced 30.27±0.05, and strain PC3 produced 29.667±0.01 μm α-KB mg^{-1} h^{-1} protein in control conditions (no metal ions in medium). However, the activity was increased by 19.28%

(43.1±0.02µm α-KB mg^{-1} h^{-1}) by strain PC1 and 23.44% (36.334±0.02 µm α-KB mg^{-1} h^{-1}) by strain PC3 in the presence of Cd metal ions. In the presence of Pb ions strain PC1 increased by 21.42% (36.98±0.02µm α-KB mg^{-1} h^{-1}), and strain PC3 increased by 27.70% (37.88±0.02µm α-KB mg^{-1} h^{-1}) and 42.32% (32.10±0.02 µm α-KB mg^{-1} h^{-1}) by strain SA. On the other hand, it was also observed the activity was unaffected by strain SA (22.56±0.02µm α-KB mg^{-1} h^{-1} protein) in the presence of Cd metal ions (Fig 4.3, Plate 4.2).

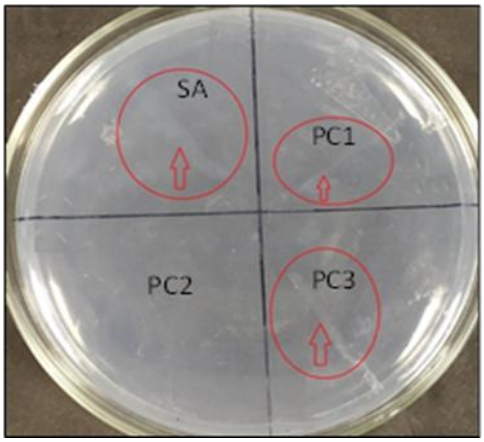

Plate 4.2 Growth of strains on DF salt media showing positive activity of ACC-deaminsae test.

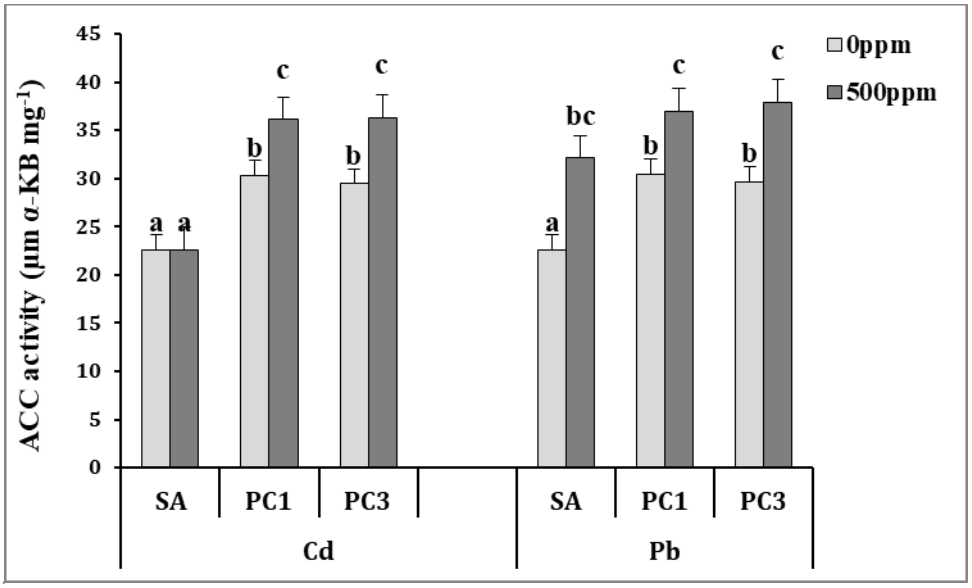

Figure 4.3 ACC deaminase production characteristic by heavy metal resistant selected bacterial strains.

4.4.2 Siderophore production

Findings from this study explained the ability of strains (SA and PC3) to produce siderophore with and without Cd and Pb ions amended media by changing the colour of the medium from blue to yellow (Plate 4.3). However, strain PC1 was found negative for siderophore production in both conditions i.e. in absence and presence of heavy metal ions. Further, quantitative assessment with strains SA and PC3 revealed that 99.01 and 54.74 µM siderophores was released into the culture supernatant in the absence of heavy metal (Cd and Pb ions) stress. Moreover, it was observed that the production of siderophore decreased by 35.79% (63.575 µM) by strain SA in Cd amended media compared to their respective control (in the absence of heavy metal ions).While, in the presence of Pb ions, the production of siderophore increased by 6.056% (105.01 µM) by the strain SA. However, in case of strain PC3, a significant ($p \leq 0.05$) reduction 49.01% (27.91 µM) and 9.27% (49.667 µM) was observed in the presence of Cd and Pb ions respectively (Fig 4.4 and Table 4.2).

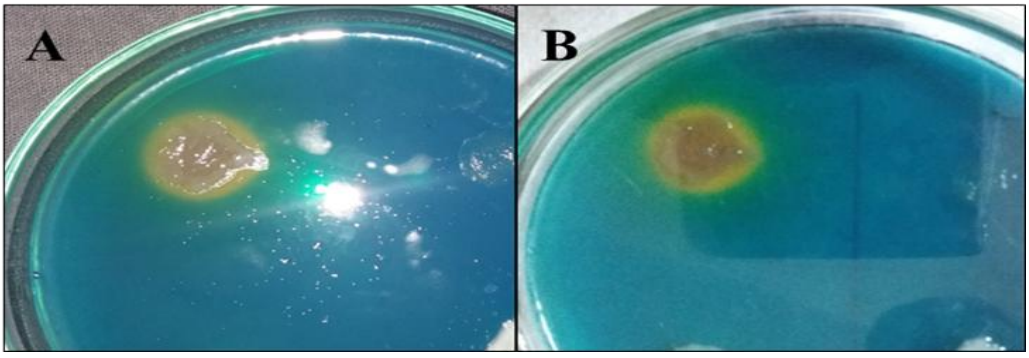

Plate 4.3 Respective culture plates showing siderophore production on defferated CAS agar media, A) PC3 and B) SA).

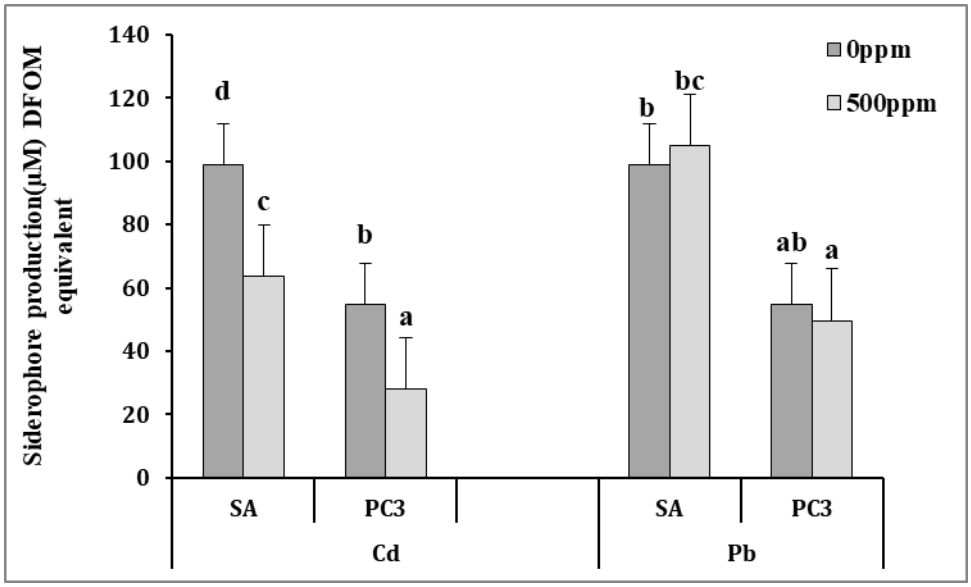

Figure 4.4 Quantitative assessment of siderophore production by selected bacterial strains in absence and presence of heavy metal (Cd and Pb).

4.4.3. Indole – 3 acetic acid (IAA) production Test

Results from this experiment revealed that all the three strains were able to produce IAA (change the medium colour in pink to red) by utilizing L-tryptophan as a precursor compound in the absence and presence of Cd and Pb (Plate 4.4). Furthermore, the quantitative estimation of IAA production was checked at different concentration of L-tryptophan (100, 300 and 500 µg mL^{-1}). It was observed, as the concentration of tryptophan increased, the production of IAA also increased. Moreover, it was observed that the highest amount of IAA was produced by strain PC1 (38.737 µg mL^{-1}) followed by strain PC3 (35.556 µg mL^{-1}) and SA (31.48 µg mL^{-1}) at 500µgml^{-1} tryptophan concentration in the absence of heavy metals (Cd and Pb). A significant reduction ($p \leq 0.05$) 37.04%, 31.9% and 38.63% was observed by strain SA, PC1 and PC3 respectively in the presence of Cd heavy metal. In the presence of Pb ions, IAA production by the isolates SA and PC1 declined significantly ($p \leq 0.05$) by 26.03% and 6.1%, respectively. Moreover, it was found

that the production of IAA increased by 3.2% by isolate PC3 when Pb ions were present in the medium (Fig 4.5 and Table 4.2).

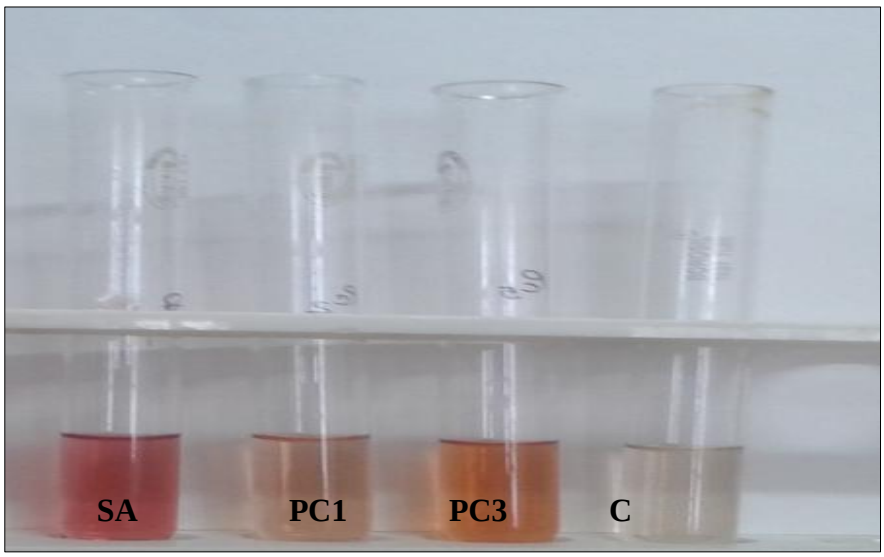

Plate 4.4 Production of IAA by the heavy metals resistant selected bacterial strains.

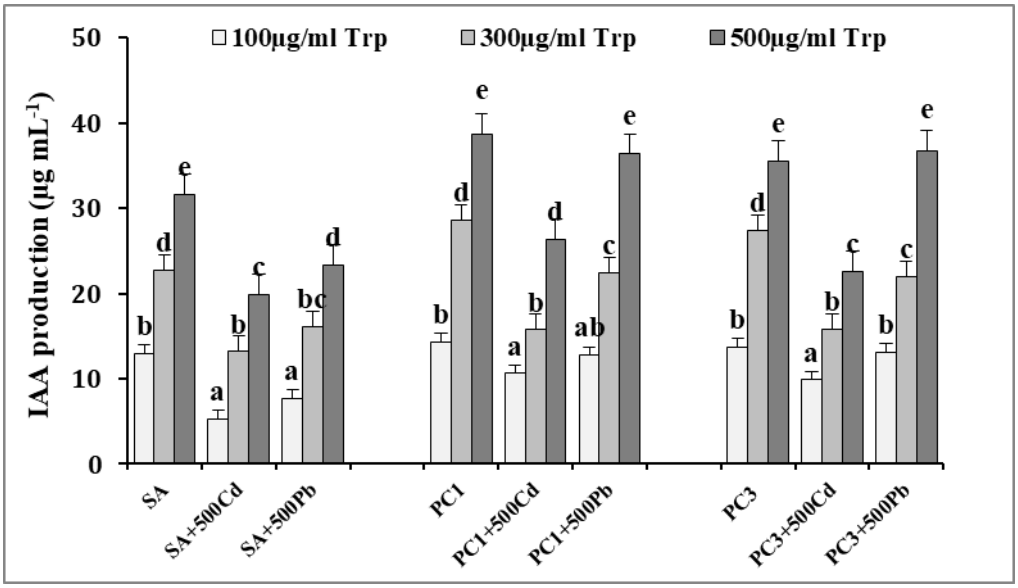

Figure 4.5 Quantitative estimation of IAA production under different concentration of tryptophan (Trp) by selected bacterial strains in the presence and absence of heavy metals (Cd and Pb).

4.4.4 Phosphate solubilization

Results from this test showed that all the selected heavy metal resistant rhizobacterial isolates were able to solubilize inorganic phosphate which was confirmed by brown

colour halo zone with or without heavy metals amended NBRIP media agar plate (Plate 4.5). Brown colour halo zone confirmed the positive tests of the result. Further quantitative assessment at different time intervals showed that solubilization was increased as the incubation period increases but at a certain time period it decreased gradually. Our results revealed that the strain SA, PC1 and PC3 could solubilize inorganic phosphate up to 148.126, 131.233 and 137.728µg/ml respectively in the absence of heavy metals (Cd and Pb) at seven days incubation period. However, when Cd ions were present in medium solubilization was decreased by 45.19%, 44.80% and 42.39% by strain SA, PC1 and PC3 respectively. While in the presence of Pb ions strain SA, PC1 and PC3 were decreased by 27.18%, 22.96% and 26.18% respectively. The results are summarized in table (4.2) and well-illustrated in figure (Fig 4.6).

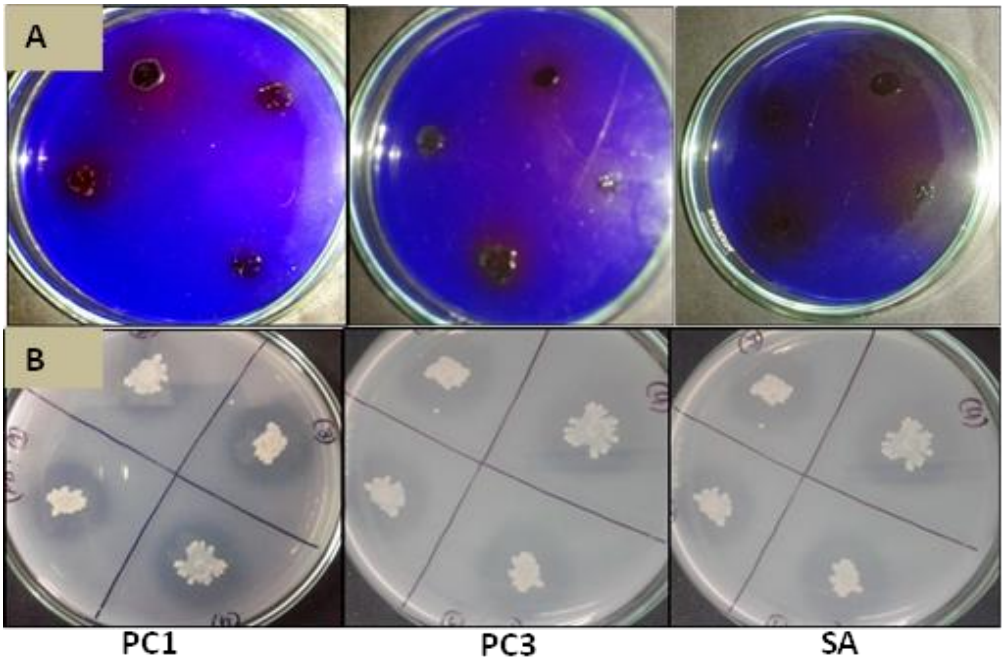

Plate 4.5 Phosphate solubilization activity of heavy metals resistant selected bacterial strains on A) NBRIP media and B) Pikovskaya agar plates.

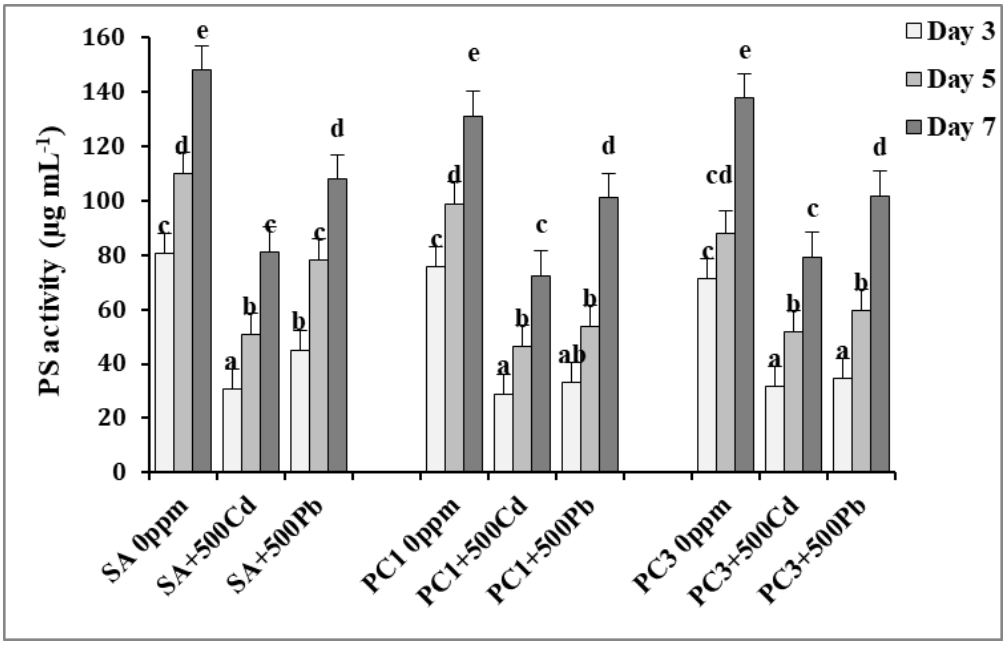

Figure 4.6 Quantitative estimation of phosphate solubilization by selected bacterial strain in presence and absence of Cd and Pb at different incubation periods.

4.4.5. HCN and Ammonia production

Findings from this experiment showed the ability of isolates to produce hydrogen cyanide (HCN) and Ammonia in the presence and absence of Cd and Pb amended media. Observed results revealed that HCN production by strains SA, PC1 and PC3 was positive in the absence of cadmium (Cd) and lead (Pb). However, in the presence of Cd, strain PC1 failed to produce HCN, while strain SA and PC3 were positive for HCN production. On the other hand, in presence of lead, all the three isolates showing positive result for HCN production (Plate 4.6).

In order to evaluate ammonia production, selected isolates were grown in peptone broth. All the three isolates were positive for ammonia production in the absence and presence of Cd and Pb (Table 4.2). Further, results are summarized in table 4.2 and well showed in figure (Fig. 4.7 and Table 4.2).

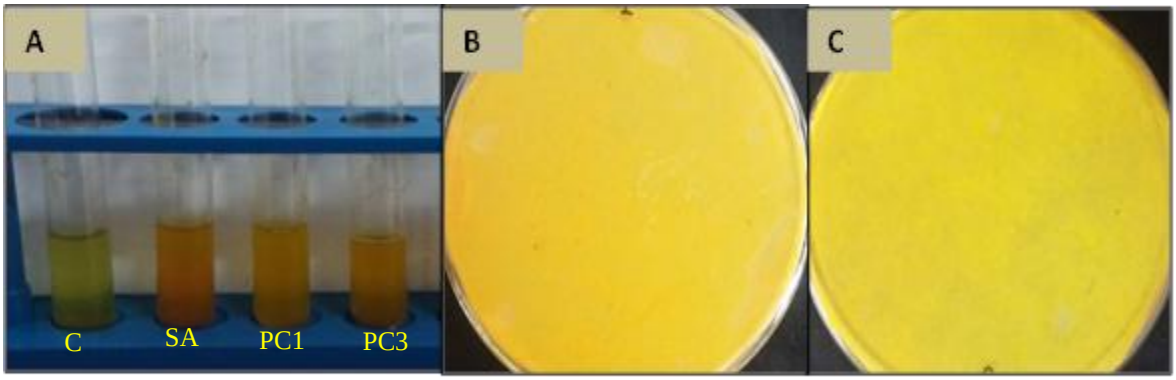

Plate 4.6 **A) Isolates showing ammonia production in tubes**
B & C) HCN production by SA and PC3 bacterial strain.

4.4.6. Extracellular polysaccharide production

The mucoid gummy colonies on LB agar plate and increased viscosity in LB broth indicated the ability of isolates to produce EPS. All the three isolates SA, PC1, PC3, were able to produce extracellular polysaccharide (EPS) in the absence and presence of Cd and Pb metal on LB agar plates (Fig. 4.7; Table 4.2). However, in case of PC3 isolate presence of cadmium and lead enhanced its production over the control while in case of SA, there was a reduction in EPS production in presence of both cadmium and lead. In brief, quantitative yield of EPS produced during submerged fermentation by isolates SA, PC1 and PC3 was 151.013, 119.271 and 137.743 µg ml $^{-1}$ respectively in the absence of heavy metal ions at five days of incubation period.

Table 4.2 Plant growth promoting traits in the presence and absence of Cd and Pb heavy metal in their specific medium. ±SD representation of three independent replicates, while different letters within each treatment indicate statistically significant differences between treatments according to Duncan's multiple comparison range test ($p<0.05$).

Isolates ↓	PGP Traits →		ACC deaminase production	IAA synthesis	Siderophore production	Phosphate solubilization	EPS production	HCN production	Ammonia production
SA	**Cd**	**0 ppm**	22.56±0.01^{a}	31.48±0.05^{c}	99.01±0.05^{b}	148.12±0.08^{d}	151.013^{d}	+++	+++
		500 ppm	22.58±0.02^{a}	19.86±0.01^{a}	63.57±0.01^{c}	81.18±0.06^{a}	103.575^{a}	+++	+++
	Pb	**0 ppm**	22.56±0.01^{a}	31.48±0.05^{c}	99.01±0.04^{d}	148.12±0.09^{d}	151.013^{d}	+++	+++
		500 ppm	32.10±0.02^{b}	23.33±0.04^{b}	105.01±0.07^{d}	107.85±0.08^{d}	145.01^{d}	+++	+++
PC1	**Cd**	**0 ppm**	30.27±0.05^{b}	38.73±0.06^{c}	ND	131.23±0.07^{b}	119.271^{b}	+++	+++
		500 ppm	43.1±0.02^{c}	26.34±0.03^{b}	ND	72.43±0.03^{a}	91.163^{a}	ND	+++
	Pb	**0 ppm**	30.27±0.05^{b}	38.73±0.06^{c}	ND	131.23±0.07^{c}	119.271^{b}	+++	+++
		500 ppm	36.98±0.02^{bc}	36.34±0.06^{c}	ND	101.09±0.06^{b}	123.372^{c}	+++	+++
PC3	**Cd**	**0 ppm**	29.667±0.01^{ab}	35.55±0.04^{b}	54.74±0.01^{b}	137.72±0.08^{c}	103.678^{a}	+++	+++
		500 ppm	36.334±0.02^{bc}	22.55±0.05^{b}	27.91±0.01^{a}	79.33±0.05^{a}	141.913^{d}	+++	+++
	Pb	**0 ppm**	29.667±0.01^{ab}	35.56±0.04^{c}	54.74±0.01^{b}	137.72±0.07^{c}	103.678^{a}	+++	+++
		500 ppm	37.88±0.02^{bc}	36.75±0.03^{c}	49.66±0.02^{b}	101.66±0.06^{b}	137.767^{d}	+++	+++

ND = Not Detected

Furthermore, it was observed that EPS production decreased by 31.41% (103.57 µg mL^{-1}) and 23.56% (91.16 µg mL^{-1}) by isolates SA and PC1 respectively in the presence of Cd. While, in case of strain PC3 the EPS production increased by 36.87% (141.913 µg mL^{-1}) in the presence of Cd. Similarly, when Pb was present in the medium, the production of EPS was increased by 21.84% and 22.13% by isolates SA and PC1 respectively.

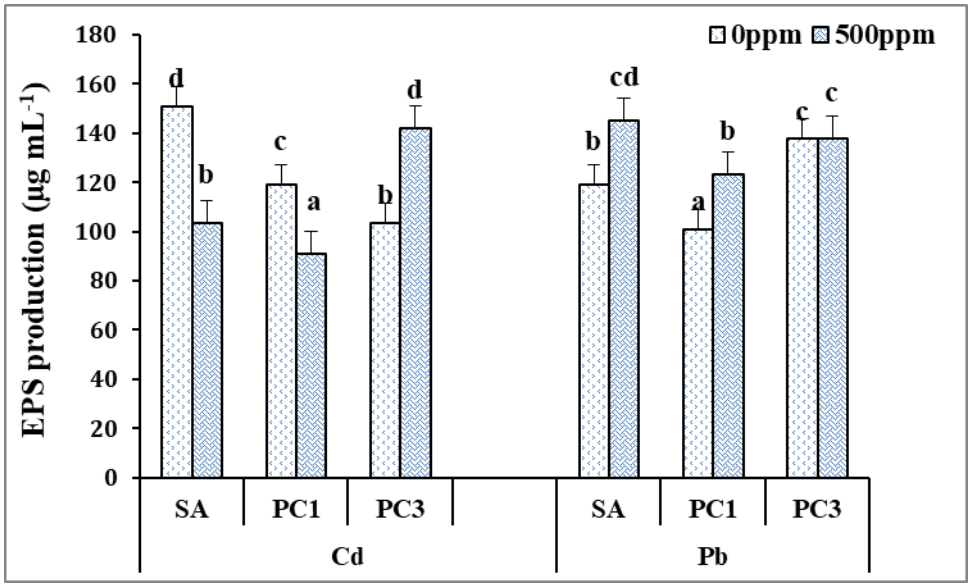

Figure 4.7 Quantitative estimation of extracellular polysaccharide (EPS) production by selected bacterial isolates under heavy metal (Cd and Pb) stress.

4.4.6.1. Fourier transform infrared radiation (FTIR) spectroscopy analysis of extracted EPS

The FTIR spectra of EPS produced by heavy metal resistant rhizobacterial isolates were analyzed and stretching vibrations were assigned to reveal the typical polymeric structural groups of the carbohydrate. Observed results showed that FTIR spectrum of EPS produced by isolates SA, PC1 and PC3 had characteristic peaks between 3401 - 805 cm^{-1}. The stretching vibration at 3402, 3072 and 3040 cm^{-1} indicating O-H (hydroxyl) group and hydrogen bonding, a characteristic of polysaccharide ring. The characteristics peak at 2850 and 2728 cm^{-1} represents to aliphatic C-H stretching of methyl or methylene groups, commonly present to hexose groups like glucose or galactose or deoxyriboses like rhamnose or fructose. Further, four peaks at 1924, 1865, 1665 and 1590 cm^{-1} were observed; in which, the first two peaks might represents N-H (amide) I and II bands, respectively. While another two spectral bands are assigned to C-N stretching vibration of proteins. The detailed description of peaks

range from 1457 to 857 as described in Table 4.5. Further, the characteristics peaks are shown in figure (Fig 4.8).

Table 4.3 Different vibrational stretching bands and their peak ranges from FTIR analysis.

Groups	**Peak ranges cm^{-1}**
O-H stretching and hydrogen bonding	3403.2
Aliphatic CH stretching (symmetric and asymmetric stretching of CH_3 and CH_2)	3077.6, 3040.5
C=O asymmetric stretching of -NH-CO-R and/or N-H bonding of H_2N-CO-R (Amide I)	1665
N-H bonding of –NH-(Amide II) and/or C=C stretching of aromatic ring	1590.8
C=O symmetric stretching of carboxylate and/or C-OH stretching of phenolic OH	1357
O-H bonding in carboxylic acid	1316.7
P=O stretching of phosphate PO_4^{3-} and/or C-O stretching of -O-COR	1232.1, 1242.0, 1250.3
C-O-C group vibrations in the cyclic structures of carbohydrates	1050.6, 1077.9
C-O-S stretching of -O-SO_4	805

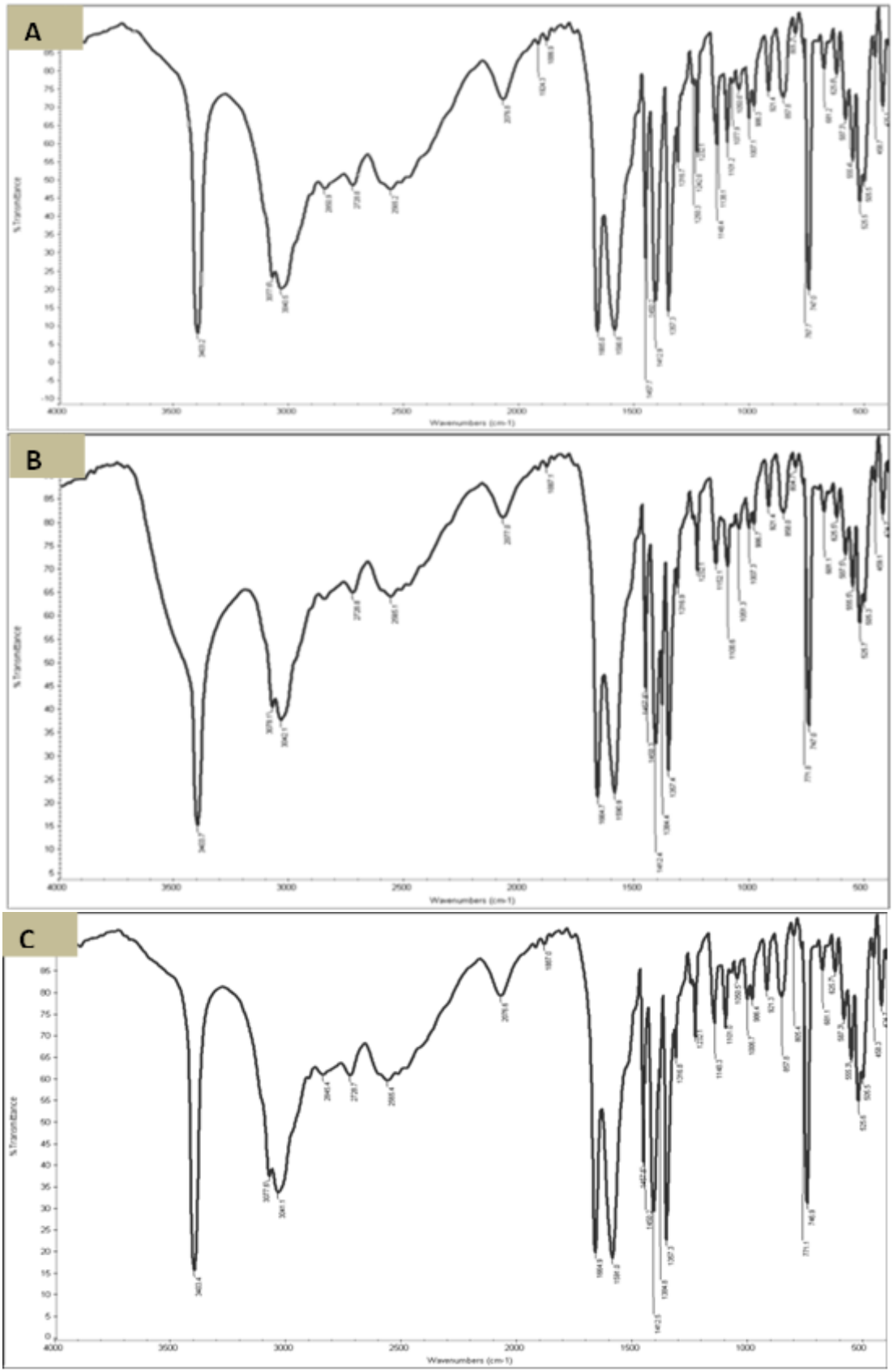

Figure 4.8 FTIR spectrums of extracted EPS produced from selected bacterial strain A) SA, B) PC1 and C) PC3.

4.5. Morphological and Biochemical characterization of selected isolates

4.5.1. Morphological: Cultural characterization & cellular Morphology

On the basis of heavy metal tolerance capability, three isolates (SA, PC1 and PC3) were found more capable as compared to 27 other isolates with respect to cadmium and lead. The three isolates were tested further for their morphological characteristics and it was found that isolate SA, possessed large hairy colony while PC1 and PC3 isolates possessed tiny round-shaped colony. Colonies formed were circular, convex and raised, margins were entire and undulate type for isolates SA, PC1 and PC3, respectively. Simple light microscopic observations showed all the isolates to be tiny rod shaped cells. Moreover, scanning electron microscopic (SEM) study of the selected isolates confirmed that all isolates were rod shaped. The light microscopic and scanning electron microscopic pictures are well illustrated in figure (Plate 4.7).

Gram reaction and motility: All the three isolates were grams negative and motile

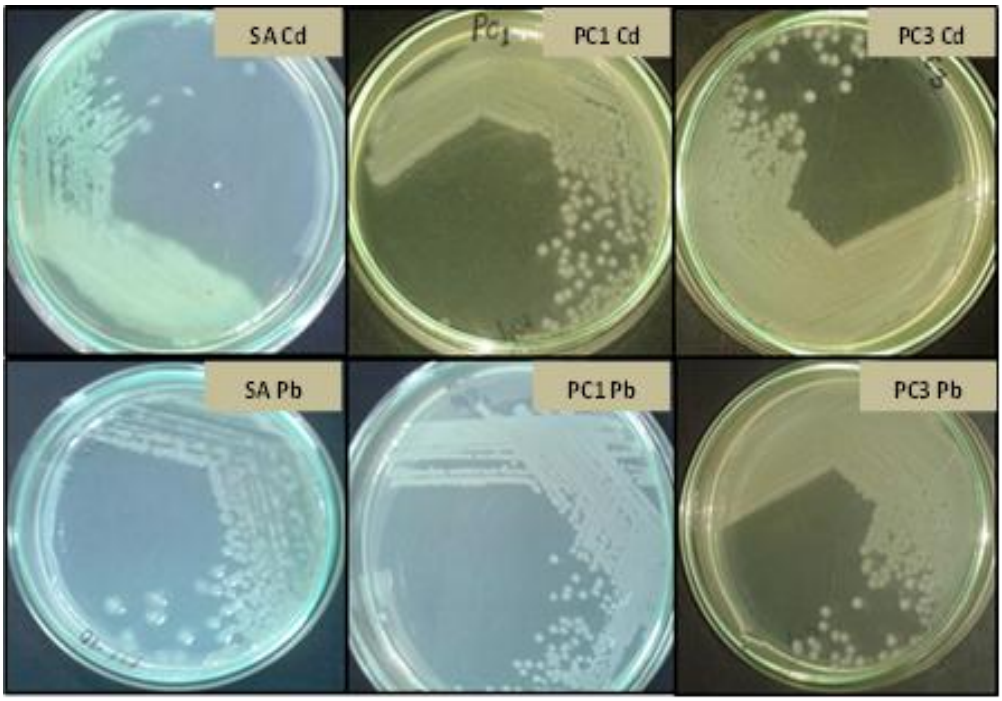

Plate. 4.7 Heavy metal (Cd and Pb) resistant test isolates.

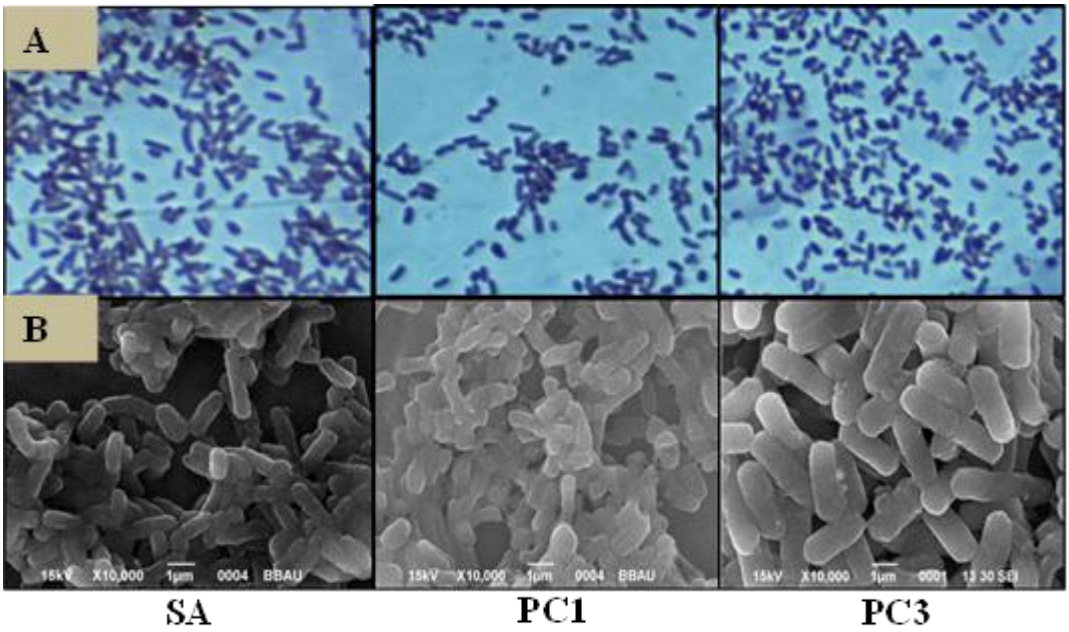

Plate 4.8 Simple light microscope and scanning electron micrographs of heavy metal (Cd and Pb) resistant selected test isolates.

4.5.2. Biochemical Characterization of the selected isolates

The selected bacterial isolates were subjected to various biochemical tests, viz. IMViC, H_2S, extracellular enzyme production: oxidase, urease, cellulose and lipase, carbohydrate utilization: glucose, fructose and lactose.

All the isolates were negative for indole and methyl red test For vogus prausker test, PC1 and PC3 were positive while isolate SA was negative, while for citrate utilization all the three were positive (Table 4.3; Plate 4.9). All the three isolates (SA, PC1 and PC3) were positive for extracellular enzymes viz., catalase, cellulase while negative for urease production test. However, in case of oxidase and lipase test, only isolate SA was positive while isolates PC1 and PC3 were negative. Carbon source utilization pattern of the test isolates varied as depicted in Table 4.3.

Table 4.4 Biochemical characterization of heavy metal (Cd and Pb) resistant test isolates.

Tests	SA	PC1	PC3
Indole test	-ve	-ve	-ve
M-R test	-ve	+ve	-ve
V-P test	-ve	+ve	+ve
Citrate utilization	+ve	+ve	+ve
H_2S production	-ve	-ve	-ve
Extracellular enzymes production			
Catalase	+ve	+ve	+ve
Oxidase	+ve	-ve	-ve
Urease	-ve	-ve	-ve
Cellulase	+ve	+ve	+ve
Lipase	+ve	-ve	-ve
Carbon source utilization pattern			
Glucose	+ve	-ve	+ve
Fructose	-ve	-ve	+ve
Lactose	-ve	+ve	-ve

"+ve" indicates positive result, "-ve" indicates negative result

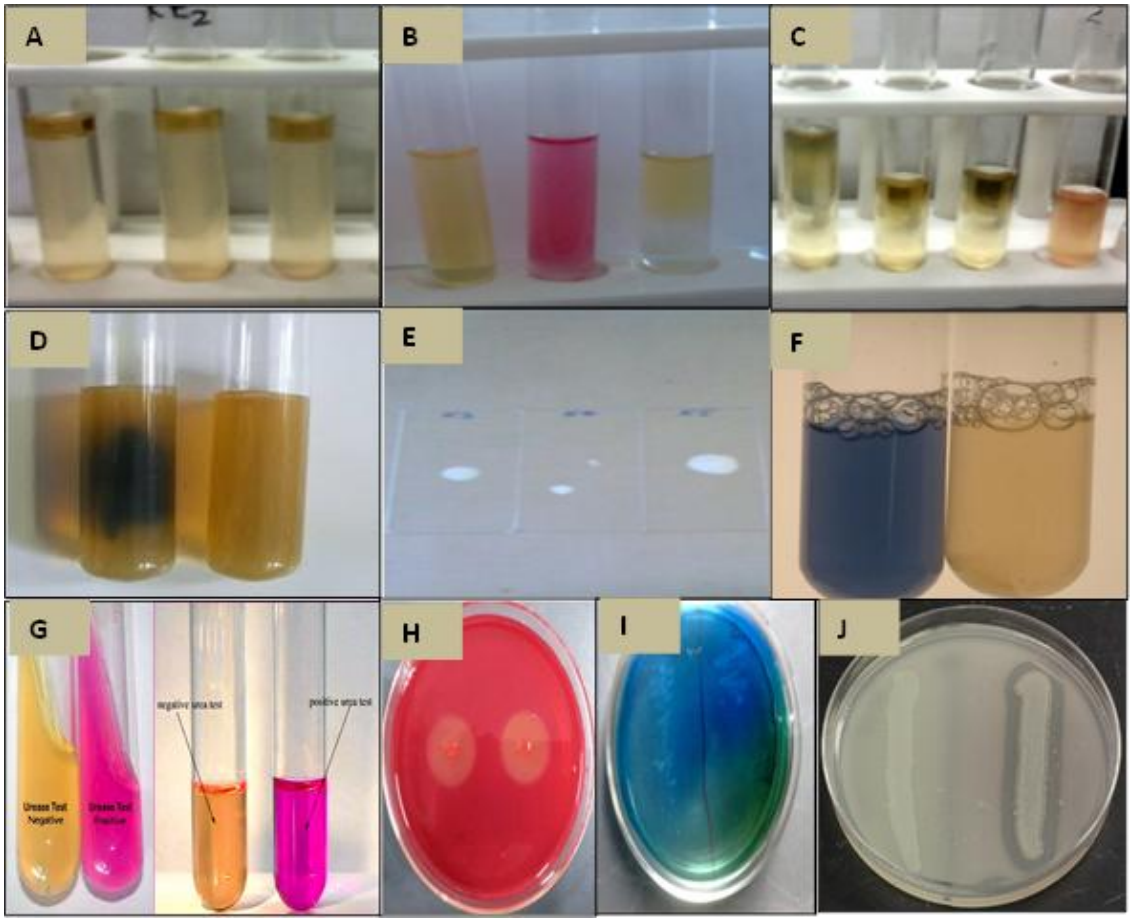

Plate 4.9 Biochemical characterization of selected isolates: (A) Indole production (B) Methyl-red test (C) Voges-Proskauer (D) H_2S production (E) Catalase production (F) Oxidase production (G) Urease production (H) Cellulase production (I) Citrate utilization (J) Lipase production.

4.5.3. Molecular Characterization and Phylogenetic analysis of rhizobacterial isolates

For molecular identification, 16S rRNA partial gene sequencing method was carried out for the selected rhizobacterial isolates. The results revealed that, the similarity value of selected isolates was more than 98% to the genera *Pseudomonas*, *Pantoea* and *Enterobacter* (Table 4.4). Further comparative analysis of the 16SrRNA sequences with already available database, it was found that strain PC1, PC3 and SA were closest to *Pantoea agglomerans*, *Enterobacter cloacae* and *Pseudomonas aeruginosa* respectively. The obtained sequences were submitted to Gene Bank

(NCBI) under the accession number KX668493 (PC1), KX668494 (PC3) KX668495 (SA). For the evolutionary relationship, a phylogenetic tree was constructed using neighbor joining (NJ) method for the isolates PC1, PC3 and SA. The results are summarized in table and illustrated in Figure (Table 4.5; Plate 4.10; Fig. 4.9, 4.10 and 4.11).

Table 4.5 Molecular characterization of rhizobacterial isolates based on 16S rRNA gene sequencing.

S. No.	Isolate code	Closest genera	% Similarity based on BLASTn	Accession number
1.	PC1	*Pantoea agglomerans*	99%	KX668493
2.	PC3	*Enterobacter cloacae*	99%	KX668494
3.	SA	*Pseudomonas aeruginosa*	100%	KX668495

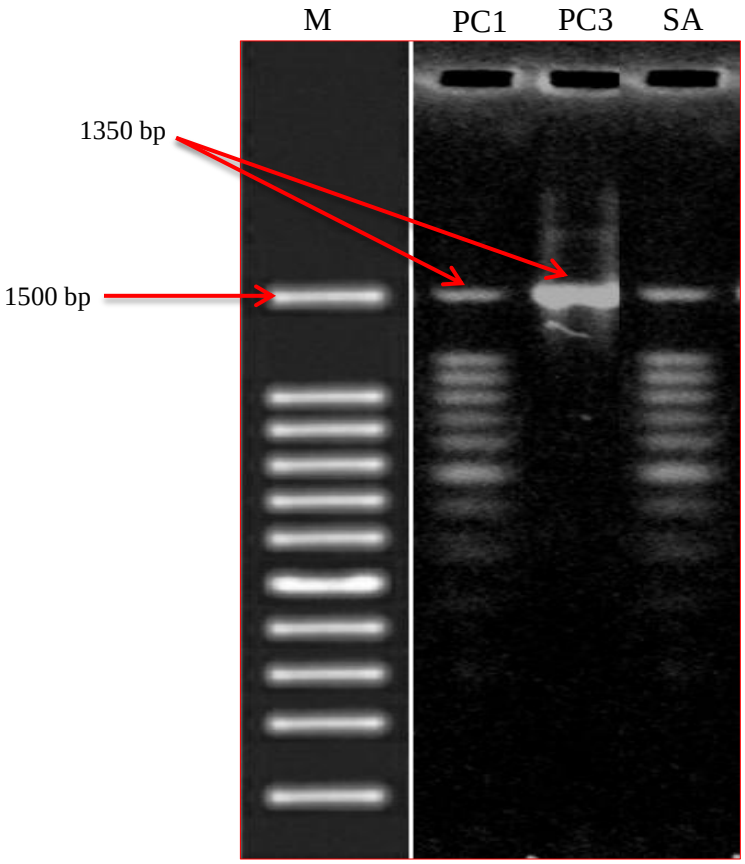

Plate 4.10 Gel electrophoretic analysis of amplified PCR product under UV light.

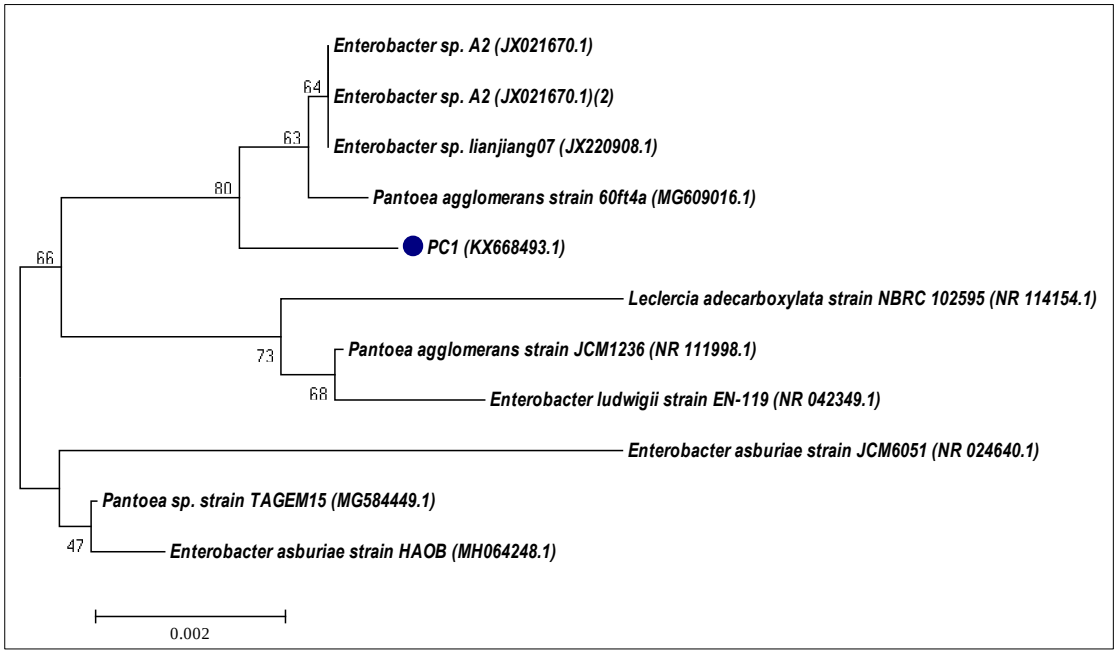

Figure 4.9 Evolutionary relationships of *Pantoea agglomerans* PC1. The optimal tree with the sum of branch length=0.16657409, and associated taxa clustered together in the bootstrap tests (100 replicates) are shown next to the branches. Bar indicates % similarity.

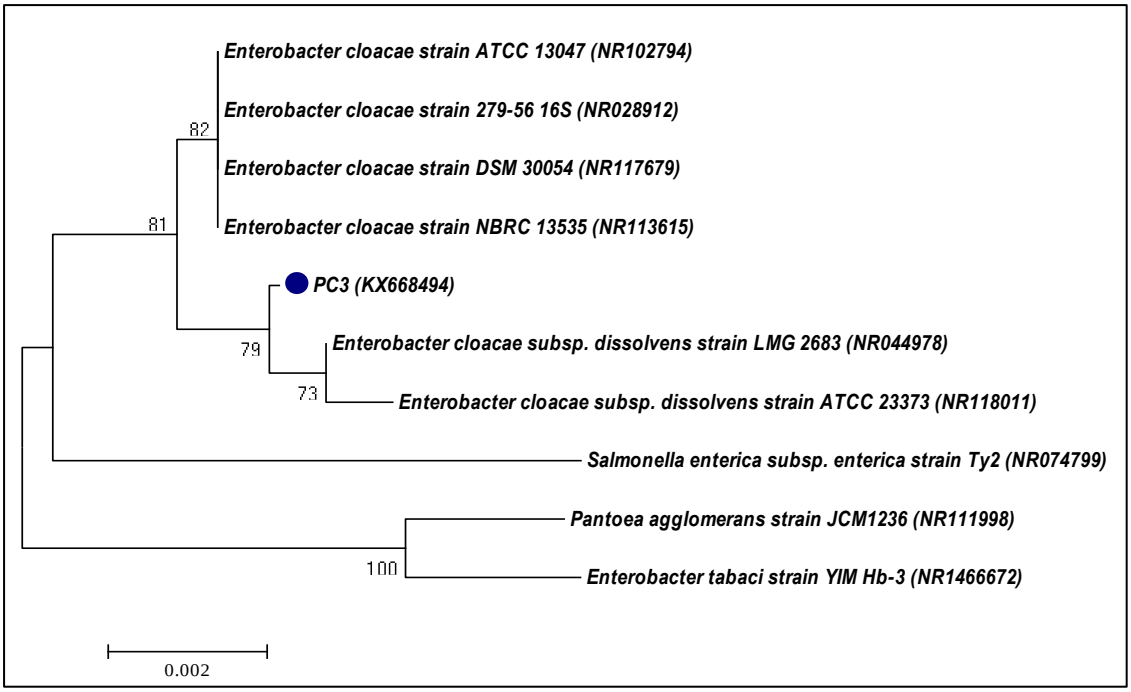

Figure 4.10 Evolutionary relationships of *Enterobacter cloacae* PC3. The optimal tree with the sum of branch length=0.16657409, and associated taxa clustered together in the bootstrap tests (100 replicates) are shown next to the branches. Bar indicates % similarity.

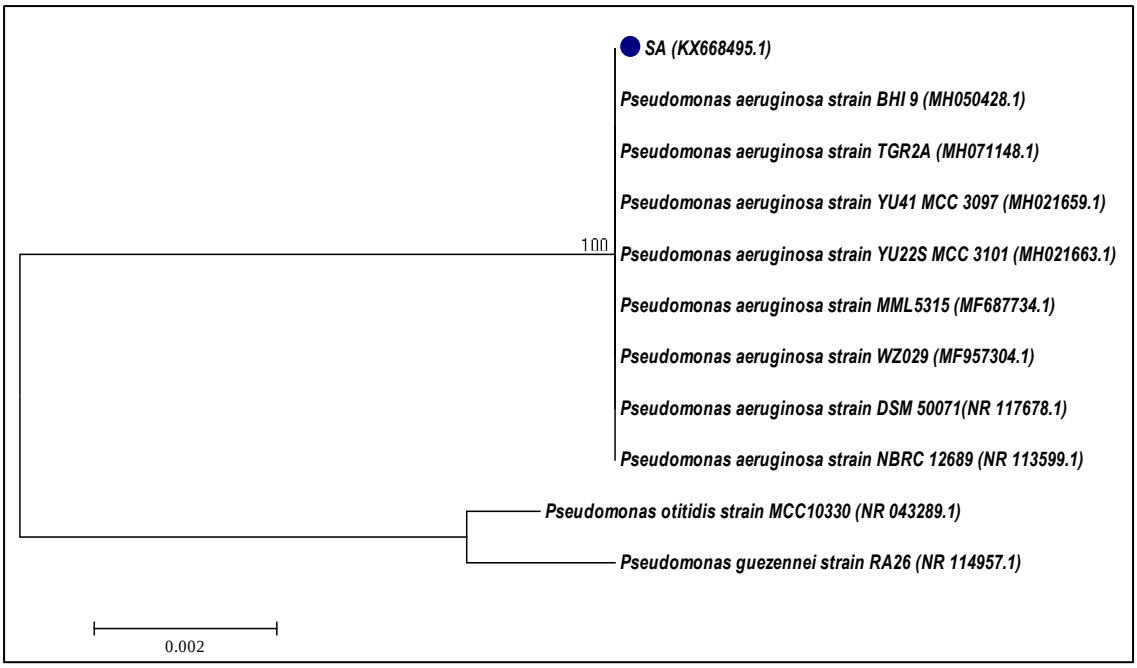

Figure 4.11 Evolutionary relationships of *Pseudomonas aeruginosa* SA. The optimal tree with the sum of branch length=0.16657409, and associated taxa clustered together in the bootstrap tests (100 replicates) are shown next to the branches. Bar indicates % similarity.

4.6. Antibiotic susceptibility test of the selected isolates

The selected isolates were tested for antibiotic sensitivity and results were interpreted by measuring the inhibition zone according to the National Committee for Clinical Laboratory Standards (NCCLS) to categorize the isolates into three categories viz., sensitive, intermediate or resistant to a particular antibiotic. The observed results showed that isolate SA was sensitive to Gentamycin, Chloramphenicol, Ceftriaxone, Ceftazidime and Ampicillin while resistant to Piperacillin and intermediate with Amikacin and Cefoxitin. Isolate PC1 was resistant to Piperacillin and Gentamycin however, sensitive to Amikacin, Chloramphenicol, Ceftriaxone, Ceftazidime and Ampicillin while intermediate for Cefoxitin. In the same way isolate PC3 was sensitive to Amikacin, Ceftriaxone, and Ceftazidime however, resistant for

Gentamycin, Chloramphenicol while intermediate to Piperacillin, Cefoxitin and Ampicillin (Plate 4.11 and Table 4.6).

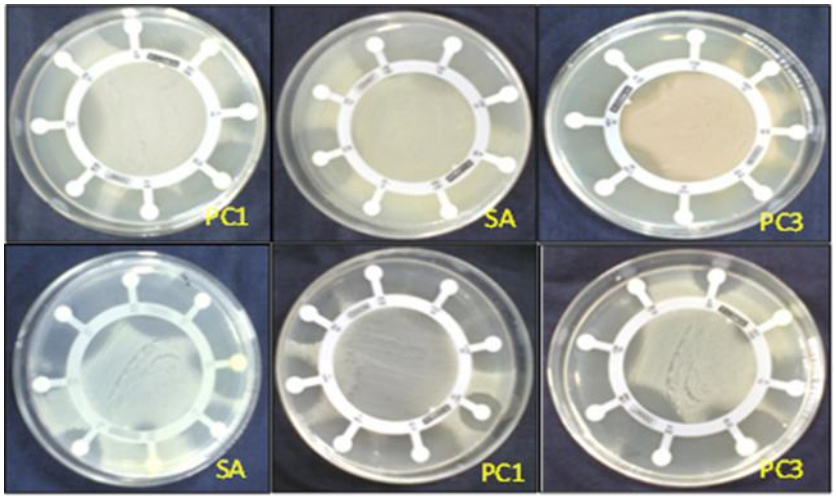

Plate 4.11 Antibiotic sensitivity response of selected bacterial strains against tested antibiotics.

Table 4.6 Antibiotic susceptibility of the selected heavy metal resistant isolates.

			Bacterial Isolates		
			SA	PC1	PC3
Antibiotics	**Antibiotics generation**	**Concentration of antibiotics**	**Zone size (mm)**		
Amikacin	3rd	30 mcg	18.01 (I)	28.12 (S)	18.22 (S)
Piperacillin	3rd	10mcg	10. 06 (R)	5.06 (R)	17.10 (I)
Gentamycin	2nd	10mcg	24.69 (S)	3.19 (R)	NZ (R)
Chloramphenicol	3rd	30mcg	23.03 (S)	26.32 (S)	NZ (R)
Ceftriaxone	3rd	30mcg	21.36 (S)	25.33 (S)	21.73 (S)
Ceftazidime	3rd	30mcg	28.39 (S)	22.95 (S)	22.14 (S)
Cefoxitin	2nd	30mcg	19.06 (I)	12.01 (I)	10.00 (I)
Ampicillin	3rd	10mcg	24.73 (S)	27.43 (S)	7.44 (I)

S – sensitive; I – intermediate; R – resistant; NZ – No zone

4.7. Development of rhizobacterial consortium

All the three strains were checked for compatibility by Agar diffusion method and were found to be compatible to each other (Plate 4.12).

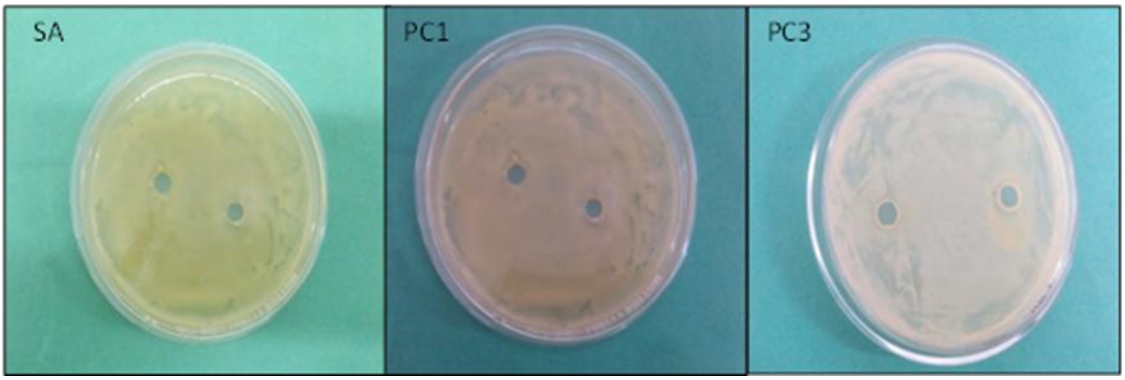

Plate 4.12 Compatibility Test of isolates SA, PC1 and PC3.

4.8. Effect of developed consortium as well as individual rhizobacterial strains on seed germination and seed vigour index of *Zea mays* L. and *Canna indica* under Cd and Pb stress

Results from this study indicated that the germination percentage and seedling vigour index (SVI) of *Zea mays* L. and *Canna indica* plants were adversely affected by Cd and Pb at 500 ppm concentration (Plate 4.13 and 4.14). It was noted that germination percentage and SVI of *Zea mays* L. were reduced by 63.4% and 72.3%, respectively in the presence of 500 ppm Pb, while in the presence of 500 ppm Cd, germination percentage and SVI reduced by 70% and 72%, respectively compared to un-amended metal control.

Similarly, in case of *Canna indica*, germination percentage and SVI were reduced by 32% and 81%, respectively in the presence of Cd. However, in the presence of Pb, germination percentage and SVI were reduced by 63% and 79%, respectively, compared to un-amended metal control. Further, in case of consortium, germination

percentage was better than individual strains SA, PC1 and PC3 in the presence of both metals (Cd and Pb) for *Zea mays* L. plant. Similar results were also noted for SVI in the presence of both metals for *Zea mays* L. plant (Table 4.4). Similarly, in case of *Canna indica* L., maximum germination percentage was recorded in case of consortium treatment followed by strains PC3, PC1 and SA in the presence of Cd. While, in the presence of Pb, similar results were found in consortium treatment. However, treatment with individual strains depicted different pattern of results (SA>PC1>PC3). Maximum SVI was recorded in consortium treatment followed by strains PC3, PC1 and SA compared to control (un-inoculated) in the presence of Cd heavy metal. Similar results were also found in the presence of Pb (Table 4.7, 4.8, 4.9 and 4.10).

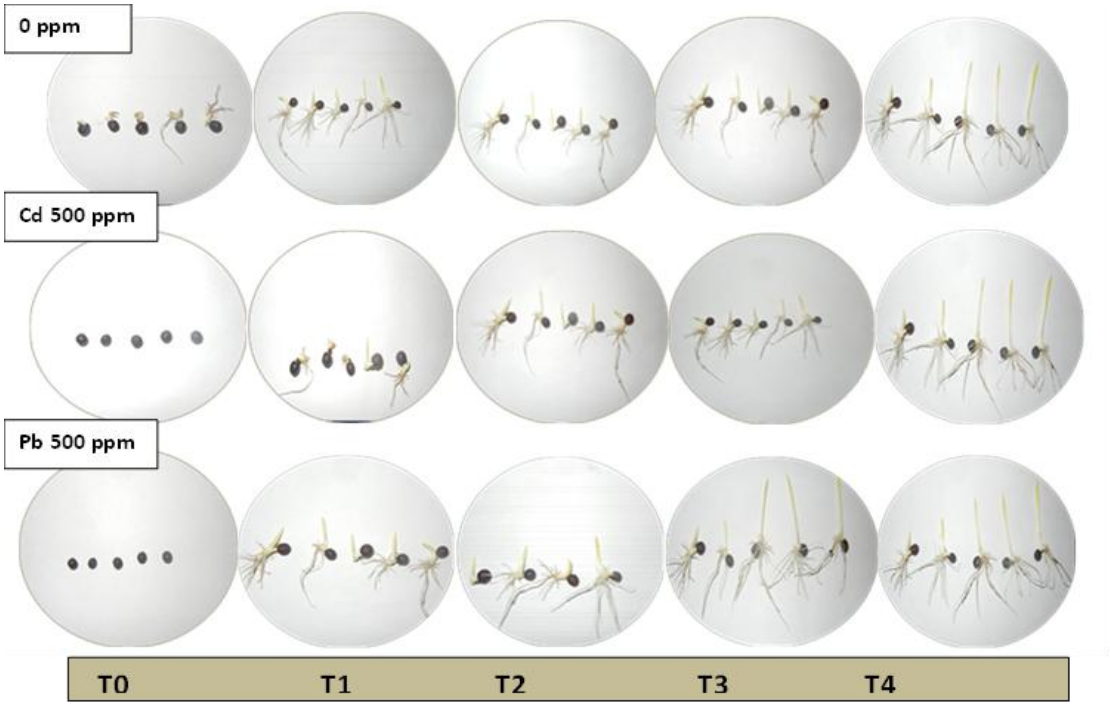

Plate 4.13 Seed germination test of *Canna indica* L. inoculated with rhizobacterial strain and developed consortium under Cd and Pb treatment.

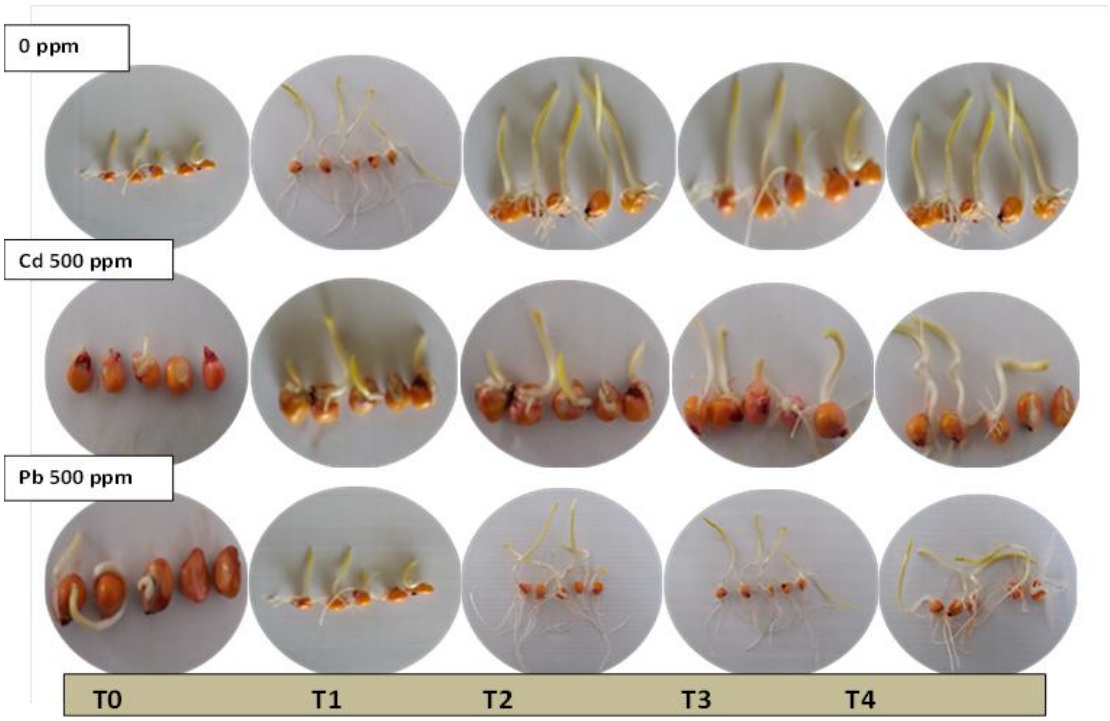

Plate 4.14 Seed germination test of *Zea mays* L. inoculated with rhizobacterial strain and developed consortium under Cd and Pb treatment.

Table 4.7 Effect of Cd stress on the Germination percent (GP) and seed vigour index (SVI) of *Zea mays* L. seedlings inoculated with strains SA, PC1, PC3 and developed consortium against control. ± SD representation of three independent replicates, while different letters within each treatment indicate statistically significant differences between treatments according to Duncan's multiple comparison range test (p<0.05).

Test	Control		SA		PC1		PC3		Consortium	
	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm
GP	83.11±0.02^{c}	25±0.03^{a}	96.16±0.09^{c}	74.3±0.04^{b}	100±0.6^{d}	76.7±0.23^{b}	100±0.5^{d}	73.2±0.04^{b}	100±0.01^{d}	91.16±0.2^{c}
SVI	1087±0.6^{b}	296±0.14^{a}	1131±0.12^{c}	567±0.4^{a}	1197±0.5^{c}	557±0.7^{a}	1153±0.2^{c}	492±0.3^{a}	1275±0.11^{c}	665±0.8^{a}

Table 4.8 Effect of Pb stress on the Germination percent (GP) and seed vigour index (SVI) of *Zea mays* L. seedlings inoculated with strains SA, PC1, PC3 and developed consortium against control. ± SD representation of three independent replicates, while different letters within each treatment indicate statistically significant differences between treatments according to Duncan's multiple comparison range test (p<0.05).

Test	Control		SA		PC1		PC3		Consortium	
	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm
GP	91.16±0.01^{d}	33.3±0.04^{a}	100±0.07	80.3±0.04^{c}	100±0.5^{d}	75.3±0.23^{b}	100±0.1^{d}	80.33±0.04^{c}	100±0.01^{d}	96.13±0.2^{d}
SVI	1180±0.8^{d}	326±0.11^{a}	1211±0.9^{d}	635±0.2^{c}	1077±0.2^{d}	587±0.5^{b}	1197±0.5^{d}	542±0.5^{b}	1155±0.11^{d}	755±0.5^{c}

Table 4.9 Effect of Cd stress on the Germination percent (GP) and seed vigour index (SVI) of *Canna indica* L. seedlings inoculated with strains SA, PC1, PC3 and developed consortium against control. ± SD representation of three independent replicates, while different letters within each treatment indicate statistically significant differences between treatments according to Duncan's multiple comparison range test (p<0.05).

Test	Control		SA		PC1		PC3		Consortium	
	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm
GP	75.13±0.05^{b}	51±0.06^{a}	100±0.11^{d}	55.67±0.4^{a}	100±0.1^{a}	66.5±0.11^{a}	100±0.2^{a}	75.13±0.4^{b}	100±0.01^{a}	86.34±0.02^{c}
SVI	908±0.5^{d}	168±0.17^{a}	931±0.07^{d}	475±0.1^{b}	1017±0.7^{e}	501±0.2^{b}	1095±0.6^{e}	521±0.1^{b}	1351±0.5^{e}	776±0.2^{c}

Table 4.10 Effect of Pb stress on the Germination percent (GP) and seed vigour index (SVI) of *Canna indica* L. seedlings inoculated with strains SA, PC1, PC3 and developed consortium against control. ± SD representation of three independent replicates, while different letters within each treatment indicate statistically significant differences between treatments according to Duncan's multiple comparison range test (p<0.05).

Test	Control		SA		PC1		PC3		Consortium	
	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm	0 ppm	500 ppm
GP	100±0.01^{d}	37±0.05^{a}	100±0.11^{d}	86.0±0.01^{c}	100±0.2^{d}	75.0±0.23^{b}	100±0.6^{d}	70.2±0.02^{b}	100±0.03^{d}	96.34±0.01^{c}
SVI	987±0.7^{c}	206±0.12^{a}	1011±0.7^{d}	533±0.2^{b}	1103±0.7^{d}	545±0.5^{b}	1109±0.7^{d}	547±0.8^{b}	1375±0.7^{d}	856±0.6^{c}

4.9. Interaction of potential strains and developed consortium with *Canna indica* and *Zea mays* L. plant under Cd and Pb stress

Pot experiments were conducted with *Canna indica* and *Zea mays* L. plants inoculated with developed consortium as well as individual stains (SA, PC1, and PC3) under semi-controlled conditions at different concentration (0, 100, 200, 300, 400, 500 ppm) of Cd and Pb contaminated soil. The physico-chemical characteristics and heavy metals content of the soil samples used for pot experiment are described in Table 4.11. Plant growth parameters were recorded at 30, 60 and 90 days after sowing (DAS).

Table 4.11 Physico-chemical characteristics of soil used during pot experiment.

Parameters	Mean value ± SD
pH	7.14±0.1
EC (dsm^{-1})	0.45±0.05
Organic Carbon (%)	1.43±0.04
Total N ($g\ Kg^{-1}$)	1.42±0.02
Total P ($g\ Kg^{-1}$)	0.87±0.001
Total K ($g\ Kg^{-1}$)	4.08±0.06
Na ($mg\ Kg^{-1}$)	3.71±0.02
Ca ($mg\ Kg^{-1}$)	2.93±0.03
S ($mg\ Kg^{-1}$)	13.37±0.27
Fe ($mg\ Kg^{-1}$)	109.45±3.1
Mn ($mg\ Kg^{-1}$)	6.97±0.07
Zn ($mg\ Kg^{-1}$)	3.02±0.05
Cu ($mg\ Kg^{-1}$)	1.79±0.12
Cd ($mg\ Kg^{-1}$)	0.06±0.001
Cr ($mg\ Kg^{-1}$)	0.006±0.001
Ni ($mg\ Kg^{-1}$)	0.012±0.005
Pb ($mg\ Kg^{-1}$)	0.69±0.02

Values are expressed as mean of three replicates ±SD (n = 3)

4.9.1 Changes in plant morphology of *Canna indica* and *Zea mays* L. under heavy metals (Cd and Pb) and rhizobacterial inoculation

The shoot length, root length, of *Zea mays* L. and *Canna indica* plant was observed at three different time intervals i.e. 30, 60, and 90 days after sowing (DAS). The results revealed that as the concentration of heavy metals (Cd and Pb) increases, physiological growth parameters of plants decrease linearly (Plate 4.15 and 4.16; Fig. 4.12 and 4.13). However, inoculation of rhizobacterial strains as well as their consortium increases the plant growth in each concentration of Pb and Cd contaminated soil. Highest shoot length and root length were observed under control treatment (without metals) as compared to cultures, but amongst culture inoculated treatments, consortium was better than individual strains. The results recorded 137.812% and 153.556%, 115.12% and 133.55%, 79.56% and 89.72%, 76.67% and 84.33%, 74.28% and 78.36% increase in shoot length of *Canna indica* plant treated with 100, 200, 300, 400 and 500 ppm of Cd and Pb respectively than their respective control at the 90 DAS (Fig. 12). On the other hand, root length increased by 78.26% and 81.33%, 72.66% and 78.55%, 63.33% and 65.77%, 59.56% and 60.33%, 59.35% and 60.22% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb concentration respectively than their respective control at the 90 DAS (Fig. 13).

Similar response were also observed in the case of *Zea may* L. plant and the shoot height increased by 112.44% and 115.31%, 99.37% and 105.01%, 78.06% and 89.66%, 71.33% and 77.47%, 70.06% and 75.49% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb, respectively than their respective control at the 90 DAS (Fig. 12).

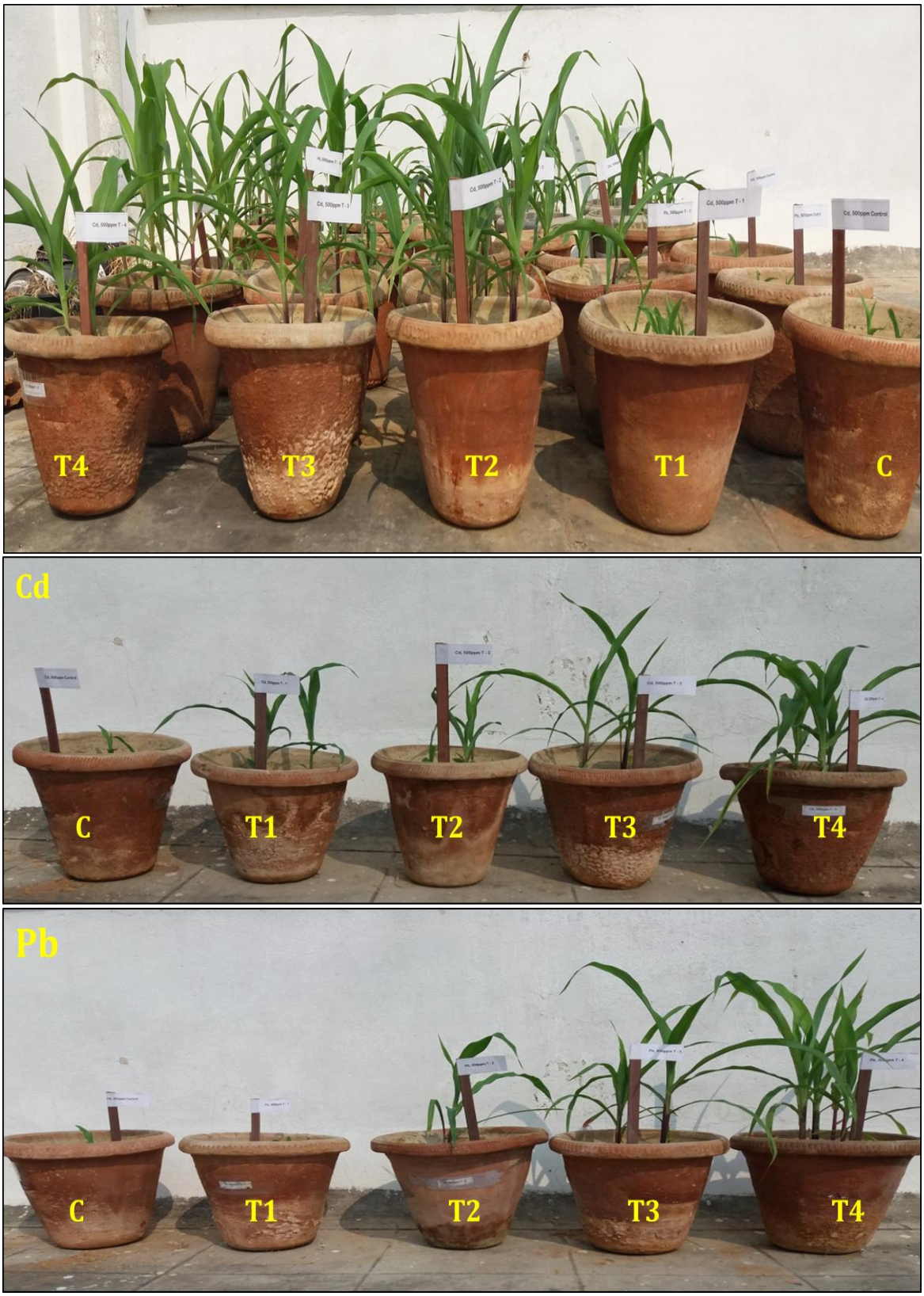

Plate 4.15 Pot experimental design for *Zea mays* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb.

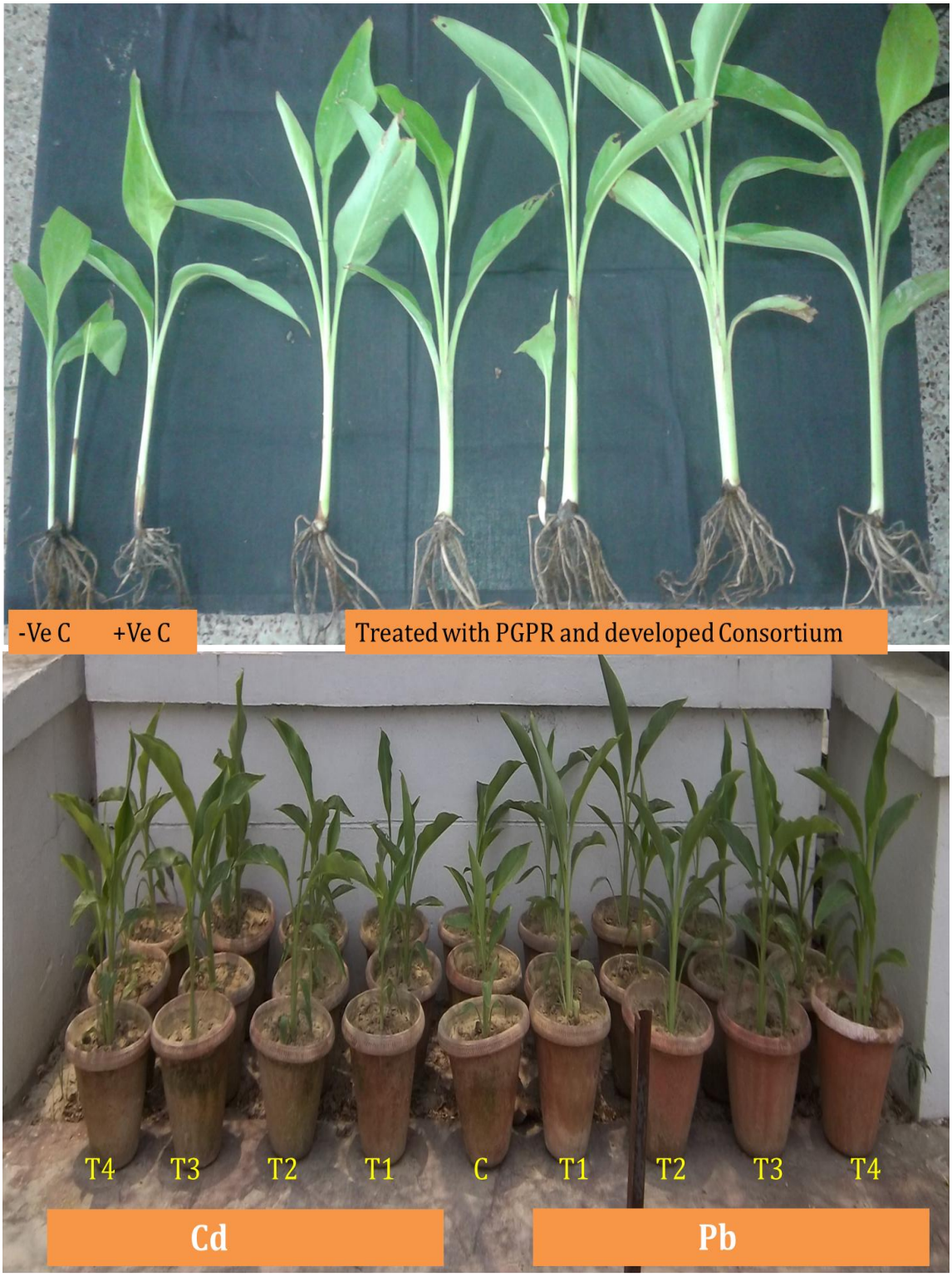

Plate 4.16 Pot experiment of *Canna indica* plant inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb.

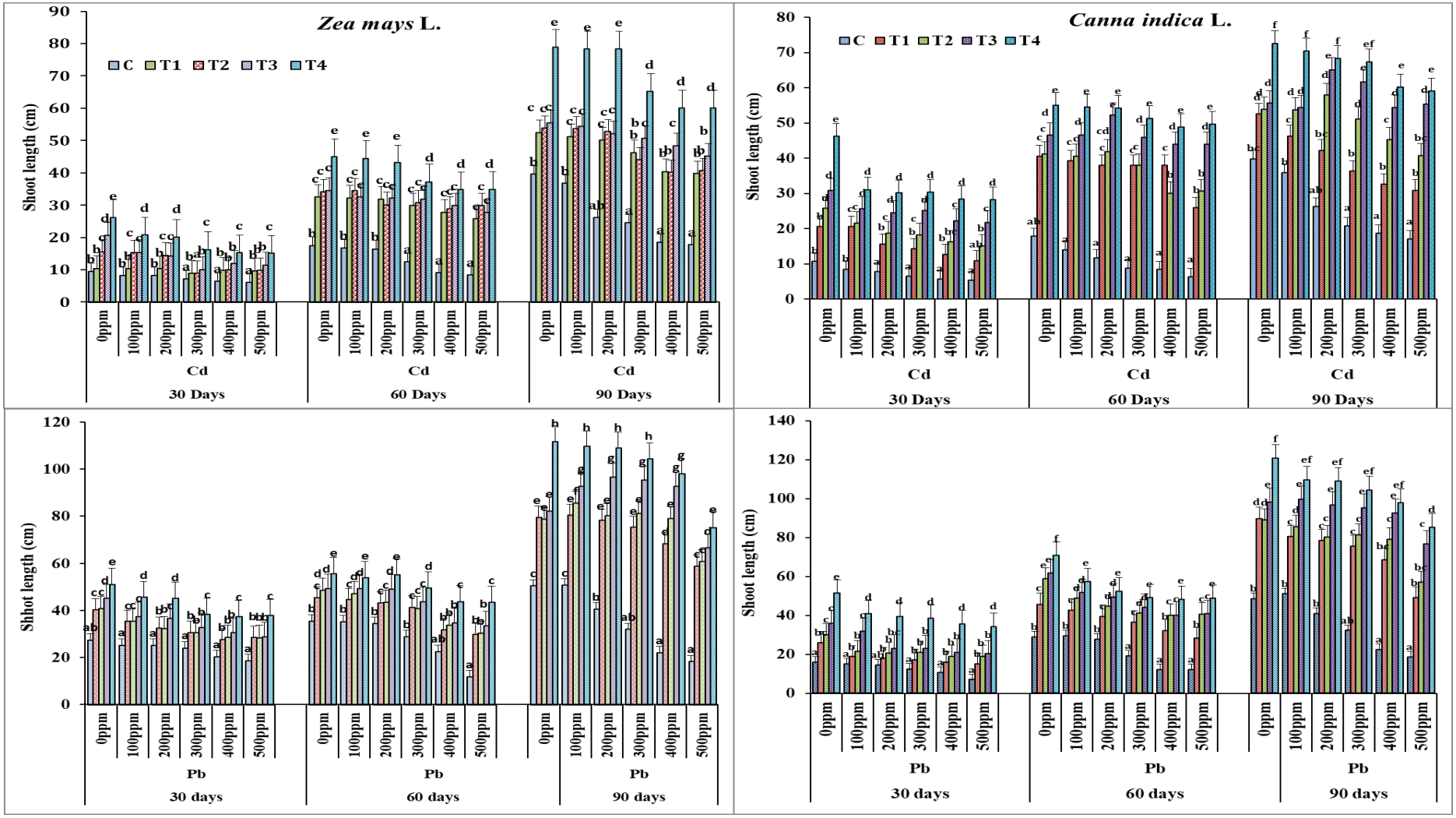

Figure 4.12 Shoot length of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

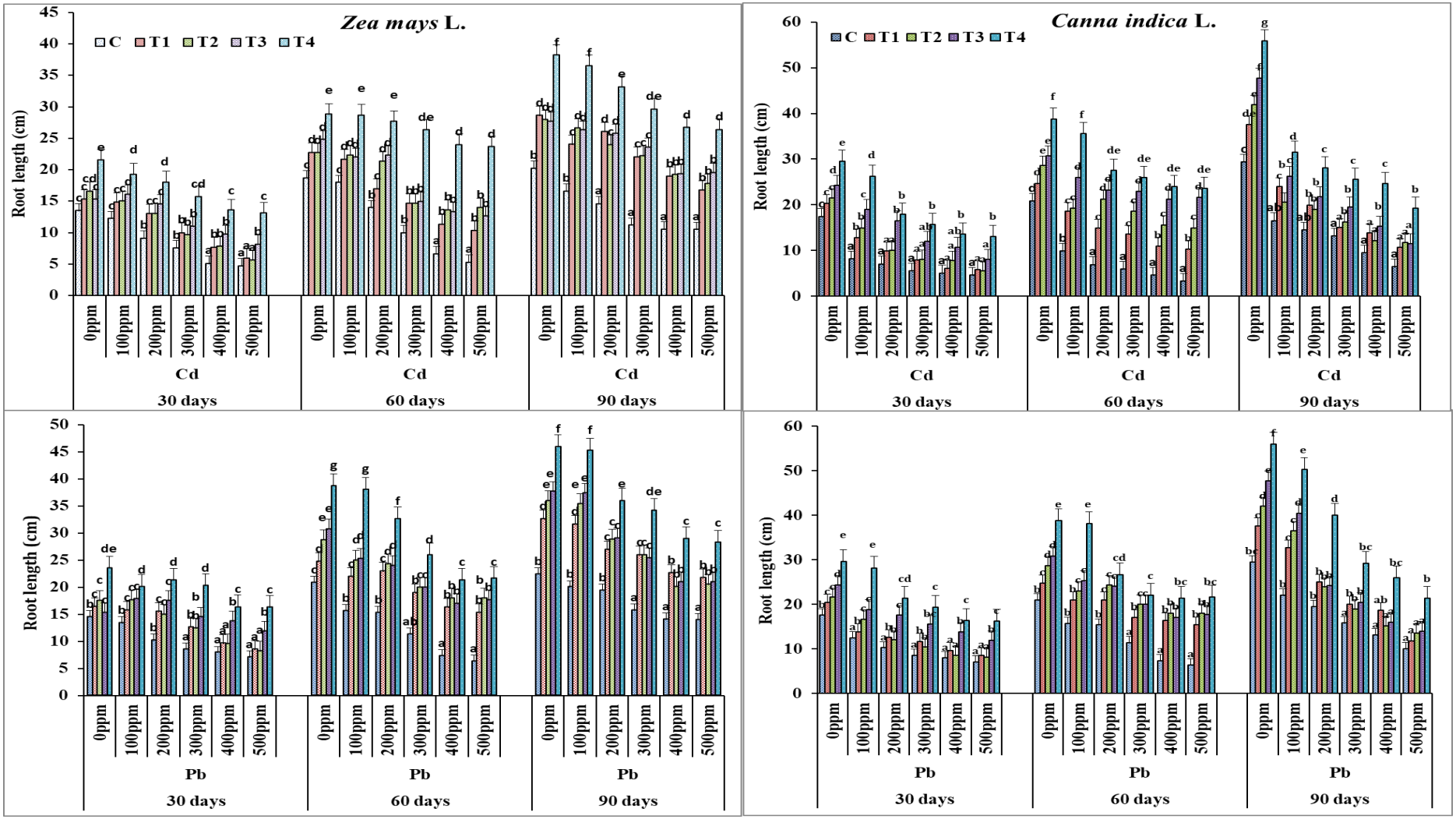

Figure 4.13 Root length of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from three independent replication.

While, root length increased by 120.08% and 126.27%, 101.33% and 110.10%, 92.66% and 103.33%, 88.76% and 102.77%, 88.05% and 102.38% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb concentration respectively than their respective control at the 90 DAS (Fig. 4.13).

4.9.2 Changes in plant biomass of *Canna indica* and *Zea mays* L. plant under heavy metals (Cd and Pb) and rhizobacterial inoculation treatment

The maximum increase of shoot dry biomass was recorded as 62.36% and 68.63%, 63.02% and 68.82%, 61.37% and 64.34%, 60.23% and 63.23%, 60.62% and 63.17% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb respectively than their respective control at the 90 DAS (Fig. 4.14). While, root dry biomass increased by 55.09% and 57.79%, 54.66% and 57.96%, 54.33% and 56.96%, 53.78% and 56.77%, 53.44% and 56.75% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb, respectively than their respective control at the 90 DAS (Fig. 4.15). The total dry biomass of *Canna indica* plant also depicted significant ($p<0.05$) increase by 65.12% and 70.06%, 63.87% and 68.27%, 63.03% and 68.06%, 61.64% and 66.56%, 61.36% and 66.44% in T4 treatment under different concentration (100, 200, 300, 400 and 500) of Cd and Pb, respectively than their respective control at the 90 DAS (Fig. 4.16).

In shoot dry biomass of *Zea may* L. there was more increase in case of consortium (T4) treated soil and recorded as 85.85% and 91.51%, 79.03% and 87.96%, 68.88% and 72.08%, 50.96% and 69.05%, 50.67% and 68.98% with 100, 200, 300, 400 and 500 ppm of Cd and Pb, respectively than their respective control at the 90 DAS (Fig. 4.14). While, root dry biomass increased by 69.06% and 61.90%, 55.03% and 55.12%, 41.37% and 48.33%, 28.66% and 32.66%, 26.56% and 30.91% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb, respectively than their

respective control at the 90 DAS (Fig. 4.15). The total dry biomass of *Zea mays* L. plant was also increased by 65.61% and 76.13%, 61.73% and 67.33%, 59.13% and 61.96%, 55.96% and 57.96%, 55.85% and 58.69% in T4 treatment under different concentration (100, 200, 300, 400 and 500) of Cd and Pb respectively than their respective control at the 90 DAS (4.16).

4.9.3. Changes in photosynthetic pigments of *Canna indica* and *Zea mays* L. plant under heavy metals (Cd and Pb) and rhizobacterial inoculation treatment

Results from this experiment revealed that different concentration of heavy metals (Cd and Pb) negatively affected the photosynthetic pigments (Chlorophyll-*a*, Chlorophyll-*b*, total chlorophyll and Carotenoid contents) of *Canna indica* and *Zea mays* L. plants. It was observed that chlorophyll (Chl-*a*, Chl-*b*, and Chl-total) and carotenoid contents decreased dramatically at higher concentration (500 ppm) of Cd and Pb. However, when rhizobacterial strains, as well as their consortium, were applied as bio-inoculum, the photosynthetic pigment increased significantly ($p<0.05$) in each concentration of Pb and Cd contaminated soil. The highest Chl-*a*, Chl-*b*, Chl-total, and carotenoid contents were observed under control treatment (without metals) while in the presence of heavy metals (Cd and Pb), T4 treatment (consortium) was showing best results (Figs. 4.17, 4.18, 4.19 and 4.20). It was observed that the Chl-*a* content of *Canna indica* plant increased by 73.53% and 77.96%, 69.86% and 69.11%, 63.35% and 68.48%, 59.87% and 65.56%, 59.88% and 66.78% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb respectively than their respective control at the 90 DAS (Fig. 4.17).

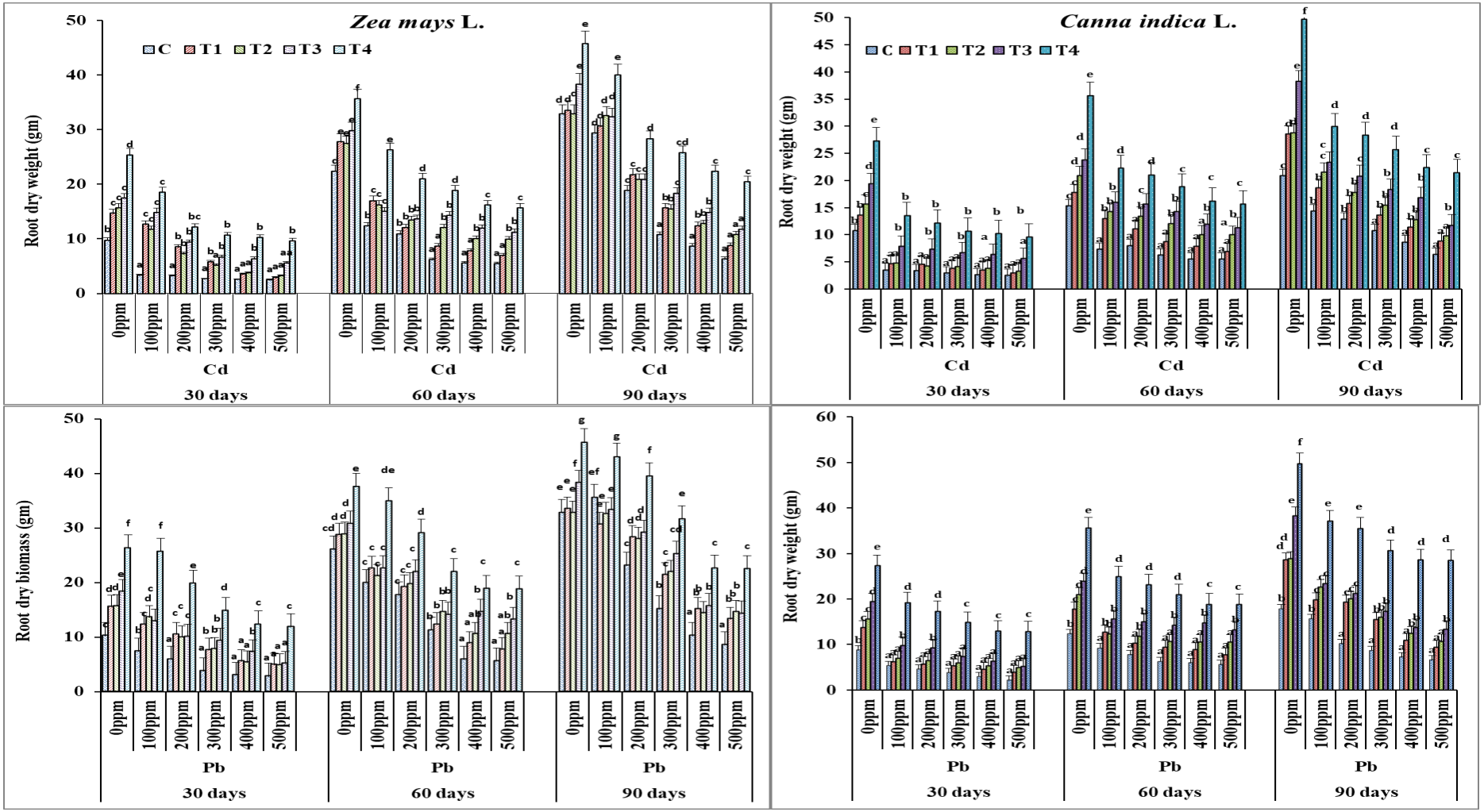

Figure 4.14 Root dry biomass of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

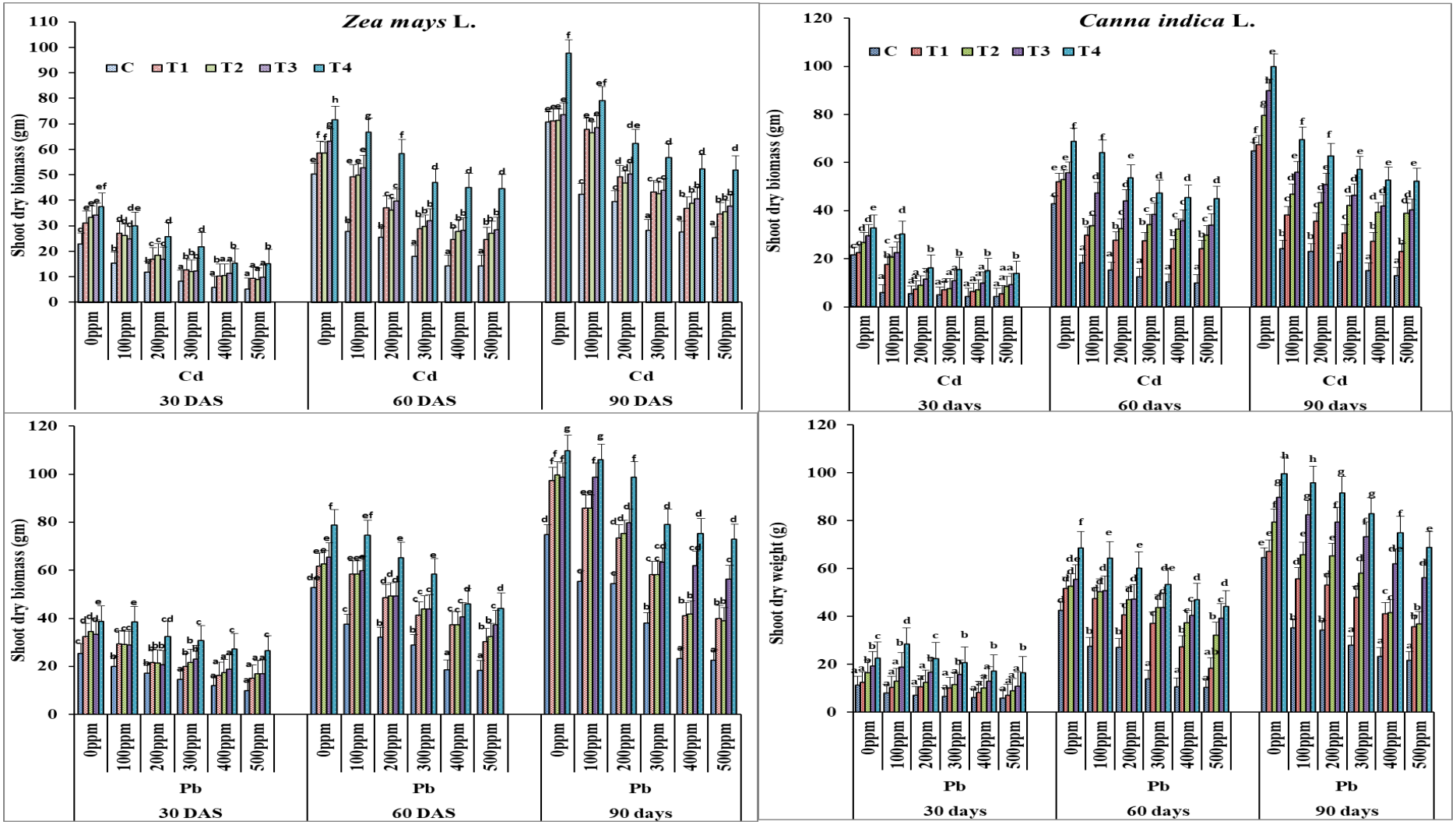

Figure 4.15 Shoot dry biomass of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

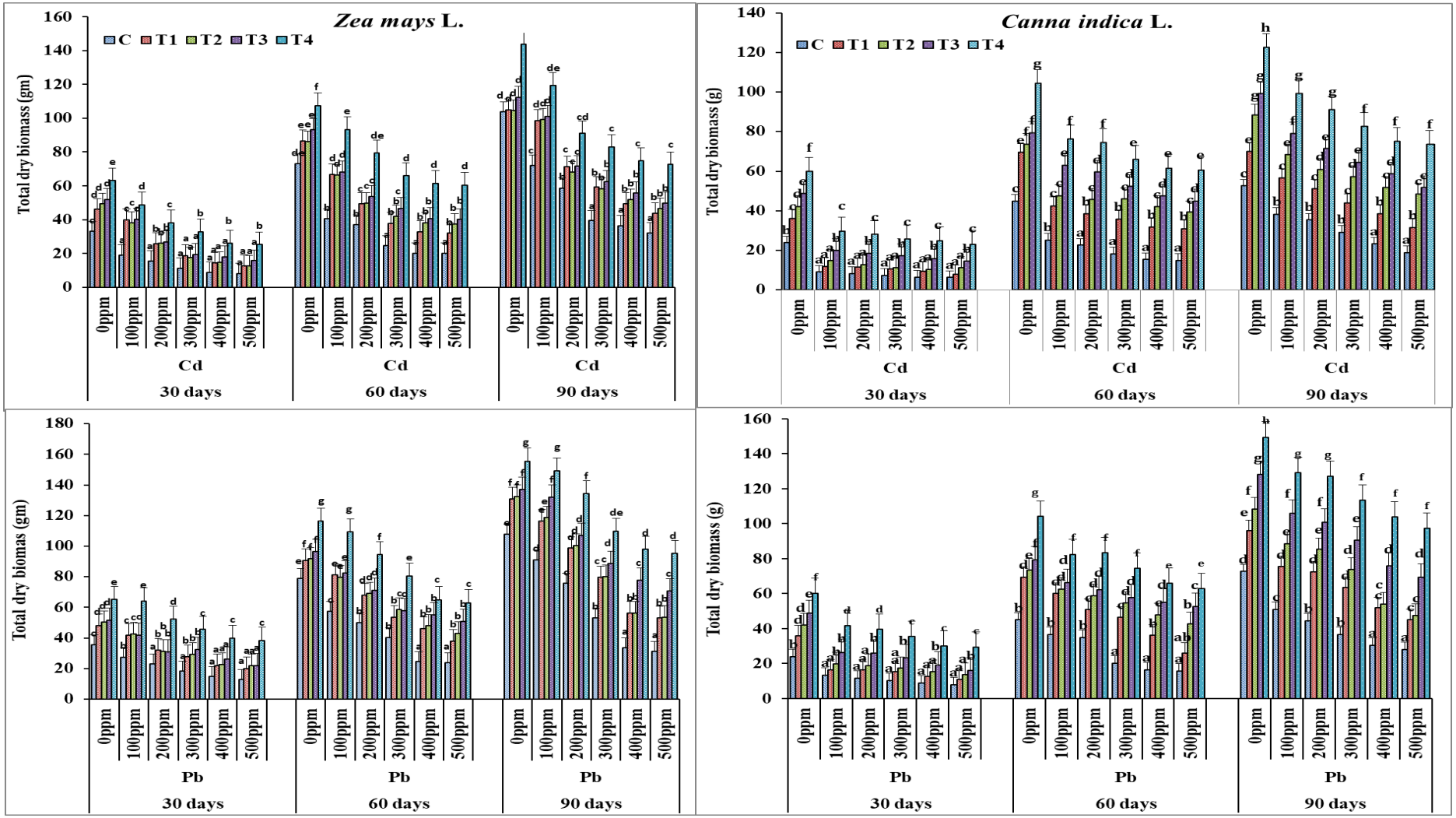

Figure 4.16 Total dry biomass of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

While, Chl-*b* increased by 99.586% and 116.55%, 78.54% and 106.84%, 77.27% and 88.03%, 56.32% and 78.70%, 56.33% and 75.01% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb, respectively than their respective control at the 90 DAS (Fig. 4.18). In case of Chl-total, the maximum increase was observed by 99.86% and 103.54%, 89.96% and 95.68%, 85.65% and 91.68%, 84.15% and 90.01%, 82.76% and 90.26% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb respectively than their respective control at 90 DAS (Fig. 4.19). The carotenoid content of *Canna indica* plant was also significantly ($p<0.05$) increased by 68.01% and 95.11%, 57.03% and 85.20%, 51.29% and 66.41%, 50.84% and 66.29%, 49.38% and 65.09% in T4 treatment under different concentration (100, 200, 300, 400 and 500) of Cd and Pb, respectively than their respective control at 90 DAS (Fig. 4.20).

Similarly in *Zea may* L. plant, Chl-*a* increased by 73.05% and 80.22%, 68.31% and 79.87%, 68.14% and 68.65%, 61.92% and 64.60%, 60.74% and 64.98% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at 90 DAS (Fig. 4.17). While, Chl-*b* increased by 92.69% and 95.55%, 87.60% and 93.50%, 80.38% and 86.57%, 78.36% and 85.79%, 74.55% and 85.92% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb concentration respectively than their respective control at 90 DAS (Fig. 4.18). In case of Chl-total, increase was recorded as 87.83% and 96.90%, 85.50% and 90.16%, 84.68% and 85.98%, 73.06% and 83.86%, 72.61% and 83.76% in T4 treatment with 100, 200, 300, 400 and 500 ppm of Cd and Pb respectively than their respective control at 90 DAS (Fig. 4.19). The carotenoid content also significantly ($p<0.05$) increased by 68.27% and 72.56%, 65.75% and 67.74%, 61.17% and 61.98%, 60.44% and 61.84%, 60.44% and 61.70% in T4 treatment under different concentration (100, 200, 300, 400 and 500) of Cd and Pb, respectively than their respective control at 90 DAS (Fig. 4.20).

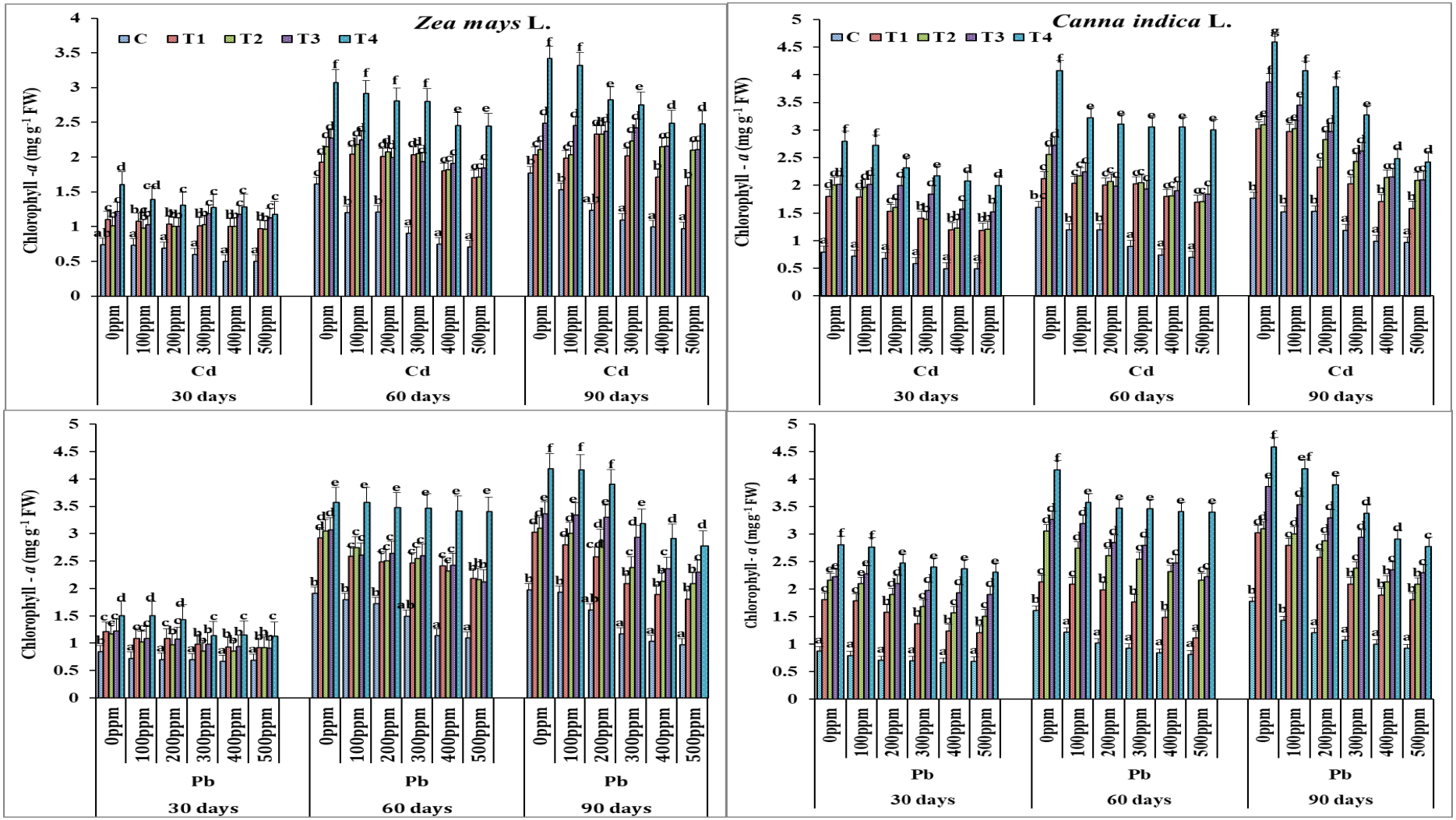

Figure 4.17 Changes in chlorophyll *a* content in leaves of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

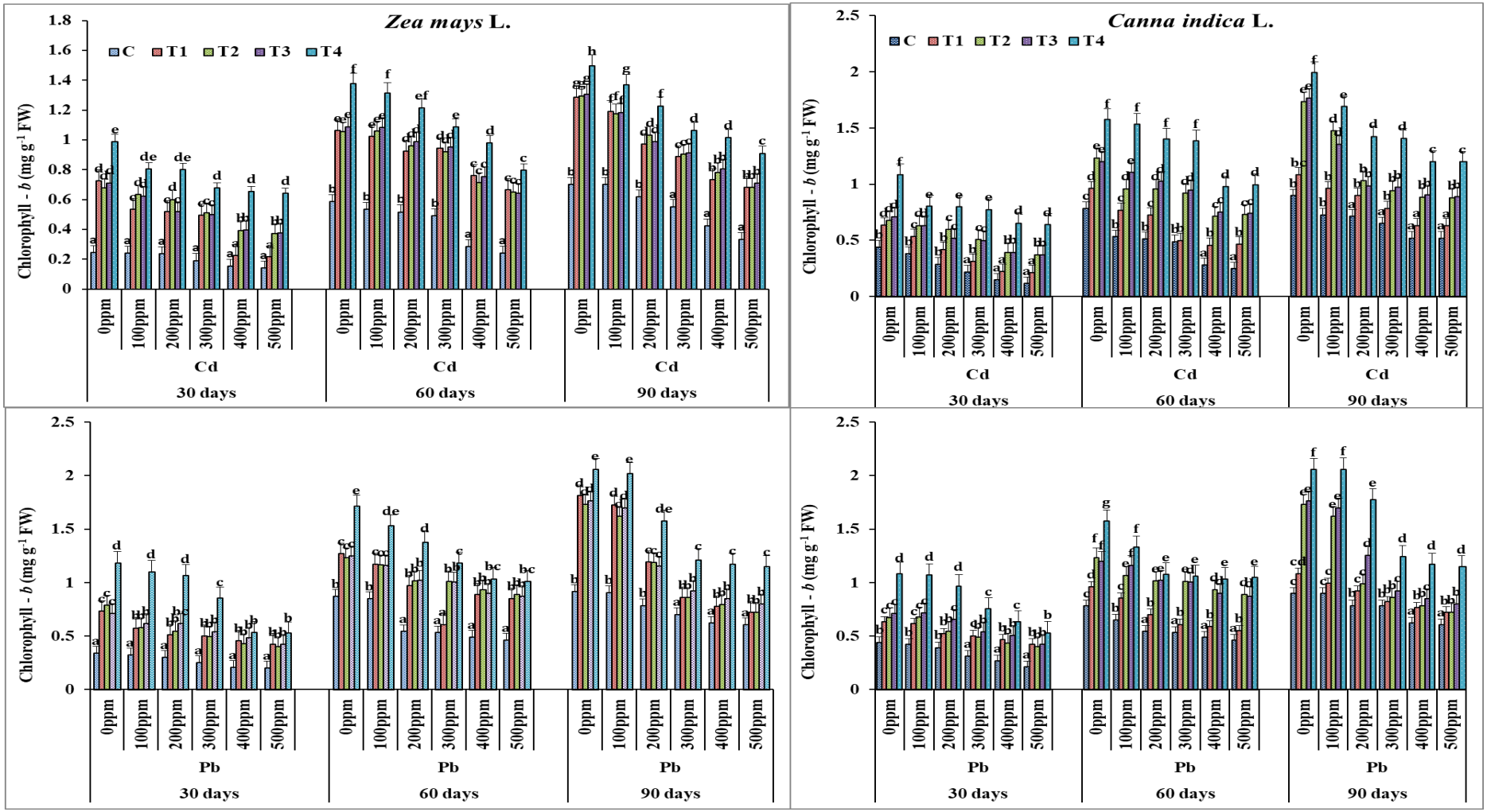

Figure 4.18 Changes in chlorophyll *b* content in leaves of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

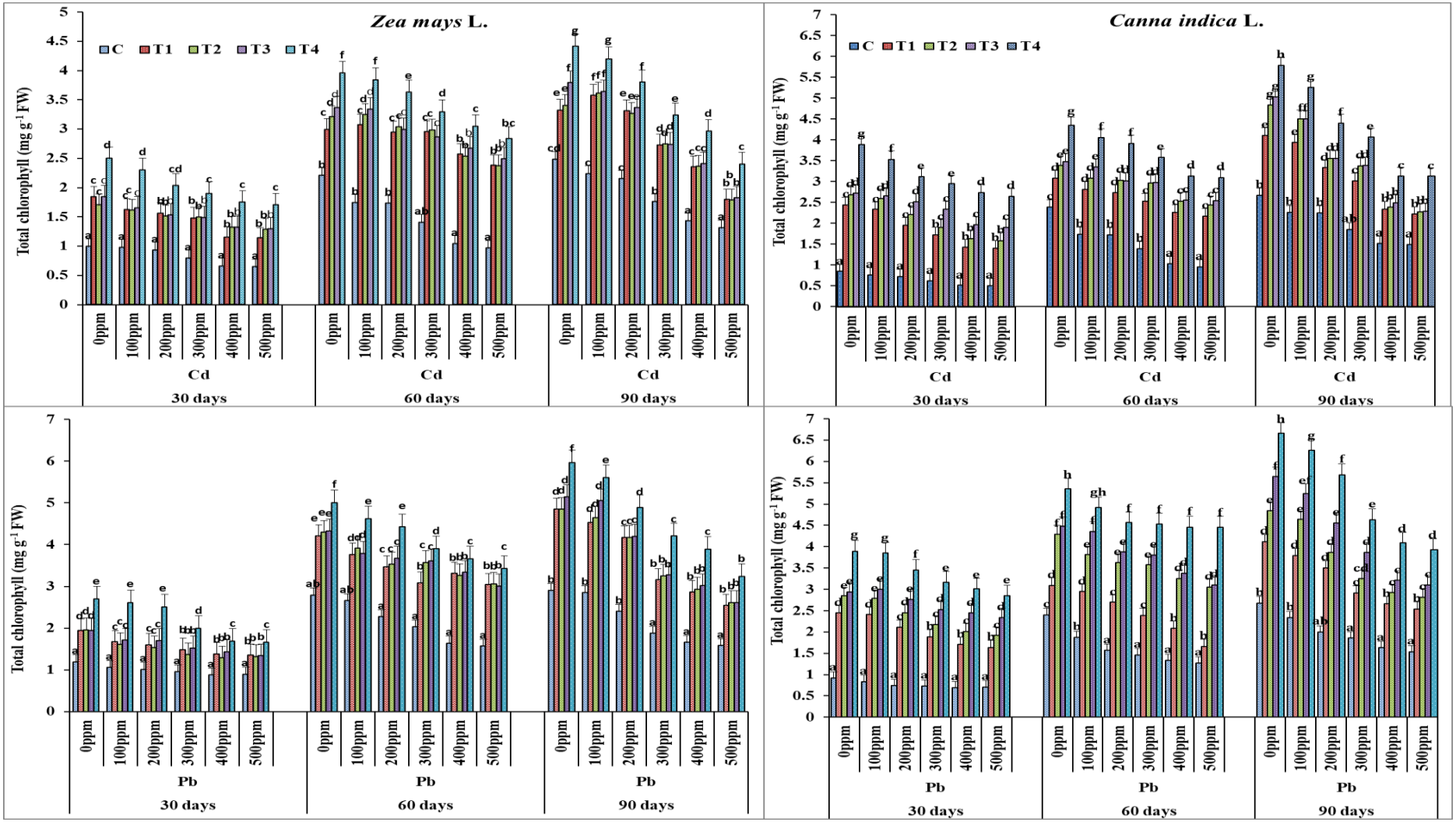

Figure 4.19 Changes in Total chlorophyll content in leaves of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

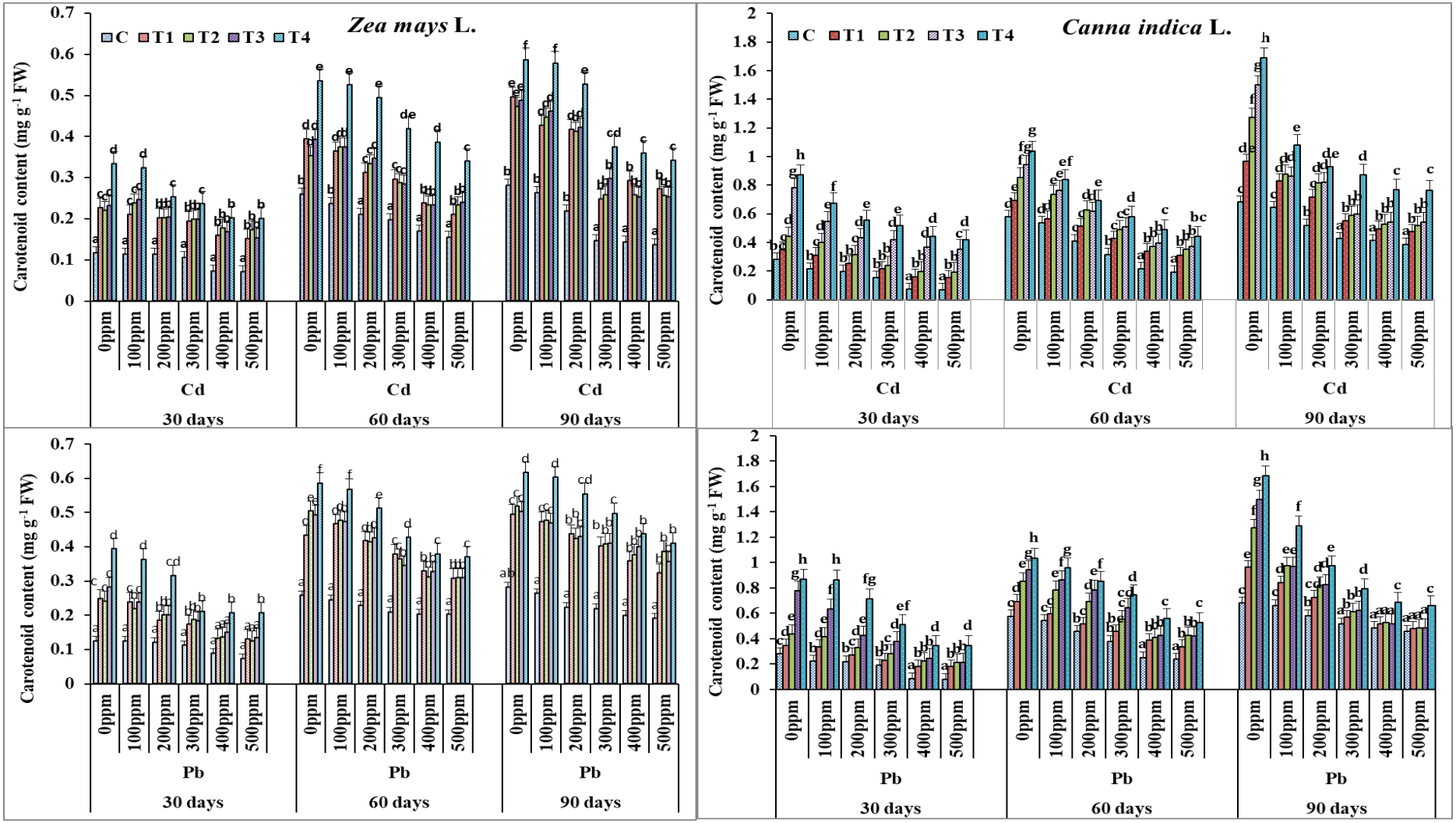

Figure 4.20 Changes in carotenoids content in leaves of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication

4.9.4 Changes in proline accumulation by *Canna indica* and *Zea mays* L. plant under heavy metals (Cd and Pb) and rhizobacterial inoculation treatment

Proline is well known for osmolytic activity which protects the cells from deleterious effects of ROS generated by cells under the influence of environmental stresses such as heavy metals, drought, and salinity. In this experiment, the foliage proline content progressively increased with increasing concentration of Cd and Pb in soil in case of both plants. The maximum proline content (24.65±0.02 and 23.18±0.01) in *Canna indica* plant was noted at 500 ppm concentration of heavy metals (Cd and Pb) under un-inoculated control at 90 DAS. However, after inoculation of rhizobacterial strains as well as their consortium, proline content decreased successively in each treatment. It was observed that the maximum reduction percent was observed in T4 treatment at 100 ppm concentration of Cd and Pb at 90 DAS (Fig. 4.21).

Likewise, in *Zea mays* L. plant the maximum proline content (27.15±0.02 and 24.33±0.01) was recorded at 500 ppm of Cd and Pb at 90 DAS. However, when rhizobacterial consortium as well as each individual strains were inoculated as a bioinoculum, the proline content reduced significantly ($p<0.05$) and maximum reduction percentage was observed in T4 treatment under 100 ppm of Cd and Pb (Fig. 4.21).

4.9.5 Uptake, accumulation and translocation of Cd and Pb by *Canna indica* and *Zea mays* L. plant treated with heavy metals and selected rhizobacteria

Uptake and accumulation of heavy metals (Cd and Pb) in shoots and roots of *Zea mays* L. and *Canna indica* L. (Figs. 4.22, 4.23 and 4.24) plants were directly correlated with the exposure time and concentration of heavy metals (Cd and Pb) in the soil. It was observed that there was a linear increase of Cd and Pb concentration (100-500 mg Kg^{-1}) with increased exposure time i.e. more the exposure time, more will be the accumulation of these heavy metals in the plant *Canna indica* and *Zea mays* L.

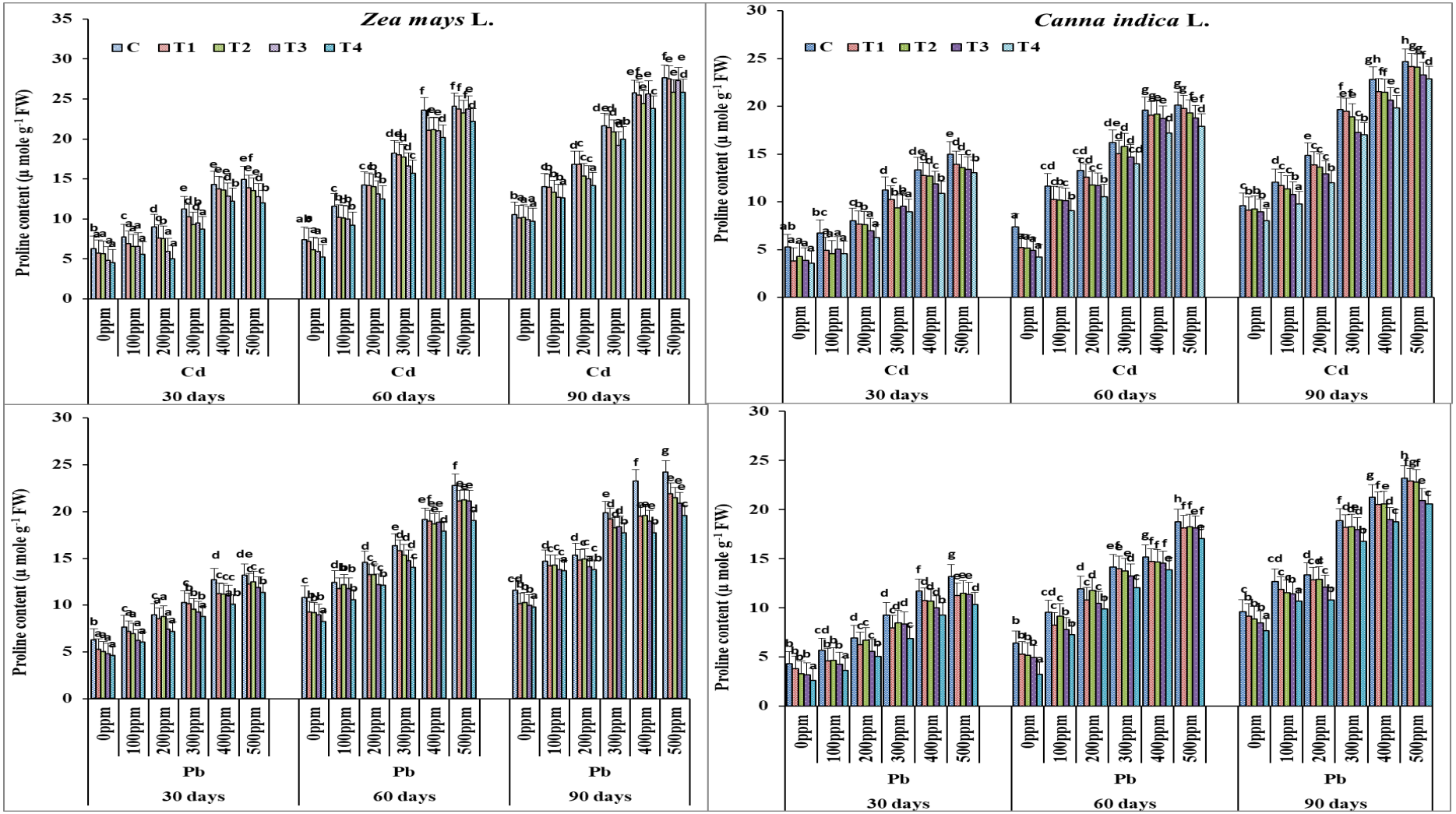

Figure 4.21 Changes in proline content in leaves of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial consortium and individual strains under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

The maximum Root Metal Content (RMC), shoot metal contents (SMC) and total metal accumulation (TMA) of *Canna indica* plant was observed at 500 ppm concentration under Cd and Pb stress at 90 DAS. Interestingly, inoculation of rhizobacterial strains as well as their consortium further increased the RMC, SMC and TMA as compared to their respective control (uninoculated plant). However, the best results were noted in consortium treatment (T4) that increased the RMC by 45.44% and 52.76%, 46.17% and 52.85%, 47.43% and 55.72%, 46.21% and 54.40%, 48.29% and 59.30% with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at 90 DAS (Fig. 4.22). On the other hand, Shoot Metal Content (SMC) in plants inoculated with rhizobacterial consortium increased by 60.08% and 62.25%, 66.60% and 65.71%, 68.17% and 65.71%, 69.24% and 65.88%, 70.40% and 65.88% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at the 90 DAS (Fig. 4.23). Moreover, the Total Metal Accumulation (TMA) of *Canna indica* plants were also highly affected by inoculation of rhizobacterial consortium and increased by 65.30% and 72.14%, 65.61% and 75.85%, 67.02% and 83.31%, 70.27% and 85.94%, 70.74% and 109.42% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at the 90 DAS (Fig. 4.24).

Similarly, when *Zea may* L. plants were inoculated with rhizobacterial strains as well as their consortium, RMC, SMC, and total metal accumulation increased linearly with exposure time and Cd and Pb concentration (Figs. 4.22 and 4.23). However, the best results were observed in consortium treatment (T4) that increased the RMC by 39.36% and 40.15%, 40.30% and 41.31%, 42.24% and 42.35%, 43.34% and 45.29%, 44.11% and 52.04% with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at 90 DAS.

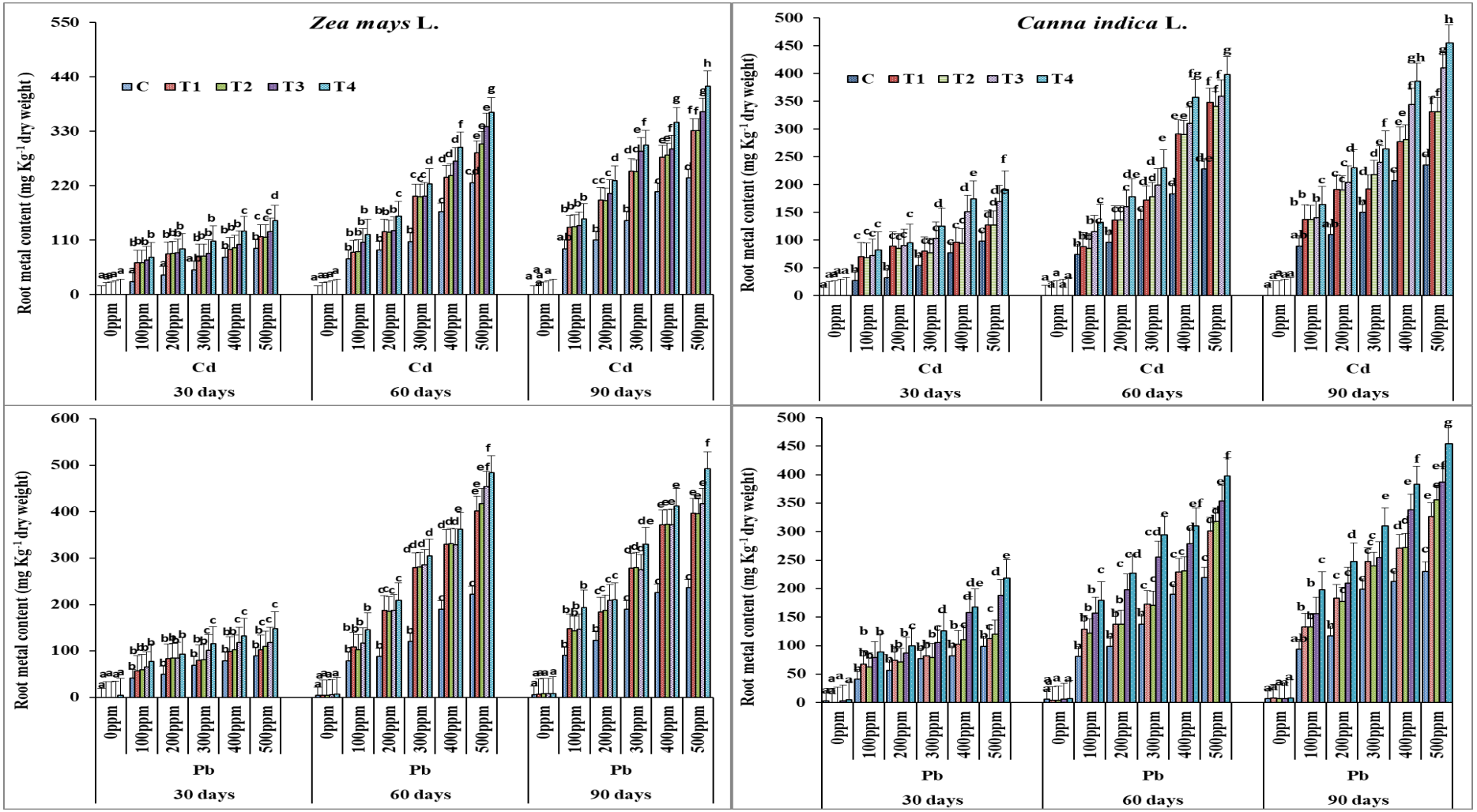

Figure 4.22 Cadmium (Cd) and Lead (Pb) concentration (mg kg^{-1} dry weight of plant) in root part of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

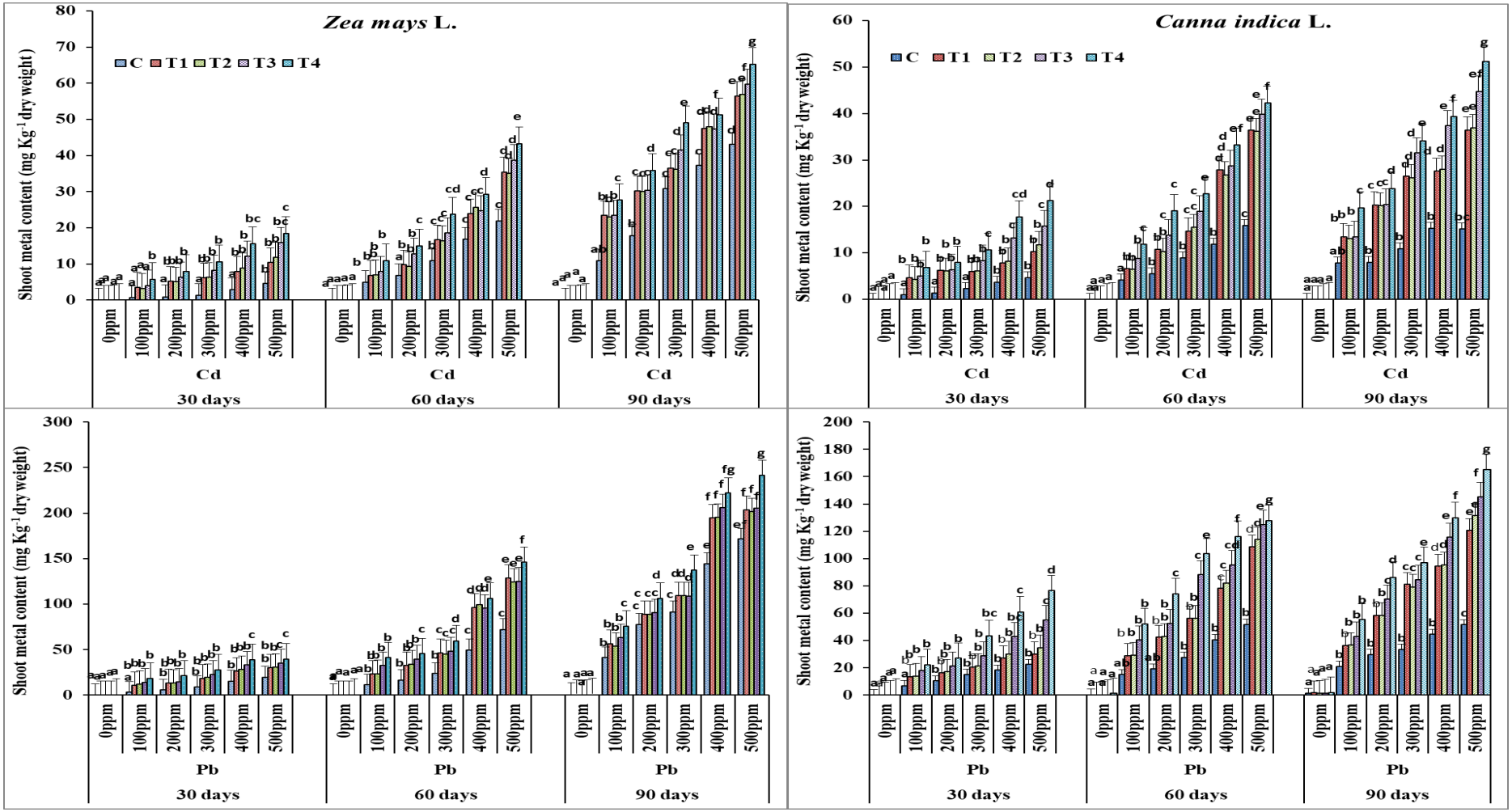

Figure 4.23 Cadmium (Cd) and Lead (Pb) concentration (mg kg^{-1} dry weight of plant) in shoot part of *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

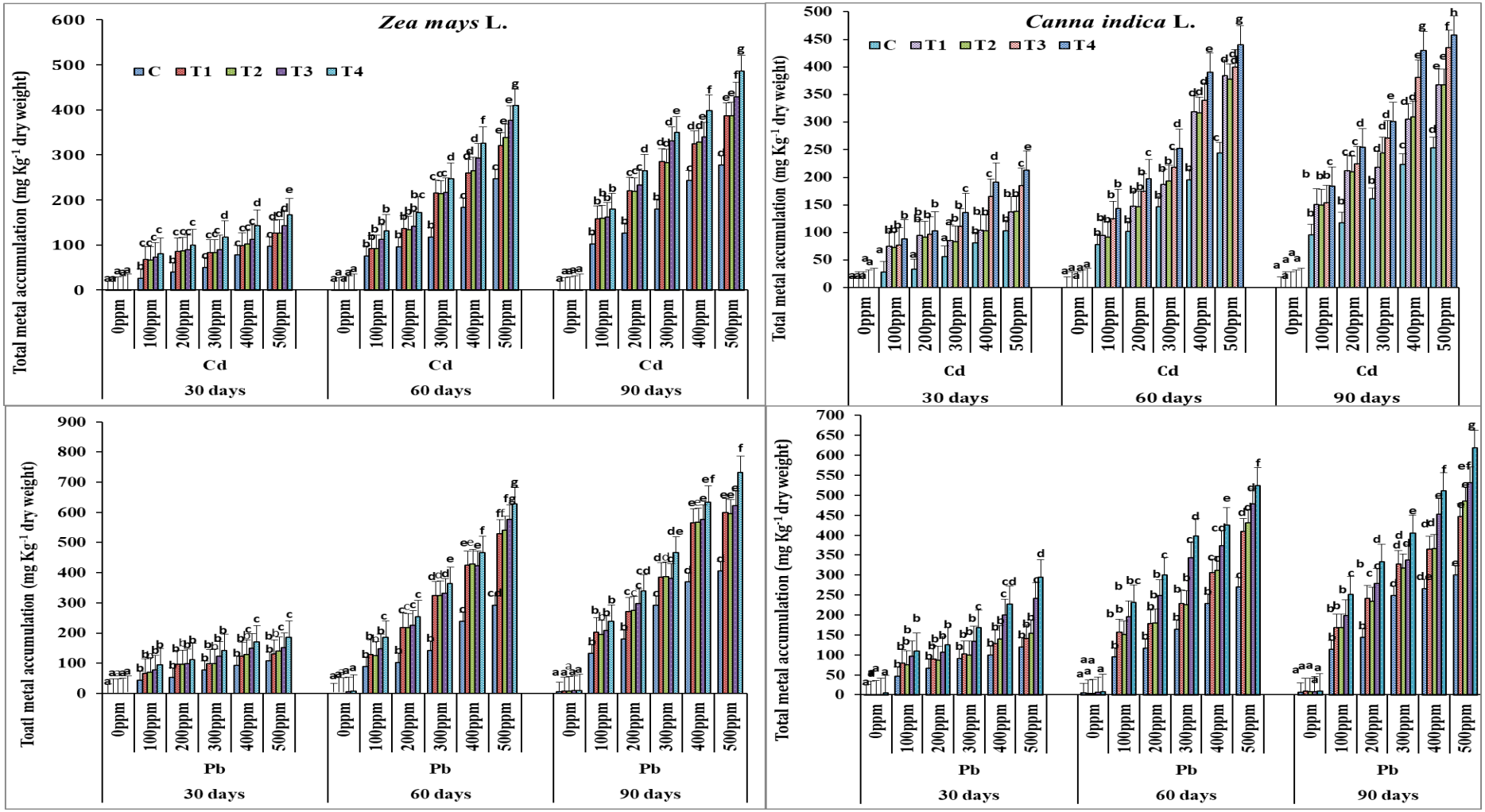

Figure 4.24 Total metals (Cd and Pb) concentration (mg kg^{-1} dry weight of plant) in *Zea mays* L. and *Canna indica* L. inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

In contrary, SMC inoculated with rhizobacterial consortium increased by 47.43% and 36.89%, 53.86% and 38.90%, 57.16% and 43.36%, 60.17% and 44.94%, 60.68% and 45.72% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb respectively than their respective control at 90 DAS. Moreover, the TMA increased by 42.64% and 44.50%, 51.96% and 46.84%, 48.46% and 37.14%, 38.68% and 41.68%, 42.74% and 44.43% in T4 treatment with 100, 200, 300, 400 and 500 ppm concentration of Cd and Pb, respectively than their respective control at 90 DAS (Fig. 4.24).

4.9.6 BCF and TF response of *Canna indica* and *Zea mays* L. plant treated with heavy metals and selected rhizobacteria

Bioconcentration factor (BCF) describes the ability of the plant to uptake metal ions from the soil. It is an important factor, required to estimate the accumulation potential of a particular plant species for remediation of heavy metal(s). Results observed from BCF test depicted that the value for *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were more than one (>1) at the concentration range from 100 ppm to 500 ppm while at 0 ppm concentration the values were below one (<1) at all exposure time (30, 60, and 90 DAS) under four different treatments (T1, T2, T3, and T4) (Fig. 4.25). The maximum BCF values for *Canna indica* plants were found to be 28.94±0.02 and 17.33±0.01 under 200 ppm Cd and 300 ppm Pb, respectively at 90 DAS in T4 treatment; while in *Zea mays* L. plant the maximum BCF values were found to be 32.08±0.04 and 16.69±0.02 at 300 ppm Cd and Pb, respectively at 90 DAS in T4 treatment. The BCF value >1 indicated that the plants have good potential for remediation of Cd and Pb contaminated soil. It was also observed that the BCF values reduced with increasing concentration of heavy metals (Cd and Pb).

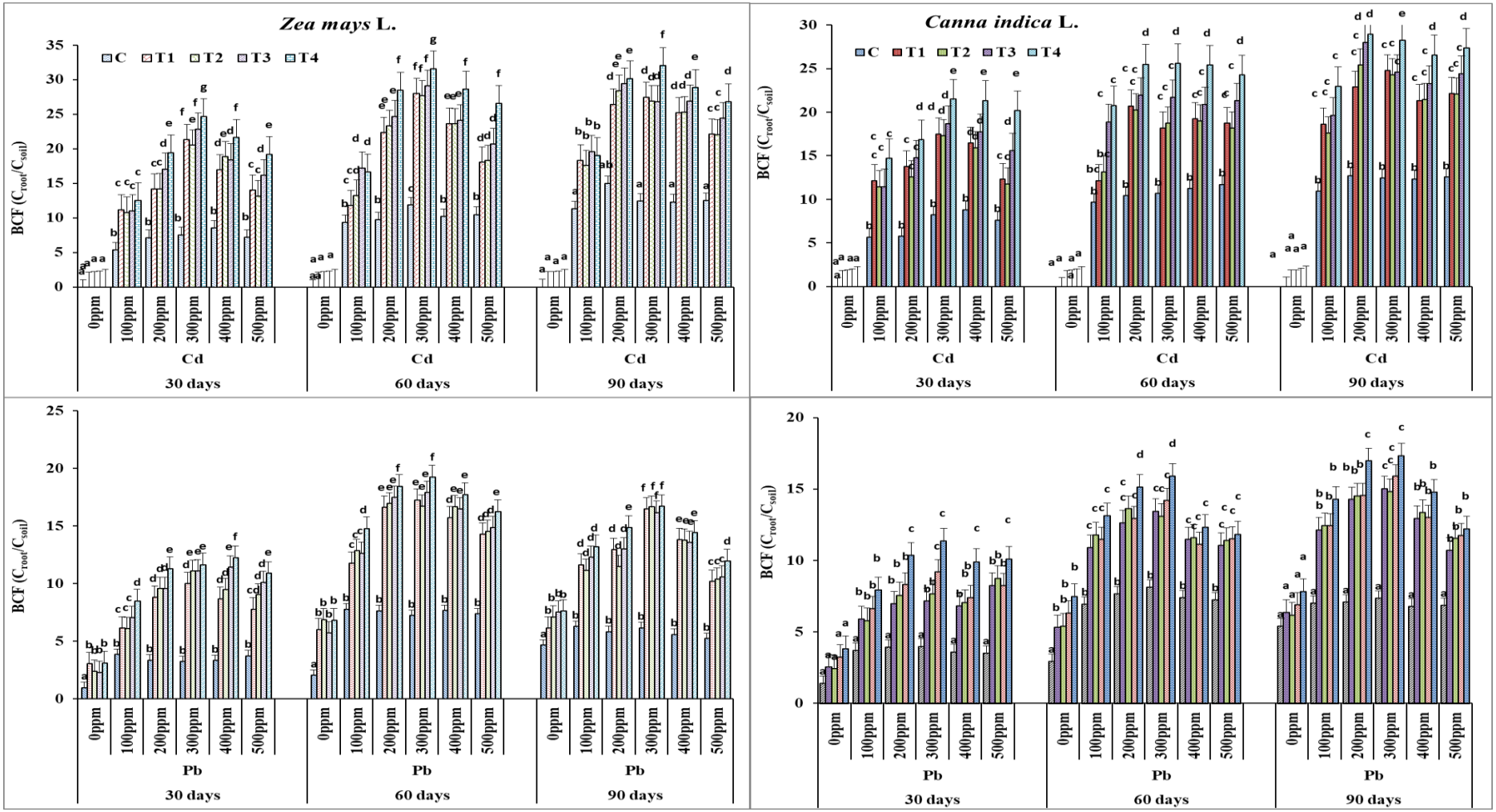

Figure 4.25 Bio-concentration factor (BCF) for Cd and Pb in *Zea mays* L. and *Canna indica* L plant tissue inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

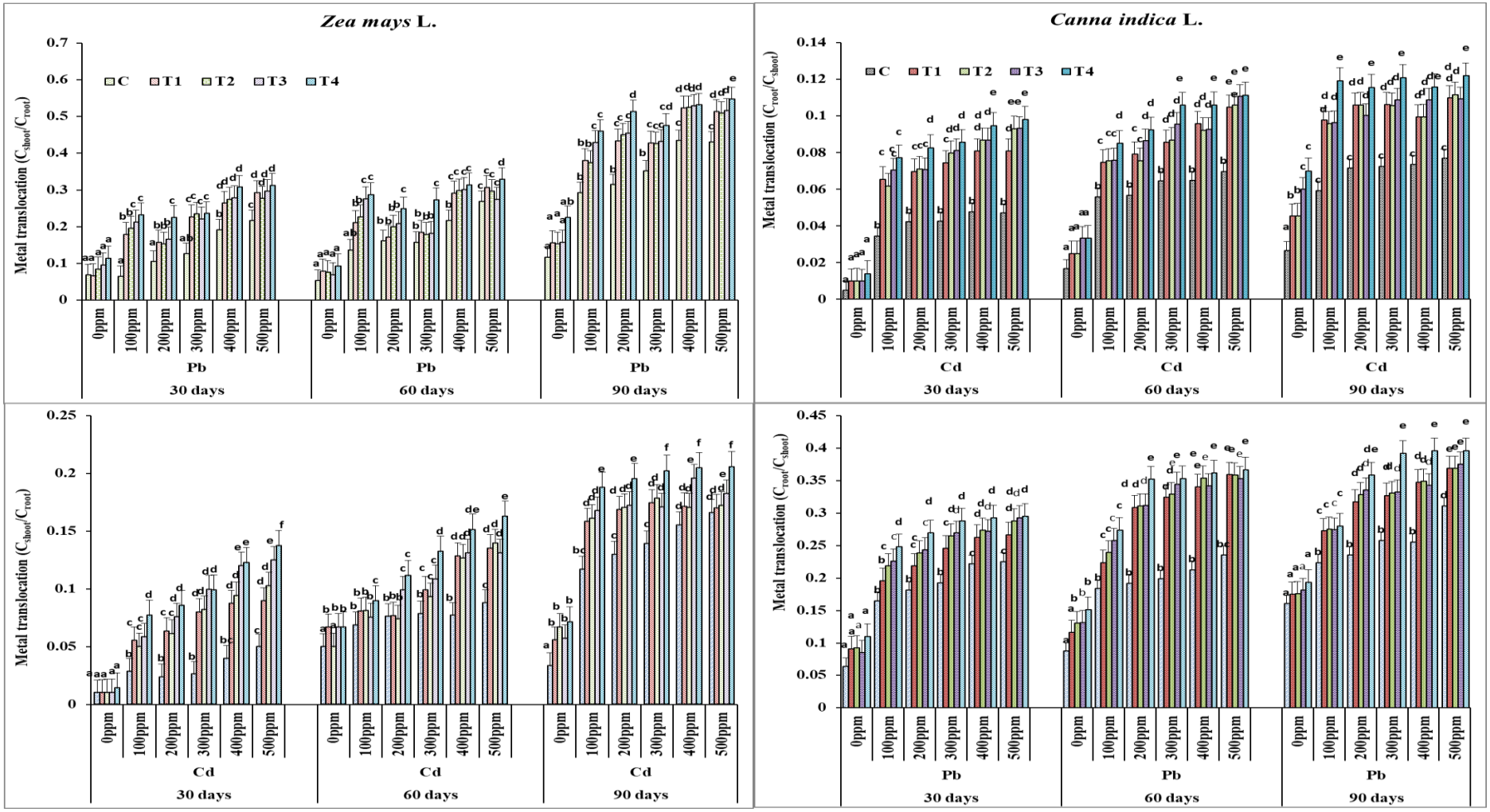

Figure 4.26 Translocation factor (TF) for Cd and Pb in *Zea mays* L. and *Canna indica* L plant tissue inoculated with rhizobacterial strains and consortium under different concentration of Cd and Pb. Bars represent ±SE from the three independent replication.

Further, the translocation factor (TF) of the plant describes the ratio of heavy metal (Cd and Pb) concentration in shoot tissue over heavy metal concentration in root tissues (Fig. 4.26). Results from this test revealed that the TF value for *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were < 1 at all concentration and exposure time (30, 60, and 90 DAS) under four different treatments (T1, T2, T3, and T4). The maximum TF for *Canna indica* plants were found to be 0.07.±0.01 and 0.03±0.05 at 500 ppm concentration of Cd and Pb at 90 DAS, respectively; while in *Zea mays* L. plant, the maximum TF values were found to be 0.16.±0.02 and 0.43±0.02 at 500 ppm concentration of Cd and Pb stress at 90 DAS respectively. Further, it was observed when rhizobacterial consortium as well as individual strains were applied, increase in TF values were recorded and the best results were found in case of T4 treatment (27.47 and 58.44%) at 90 DAS under 500 ppm concentration of Cd and Pb respectively for *Canna indica* plant. While in case of *Zea mays* L., TF values increased by 23.91 and 27.27% in T4 treatment at 90 DAS under 500 ppm concentrations of Cd and Pb respectively (Fig 4.26). Increase in TF value in the presence of rhizobacterial consortium is indicative of phyto-extraction process of heavy metal contaminated soil.

4.10 Comparative study for heavy metal (Cd and Pb) phytoextraction strategy of *Canna indica* and *Zea mays* L. plant treated with heavy metals and selected rhizobacteria

Principal component analysis (PCA) was performed for comparative analysis of two different plants, *Canna indica* and *Zea mays* L. for Cd and Pb accumulation treated with rhizobacterial consortium as well as each individual strains at different time intervals. The total variables of principal component analysis were the percentage of different parameters such as total dry biomass (TDB), root metal content (RMC), shoot metal content (SMC), translocation factor (TF), bio-concentration factor (BCF) and total metal accumulation (TMA) under different concentration of Cd and Pb

treated with bacterial consortium at different time intervals. The results of PCA yielded twelve components that explained 100% of the total variance in the data and first two components had eigen value more than 1 (which is more significant), and together they described 90.334 and 87.89 of the variance of the data from Cd and Pb plot respectively (Figs. 4.27 and 4.28). In the case of Cd, loading factor with score plot indicates that component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under high concentration of Cd concentration at 90 DAS. Component -1 explains 73.76% of the variance of the experimental data. The second principle component (PC2) seems to represent the positive association amongst TDB and SMC under control condition (0 ppm) and lower concentration of Cd concentration (upto 200) at all the sampling periods. TF of *Zea mays* was also positively loaded on PC2. TDB of both the plant species in T4 treatment under control condition (Cd 0) shows >0.5 loading value on PC2. However, other variables i.e. RMC, BCF, TMA shows negative correlation under control treatment. PC2 explains 16.574% of the variance of the results (Fig. 4.27a and b).

In the case of Pb, loading factor with score plot indicate that the component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under higher concentration of Cd concentration at 60 and 90 DAS. PC1 explains 70.43% variance of the total experimental data. The second principle component (PC2) seems to represent the positive association amongst TDB, BCF under control condition (0 ppm) and lower concentration of Pb concentration (upto 200) at all the sampling periods. TF of *Zea mays* were also positively loaded on PC2. While TDB of both the plant species in T4 treatment under control condition (Cd 0) at 60 and 90 DAS shows >0.5 loading value at component-2. However, other variables *i.e.* RMC, SMC, TMA were showing negative association with control treatment. PC2 explains 17.46 % of the variance of the results (Fig. 4.28a and b).

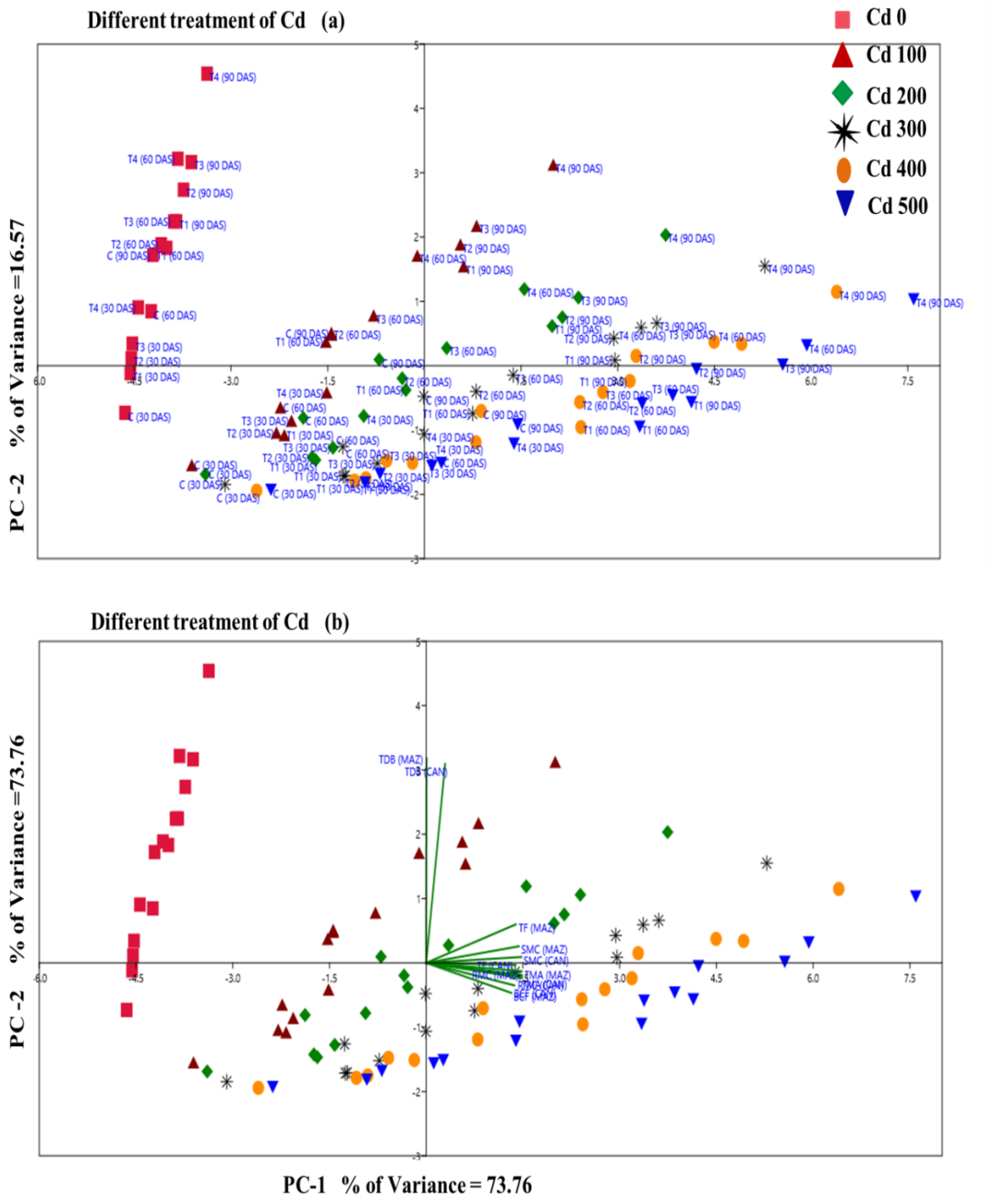

Figure 4.27. Score plot (a) and Loading plots (b) from a PCA (PC1 and PC2) of TDB, SMC, RMC, TMA, BCF and TF *Zea mays* L. and *Canna indica* L plant tissue inoculated with rhizobacterial strains and consortium under different concentration of cadmium (Cd).

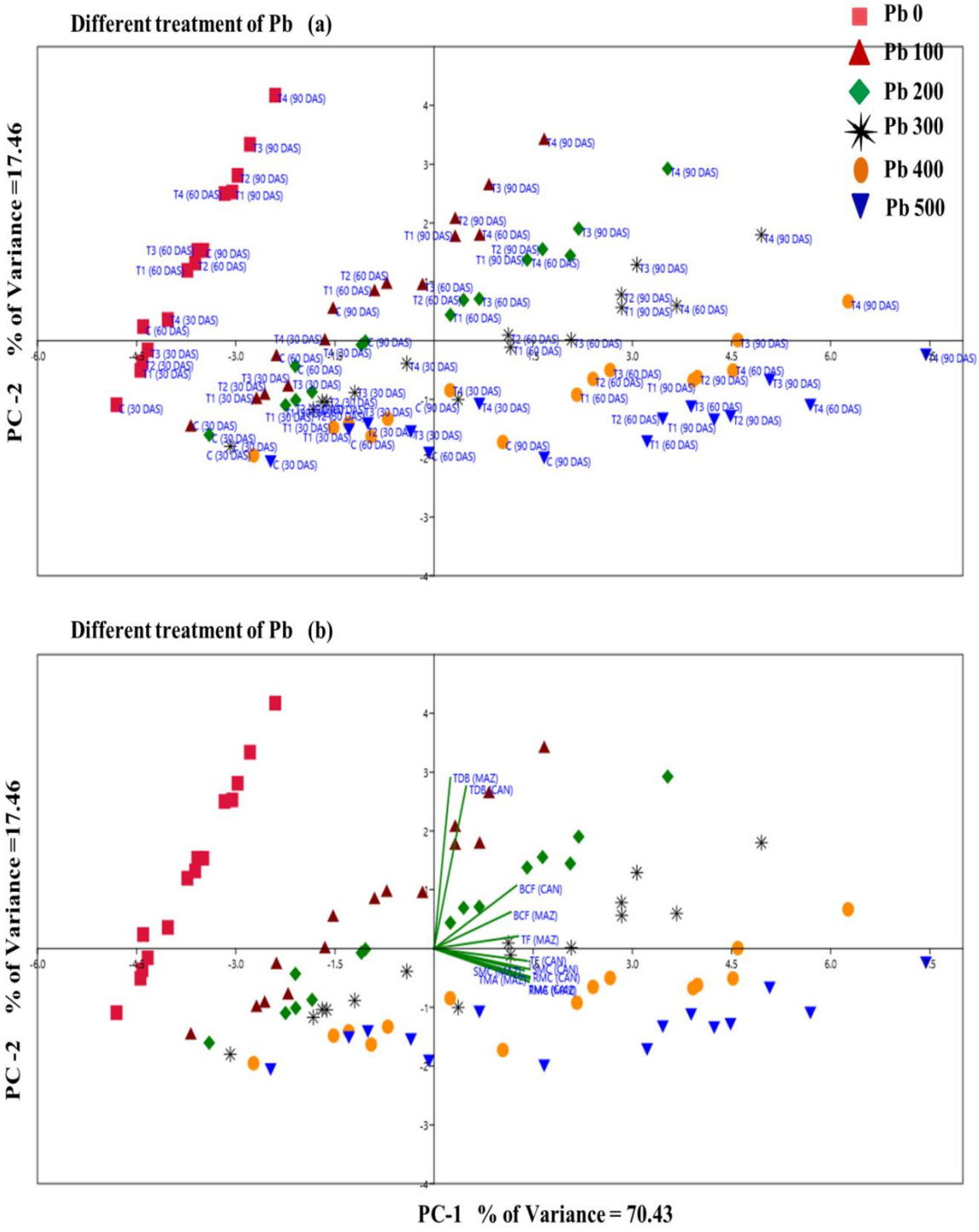

Figure 4.28. Score plot (a) and Loading plots (b) from a PCA (PC1 and PC2) of TDB, SMC, RMC, TMA, BCF and TF *Zea mays* L. and *Canna indica* L plant tissue inoculated with rhizobacterial strains and consortium under different concentration of lead (Pb).

Chapter 5

Discussion

CHAPTER-5

DISCUSSION

Soil pH and other soil properties are important in soil processes responsible for solubility of heavy metals in soil and their transportation (Mathews- Amune and Kakulus, 2013). At high pH, metals tend to form metal mineral phosphates and carbonates which are insoluble, while at low pH they tend to be found as free ionic species or as soluble organo metals and are in more bio-available form (Rensing and Maier, 2003; Hoffman, 2007; Egbenda et al., 2015). The range of pH values obtained in this study was in the acidic to normal mainly due to the contribution of acidic substances from nearby industries. Due to the acidic range of the soil sampling sites, metals present in the soil may have been in more bio-available form for the plants to cause toxicity due to solubilization and hence chances of getting heavy metal resistant microbial population will be more in these type of contaminated soils (Osakwe and Okolie, 2015; Rahamn et al., 2015).

According to Ouyang (2003), total organic carbon is a measure of organic content in soil and contributes significantly to the acidity of soil through organic acids and biological activities through the complexion of metals (Zoumis et al., 2001). High total organic carbon content entails larger adsorption surfaces and more metals are adsorbed to organic material (Nelson and Sommers, 1982; Osakwe and Okolie, 2015). In present study, organic carbon content in sampling area ranged from 0.5 to 1.97%. These level of organic carbon are far lower than those reported by Olayinka et al., (2017) and Chaudhari et al., (2013), but similar to the results reported by Osakwe and Okolie (2015) and Rahaman et al., (2015). Soils, low in organic carbon are possibly because of high temperature and good aeration in the soil which increased the rate of oxidation of organic matter (Singh and Mishra, 2012).

The electrical conductivity value of the contaminated soil samples ranged from 0.78 to 1.04 dS m^{-1}. The range of values obtained in the present study is higher than that reported by Singh and Mishra (2012) and Osakwe and Okolie (2015) but lower than the values reported by Inobeme (2014). The disparity in the electrical conductivity values could be attributed to the significant presence of metal ionisable materials or differences in the soluble salt content of the soils (Inobeme et al., 2014; Osakwe and Okolie, 2015).On the basis of limits suggested by Muhr et al., (1965) for judging salt problem of soils, most of the samples were found normal (EC < 1.0 dS m^{-1}). The normal electrical conductivity may be ascribed to leaching of salts to lower horizons (Singh and Mishra, 2012).

Total nitrogen status varied from 15 to 32 ppm with an average value of 27.714 ppm. On the basis of the ratings suggested by WHO (2002), all the soil samples were found to be low (<25-30 ppm). Low nitrogen status in the soils could be due to low amount of organic carbon in the soil. A significant positive correlation was reported between organic carbon and available nitrogen by Singh and Mishra (2012).

The highest value of phosphorus was found in soil collected from Panki Power Plant, Kanpur (PC) site and the lowest was observed in soil collected from Sarojini Nagar Industrial area, Lucknow (SA) site. On the basis of the limits suggested by Muhr et al., (1965) i.e. 8 ppm, all the soil samples were higher in soil phosphorus status. Similar results for high amount of phosphorus in soil have been reported by Osakwe and Okolie (2015). This high amount of phosphorous is attributed to the presence of organic matter which on decomposition yields more of phosphorous. According to Singh et al., (2012), about 50% of phosphorus (P) is found in organic form and decomposition of organic matter produces humus which further prevents the complexation of phosphorus with Al and Fe. Although, Phosphorus is used for crop

production as a macro nutrients but excessive amount of phosphorus in soil may cause detrimental effects in crops production (Rahman et al., 2015; Osakwe and Okolie, 2015).

Status of available potassium in the soil samples ranged between 111 and 301 ppm. Similarly, Muhr et al., (1965) also reported higher rate of potassium (50-120 ppm) range in 99% of the soil samples studied in heavy metal contaminated soils. Similar results of higher potassium in soil have also been reported by Rahman et al., (2015). It might be due to the presence of most of the mica (biotite and muscovite) in finer fractions in the collected soil (Singh and Mishra, 2012).

Contamination of soils by heavy metals is the most serious environmental problem and has significant implications for human health process (Dang et al., 2002; Obiajunwa et al., 2002). Sources such as atmospheric deposition, waste disposal, fertilizer application and wastewater in agricultural land constitute the major anthropogenic inputs (Krishna and Govil, 2007). The levels of heavy metals in the soil from present study were compared to the Indian Standard (Codex Alimentarious Commission, 1996; WHO, 2000; 2002; European Union, 2000). Concentration of Fe, Zn, Cd and Pb were found over the standard limit in which Cd and Pb were present in higher values in each sampling site.

Similar levels of Zn as obtained in this study were at par with that reported by Inobeme et al., (2014) and Olayinka et al., (2017). The concentrations of Zn in most of sampling sites (SA, PC and VA) in this study were relatively higher than level reported by Osakwe and Okolie (2015) and Rahman et al., (2015). Zn is involved in various metabolic activities of many organisms and is also one of the micronutrients essential for normal plant growth, but its increased level can cause many health disorders. Zn can interrupt the activity of microorganisms and earthworms, thus

retarding the breakdown of organic matter (Greany, 2005). The sources of Fe and Zn in present study sites are mainly due to burning of fossil fuel and anthropogenic activities such as disposal of solid waste sludge.

Cadmium concentration in the soil samples in the present study ranged from 1.03 to 5.03 ppm and the highest concentration was in soil sample collected from Sarojini Nagar Industrial area, Lucknow (SA) site. The concentration of Cd obtained at this site may be due to the dumping of Poly vinyl chloride (PVC), Ni-Cd batteries etc. Concentrations of Cd obtained in this study sites were similar to concentrations reported from soil samples from fuel filling station (Dauda and Odoh, 2012) and soil samples collected from dumpsites, abattoirs, mechanic workshops, petrol stations, hospital incinerator sites, etc. (Olayinka et al., 2017). Lead concentration greater than 1.0 ppm is generally indicated as a local source of pollution. In present study Pb levels in all the sites were in the range of 25.78 to 98.21 ppm, a range quite higher than the permissible limits. Similar range of values for lead has been reported by Olayinka et al., (2017) soil collected from petrol stations. However the lead levels observed in this study are significantly higher than those reported by Osakwe and Okolie (2015) and lower than those of other similar study reported by Tasrina et al., (2015). The concentration of lead in the soil is likely to have been derived from vehicle exhaust fumes containing some Pb rich aerosols (Zakir et al., 2014; Osakwe and Okolie, 2015).

Chromium, nickel and copper were found below the standard limits but their presence is associated with the industries like chrome plating, paint etc. present near soil sample collection sites as reported at other sites in other reports also of Al-Khashman, 2007; Iwegbue, 2013 and Olayinka et al., 2017.

Contamination of soils by heavy metal has negative impact on plant growth. Root-associated rhizobacteria have the ability to tolerate extreme environment and have the potency to grow under heavy metal stress (Pramanik et al., 2018a and b; Mitra et al., 2018). In the current scenario, application of rhizobacteria in the remediation of heavy metal is due to its ability to protect plants against heavy metal toxicity as well as enhance plant growth. One of the primary goal of this work was to determine the heavy metals (Cd and Pb) resistant rhizobacterial population in the contaminated site, to get the bacteria having the capacity to enhance plant growth (plant growth promontory) under heavy metals (Cd and Pb) stress condition. While the influence of the Cd and Pb on the microbial population is not precisely known to date, previous studies have demonstrated that heavy metals can significantly alter the microbial activity and their characteristics in soil (Tsai et al., 2015; Singh et al., 2015). In the present study, about 30 heavy meals (Cd and Pb) resistant bacterial strains were recovered from the heavy metal contaminated sites. The highest number of Cd resistant bacteria was observed in SA sample while the highest number of Pb resistant bacteria was found in JA coded sample.

Three different rhizobacterial strains i.e. SA, PC1 and PC3 have been characterized for multi-metal tolerance at higher concentration of Cd and Pb (up to 500 ppm) among the 30 isolates along with PGP characteristics. Pishchik et al., (2009) have reported that *Pantoea agglomerance* have the ability to grow in Cd contaminated environment. Moreover, both bacteria have also been reported for their ability to promote plant growth by different methods (Singh et al., 2015; Uzair et al., 2018). The present findings are in accordance with the findings of Nath et al., (2012). They reported five isolates (*Pseudomonas* sp., *Klebsella* sp., *Staphylococcus* sp., *Proteus* sp. and *Bacillus* sp.), that exhibited high resistance to Cd (1800 µg mL^{-1}) and Pb

(1200 µg mL^{-1}) out of 30 metal resistant bacterial isolates, isolated from plant rhizosphere collected from contaminated fields nearby petrol pumps, garages, industrial and garbage dumping sites of Barak Valley region of Assam, India. 13 different rhizobacterial isolates were reported for their resistance capability on Cr, Pb, Co, Ni and Fe isolated from the banks of Kestopur Cannal (Gupta et al., 2012). Our findings are also in agreement with the results reported by Ryan et al., (2005), who found that Zn, Cu and As resistant bacteria dominated Zn, Cu and As contaminated site. Several other Cd resistant PGPR under the genus *Ochrobactrum* (Pandey et al., 2010), *Stenotrophomonas*, *Serratia*, *Bacillus* (Ahmad et al., 2014), *Bradyrhizobium* (Guo and Chi, 2014), *Klebsiella* (Pramanik et al., 2017) and *Enterobacter* sp. (Pramanik et al., 2018 a; Mitra et al., 2018) were previously isolated from Cd-contaminated soil. Similarly, Abdelkrim et al., (2018) reported 12 different Pb resistant PGPR belonging *to Rhizobium leguminosarum, Streptococcus meliloti, Pseudomonas sp., Pseudomonas fluorescens, Luteibacter sp., Variovorax sp., Bacillus simplex, and Bacillus megaterium,* isolated from multi metal contaminated site.

On the basis of Morphological, biochemical and 16S rDNA analysis, heavy metal (Cd and Pb) resistant, selected isolates were identified as *Pseudomonas aeruginosa*, *Pantoea agglomerance* and *Enterobacter cloacea* coded as SA, PC1 and PC3, respectively. Saif and Khan, (2017) reported the successful isolation of heavy metal (Pb, Ni, Cr, Cd and Zn) resistant PGPR's and identified them as *Pseudomonas* spp., *Bacillus* spp. and *Azotobacter* spp. Similarly Pramanik et al., (2018a) also reported the isolation and characterization of heavy metal resistant PGPR and identified them as *Enterobacter* species. In other study, reported by Pishchik et al., (2009) and Paredes Páliz et al., (2018), *Pantoea agglomerans* has been isolated and characterized as heavy metal resistant PGPR.

To characterize any rhizobacteria as plant growth promotory, it should possess attributes like production of diverse metabolites including ACC deaminase, siderophores, indole acetic acid (IAA), ammonia, HCN, and activities such as phosphate solubilisation (El-Deeb et al., 2012; Goswami et al., 2014). In the present study, selected heavy metal resistant rhizobacteria were showing multiple plant growth promoting traits.

It is now widely accepted that PGPR having ACC deaminase activity under heavy metal stress is known to cleave ACC that lowers the stress ethylene level; thereby resulting in the enhancement of plant's tolerance level to grow under heavy metal stress (Sharma and Archana, 2016). The selected isolates (SA, PC1 and PC3) were found positive for ACC deaminase and it was also recorded that strain *Enterobacter cloacea* (PC3) showed higher production of ACC deaminase amongst all three strains. In addition to this, Cd induced the level of ACC deaminase which is essential to reduce the stress caused by increased ethylene level. Similar findings for *Enterobacter* sp. have been reported by Mitra et al., (2018). Similarly, Carlos et al., (2016) reported, ACC-utilizing, *Pseudomonas brassicacearum* and *Pseudomoas marginalis* that protect pea plants from growth inhibition due to elevated Cd concentrations in soil and produce higher ACC deaminase activity in the presence of Pb and Cd than control. The selected isolates also corroborated the similar findings in the presence of Pb and Cd metal ions.

Siderophore producing rhizobacteria play an important role in successful survival and growth of plants in metal contaminated soil by alleviating the metal toxicity and supplying the plant with nutrients, particularly Fe (Etesami, 2018). In the present study, *Pseudomonas aeuroginosa* (SA) and *Enterobacter cloacea* (PC3) exhibited the ability to produce maximum quantity of siderophore either in presence or absence of

lead while in case of cadmium stress, siderophore production was reduced. In case of PC3 strain, there was production of siderophore in the presence of cadmium and lead but it was less as compared to control. These results are not in conformity as reported earlier. Earlier reports show a stimulatory effect of heavy metals on siderophore biosynthesis in various rhizobacterial strains. For instance, Hesse et al., (2018) observed an increased production of siderophore in wild type of *Pseudomonas aeuroginosa* in the presence of copper. They have also observed that siderophore production was greater in contaminated soil in comparison to uncontaminated soil. The study reflected that the siderophore production was higher in the presence of Pb in comparison to control, same trend was found in present study in case of strain *Pseudomonas aeuroginosa* (SA). It may be attributed to the fact that some metal ions have been shown to stimulate siderophore production *viz.* Zn, Cu and Mn resulting in enhanced siderophore production (Dimkpa et al., 2008). Result from present study of sidrophore production by *Enterobacter cloaceae* (PC3) was in accordance with the findings of Das et al., (2017) in the presence of Pb heavy metal. Maleki et al., (2018) also reported siderophore production by a novel strain of *Enterobacter cloacea* species. The increased production of siderophore by *Streptomyces* in the presence of metals can be explained by the fact that metal ions compete for siderophore binding with the trace amounts of iron present, necessitating increased siderophore production to obtain equivalent levels of iron to circumvent, or at least alleviate metal-induced Fe deficiency (Dimkpa et al., 2008). It might explain the ability of survivability and heavy metal phytoextraction of *Pseudomonas aeruginosa* and *Enterobacter cloacea* strains under heavy metal stress condition through siderophore production. Reduction in the present study can be attributed to the reason that 500 ppm concentration may have been not very supportive to induce siderophore production (Shi et al., 2017).

Indole acetic acid (IAA) is well known to be involved in cell division, cell enlargement, tissue differentiation, lateral as well as adventitious root initiation, and resistant to stressful condition. The addition of IAA to soil can enhance the uptake of metal in plant roots (Yu et al., 2014). In the present study, heavy metal tolerant selected isolates, *Pseudomonas aeuroginosa* (SA), *Pantoea agglomerance* (PC1) and *Enterobacter cloacea* (PC3) were found to produce a copious amount of IAA in both conditions *i.e.* in absence and presence of Cd and Pb. However, application of Tryptophan triggers the production of IAA (up to 40 µg mL^{-1} under 500 µg mL^{-1} of tryptophan) even in the presence of heavy metals (Cd and Pb) stress condition. The result was in accordance with the findings of Singh et al., (2015). Similar findings have been reported by Karthik et al., (2017) in presence of Cr heavy metal. IAA is derived mainly from tryptophan through multiple enzymatic pathways by many different genera of PGPR (Husen et al., 2016; Govindasamy et al., 2017). According to Yu et al., (2014), the abundance of isolates producing more than 20 µg mL^{-1} of IAA suggested that the plant growth promoting ability of the isolates might assist in the phytoremediation of contaminated soils.

Phosphate solubilization is one of the promising traits for plant growth promotion and heavy metal remediation as microorganisms solubilize inorganic phosphate into organic form making it available for plants. The selected strains SA, PC1 and PC3 exhibited phosphate solubilizing activity in NBRIP medium containing Cd and Pb. Similar results were reported by Biswas et al., (2018) for three Cu and Zn resistant bacterial strains *Bacillus megaterium* (MF 589715), *Staphylococcus haemolyticus* (MF 589716) and *Bacillus licheniformis* (MF 589720) exhibiting phosphate solubilisation activity. The oxidation-reduction reaction with BPB (bromophenol blue) dye might be due to drop of pH caused by the excretion of organic acids, which

might be responsible for the phosphate solubilization (Ma et al., 2016). Oves et al., (2017) optimized phosphate solubilization with *Ensifer adhaerens* OS3 in presence of Cd, Cr, Cu, Ni and Zn, but higher solubilization was observed in control (without heavy metals).

HCN plays an important role in disease suppression (Wei et al., 1991); in this study, all the strains were recorded to be positive for HCN production in control and in the presence of Cd and Pb metal, while presence of Cd heavy metal inhibits the production of HCN by *Pantoea agglomerance* (PC1). Singh et al., (2015) reported *Pseudomonas putida* as HCN producer while *Bacillus* sp. observed as negative under heavy metal stress condition. Similar results have also been reported by Saif and Khan, (2017) for HCN production (*Pseudomonas, Bacillus, Rhizobium* and *Azotobacter* species). In contrary, Verma et al., (2015) reported the negative response of rhizobacterial isolates for HCN production in the presence of Cd heavy metals.

Ammonia production is another important PGP trait, where an organism can break down complex nitrogenous materials like peptones to release ammonia in soil which is taken up by plant as a nutrient source (Yao et al., 2011). Accumulation of ammonia can occur in nitrogen rich soil, may increase the pH creating an alkaline condition (at pH 9-9.5) responsible for suppressing the growth of certain fungi, nitrobacteria due to it potent inhibition effect besides inhibiting germination of spores of many fungi (Jha et al., 2012; Saif and Khan, 2017). In the present study, all the three strains were ammonia producers. The result was in accordance with the findings of Singh et al., (2015) on *Pseudomonas putida* and *Bacillus* species. The result was in parity with the studies done by Passari et al., (2015) on endophytic actinomycetes.

Present study also observed the exopolysaccharide (EPS) production capability of heavy metals resistant selected PGPR strains. Result showed that all the stains were

positive to secrete EPS in presence and absence of heavy metals (Cd and Pb). Presence of heavy metal triggers the EPS production due to the induced defence mechanism by bacterial strains under metal stress condition. The results were in accordance with the findings of Subudhi et al., (2016) Kaplan et al., (1987), who reported the EPS production strategy of heavy metal resistant *Achromobacter xylosoxidans*. Similarly, Castellane et al., (2015) reported the screening of EPS production by rhizobacterial species having the characteristics of plant growth promotion. Recently Karthik et al., (2017) reported the EPS production and PGP characterization of heavy metals (Cr) resistant rhizobacterial strain. Although some reports are available for the EPS production by *Pantoea agglomerans;* interestingly this is the first study reported for EPS production by *Enterobacter cloacea* species in the presence of heavy metals (Cd and Pb).

The composition of the EPS matrix secreted by rhizobacteria is reported to be very complex, containing proteins, carbohydrates, nucleic acids, lipids, amphiphilic molecules and humic substances (Flemming et al., 2007). In the present study, EPS was characterized through FTIR and presence of amide group, glucose and protein were analysed. The result was in accordance with the findings of Kaplan et al., (1987). They reported that the sulphate ester group plays a minor role in metal chelation as compared to uronic acid. In addition, the presence of acidic sugars in the EPS may be important, considering the heavy metal-binding properties of this polymer.

Apart from PGP activity and heavy-metal resistance, the selected isolates were also found resistant towards several antibiotics such as Piperacillin, Gentamycin and Chloramphenicol, while showing intermediate response for Cefoxitin antibiotic. Among the species *Pantoea agglomerance* (PC1) and *Enterobacter cloacea* (PC3)

were showing resistance pattern for most of the antibiotics. Similarly, Cd resistant *Enterobacter* species have also been reported to exhibit resistance to a range of antibiotics (Pramanik et al., 2018a). Chaudhari et al., (2016) found *Citrobacter freundii* strain NK2 as heavy metal (Pb and Cu) resistant species and showed resistance to Kanamycin, Chloramphenicol while sensitive to Ampicillin and Neomycin commercial antibiotics. These results are attributed to the fact that the genetic elements that are responsible for heavy metal resistance might be on the same locus (Hobman and Crossman, 2015). These genetic elements played a key role in assisting multi-drug resistant and horizontal gene transfer, through co-carriage and/or co-selection of antibiotic resistance along with metal tolerance (Martinez et al., 2009; Pramanik et al., 2018b). Wani and Irene (2014) have reported that the importance of combined heavy metal and antibiotic resistance might be the basis in bacterial survival.

To prepare successful microbial consortium, bacterial cultures must be compatible with each other without any antagonism among them in order to concomitantly perform all the metabolism required for plant growth promotion and heavy metal tolerance (Sarkar et al., 2013). Mnif et al., (2015) reported that while developing a consortium for environmental application, it is not necessary that all the individuals strains should have similar ability *i.e.* IAA production, Phosphate solubilisation activity etc. As in the present study, all the three rhizobacterial strains were showing multiple PGP characteristics and also heavy metal resistance (Cd and Pb) with minor differences. All the strains showed compatibility to grow with each other which is one of the important step for consortium development. Paredes Páliz et al., (2017) reported development of consortium of Gram-negative *Pantoea agglomerans* RSO6

and RSO7 with Gram-positive *Bacillus aryabhattai* RSO25 strain and recorded its positive impacts on plant growth.

Further, seed germination and seedling growth are presumed as an indicator of heavy metal stress on plant growth and survivability of plant (Yuan and Huang, 2016). Present result suggests that Cd and Pb metal ions negatively affected germination and early growth of both plant *Canna indica* and *Zea mays* seedlings. At the highest concentration (500 mg L^{-1}) of Cd and Pb metal ions, a comparatively greater reduction in seed germination and seedling growth were recorded in both (*Canna indica* and *Zea mays* L.) plant seedlings. Reduction in germination and seedling growth might be due to the reduction in mitotic cell division in the meristematic zone of root suggested by Lequeux et al., (2010) in *Arabidopsis thaliana*. Moreover, reduction in root length might be due to the accumulation of metal ions in meristematic cells that reduces the mitotic cell division especially blocking the metaphase in meristematic tissues (Yuan and Huang, 2016). Another crucial cause in decreased seedling growth under heavy metal stress might be the production of enzymes in the cotyledons and endosperm cells that begin to digest and store food, which is converted into soluble form and transported to the radicle plumule tips. For instance, amylase converts starch into sugar and protease breaks the protein (Bona et al., 2016). Therefore, when enzymatic activities were disturbed, the food did not reach the radical and plumule and in this way, the seedling growth was affected negatively. This is the result of fact that bacteria in polluted soils can increase phytohormone and siderophore production (Ahemad et al., 2015), which matches with the findings from the present study. Results were showing an increase in the germination percentage and seed vigour index inoculated with indigenous strain of SA, PC1, PC3 and developed consortium in the presence of heavy metals and the best result were

observed under consortium treatment. Similarly, Manjunath et al., (2011) reported the appropriate response of PGPR consortium than individual strains in seed germination and plant growth of wheat. This result attribute the importance of PGP activity depicted by bacterial strains *viz.* ACC deaminase activity, IAA production, siderophore production and phosphate solubilization which detoxify the heavy metal effects on seedling either by absorption, chelation or transformation mechanisms and increase the seed germination rate (Sasirekha and Srividya, 2016; Oves et al., 2017; Roman-Ponce et al., 2017). Bacterial strains possessing ACC deaminase are able to reduce ethylene production in stressed plants resulting from a decrease in its precursor ACC, thus enhancing the root elongation and the growth of plants (Belimov et al., 2009).

Investigation was also taken up for the interactive effects of heavy metals (Cd and Pb) with individual strains SA, PC1, PC3 and consortium on *Zea mays* L. and *Canna indica* plant species at different time interval. The physical and chemical properties of the soil used for pot experiment revealed un-contamination of heavy metals (Cd and Pb) in it, as it was collected from the garden field. The result was in accordance with the work of Mitra et al., (2018) who conducted the pot experiment for heavy metal phytoremediation using least heavy metal contaminated soil.

The root-shoot length and biomasses were directly related to plant growth which was observed to decrease consequently under Cd and Pb stress, while in presence of three test isolates and consortium, there was an increasing trend in all the three parameters where consortium was better than individual strains. This reduction in root-shoot length and biomass is attributed to the toxic effect of Cd and Pb that can impair the plant growth causing disturbances in nutrient uptake and other metabolic and physiological activities of plants and consequently growth inhibition. In general,

increasing metal concentration in the soil exerts a severe effect on root growth and functions, resulting in a diminished uptake of water and nutrients and in ensuing reduction in fresh/dry weight (El-Tayeb, 2006; Hao et al., 2012). Moreover, the increased plant length and biomass under metal stress condition inoculated with PGPR strains and consortium depicted reduction in toxicity through diverse mechanisms such as ACC deaminase, IAA synthesis and phosphate solubilisation (Liu et al., 2017; Roman-Ponce et al., 2017). The results are attributed to the ACC deaminase producing ability of the inoculated strains. A study reported by Cheng et al., (2007) described that ACC deaminase activity might be a key factor to promote the excellent plant growth because they decrease plant stress by efficiently blocking ethylene production (Cheng et al., 2007). This result was in accordance with the findings of Kamran et al., (2015). A similar effect of root protection to metal exposure was observed by Rajkumar et al., (2008) for sunflower plants inoculated with the PGPR *Bacillus weihenstephanensis* in the presence of Zn, Ni and Cu. Similarly, increase of the root-shoot length and biomasses under heavy metal (Pb, As, Cu and Zn) stress were also found to increase after inoculation of PGPR (*Microbacterium* sp. and *Curtobacterium* sp.) as reported by Roman-Ponce et al., (2017). A study conducted by Mitra et al., (2018) also reported that bioinoculation of *Enterobacter* sp. enhance the rice seedling height and biomass under cadmium stress condition.

Photosynthetic pigments such as chlorophyll ‘a’ and chlorophyll ‘b’ are the basis of photosynthesis and it is a vital process for plant growth (Kumar et al., 2018). It is reported that the inter-venal chlorosis of leaves is the first visible symptom of metal phytotoxicity and is closely related to chlorophyll content (Aibibu et al., 2010; Moreira et al., 2014). Present study findings with Chlorophyll content reveals that, at lower concentration of Cd and Pb, both chlorophyll ‘a’ and ‘b’ contents of leaves

were higher. However, with increase in concentration of Cd and Pb in soil, chlorophyll content declined. This result might be due to the inhibition of either photosynthetic activity or biosynthesis of chlorophyll (Kamran et al., 2015; Mitra et al., 2018) or due to the blockage of photosynthetic electron transport chain (Mitra et al., 2018). However inoculation of *Pseudomonas aeruginosa* (SA), *Pantoea agglomerans* (PC1), *Enterobacter cloacae* (PC3) and their consortium showed marked increase in Chl-*a*, Chl-*b* and Chl-total content in both the experimental plant under each concentration of Cd and Pb treatment to overcome the heavy metal induced stress. Such types of results are expected due to hypothesis that presence of PGPR accelerates the uptake of iron in the plant with their siderophore production activity, which could enhance the chlorophyll contents in the PGPR-inoculated plants. Kamran et al., (2015) discussed that iron is an important factor in photosynthesis for different cellular activities, and its uptake is influenced by the Cd contents in the growing medium. However, microbial iron siderophore complexes can be taken up by inoculated plants as a source of iron. Similar results were observed in *Zea mays* L. (Moreira et al., 2014), *Eruca sativa* (Kamran et al., 2015) and rice seedling (Mitra et al., 2018) exposed to Cd stress conditions. Similarly, Janmohammadi et al., (2013) reported the influence of PGPR inoculation in enhancing chlorophyll content in wheat plant in presence of lead. Similar to the chlorophyll inhibition pattern, present study also investigated the inhibition of carotenoid content in both the plant leaves under the toxic effects of Cd and Pb. Marked distortion of chloroplast ultrastructure leads to disturbed shape and inflated thylakoids and this decrease in carotenoid content leads to negative effects on photosynthetic performance which ultimately reduces the plant growth (Parmar et al., 2013). However, in the present study, inoculation of PGPR strains (SA, PC1 and PC3) and consortium consequently increased the carotenoid

content and helped plant, overcome the toxic effects of Cd and Pb. Similar result have also been reported by Ehsan et al., (2014) in *Brassica napus* under the Cd stress condition treated with citric acid.

Proline is well documented to serve as an osmoprotectant and plays an important role in stabilizing proteins and molecular membrane which guards plant from the deleterious effects of ROS generated by plants under the influence of heavy metal stress (Kamran et al., 2015; Rizvi et al., 2019). Proline, also acts as scavenger of free radical (ROS); besides this, proline assists plant in maintaining osmotic balance and homeostasis (Ahmad et al., 2012; Kamran et al., 2015; Rizvi et al., 2019). Therefore, during abiotic stress condition plants increase synthesis and accumulation of osmolytes such as proline for maintaining tissue water content as well as plant growth (Mitra et al., 2018; Rizvi et al., 2019). Similarly, in presented study, proline accumulation in *Zea mays* and *Canna indica* plant species were enhanced with the increasing concentration of cadmium and lead. These findings are attributed to the fact that due to reduced degradation and/or *de novo* synthesis of proline. Similarly, Pramanik et al. (2018b) reported a significant accumulation of proline content in rice seedling plants that were exposed to varying concentrations of cadmium in soils. However, after inoculation of *Enterobacter aerogenes* MCC 3092, there was a decrease in the plant proline as also observed in the present study.

Present study investigated the decrease in proline content of *Zea mays* L. and *Canna indica* plants after inoculation of *Pseudomonas aeruginosa* (SA), *Pantoea agglomerans* (PC1), *Enterobacter cloacae* (PC3) and consortium under Cd and Pb stress. The highest reduction of proline was shown under consortium treatment which indicated the successful alleviation of Cd and Pb stress by inoculated strains and consortium. Moreover, IAA producing, phosphate solubilizing and ACC deaminase

producing *Enterobacter aerogenes* strain MCC 3092 alleviated Cd toxicity in rice seedling by stimulating antioxidant enzymes (Pramanik et al., 2018a) which corroborated the present work. Similar results were also reported by Janmohammadi et al., (2013) in wheat inoculated with PGPR strains under Pb stress. Rizvi et al., (2019) also reported the decrease of proline content in *Pennisetum glaucum* (Bajra) inoculated with *Bacillus* sp. under Pb and Ni stress condition.

In contrary of the present study, Kamran et al., (2015) reported a consistently increase of proline content in *Eruca sativa* plant inoculated with *Pseudomonas putida* under Cd stressed condition. This process is governed by PGPR-assisted tolerant plant species, which inhibits the phyto-toxic effect for a wide range of pollutants and are expected to enhance the metal uptake by producing different degrading enzymes, organic acid, iron chelators, and siderosphore (Oves et al., 2013; Kamran et al., 2015). Similar results have also been reported by Irfan et al., (2014) and Mitra et al., (2018).

Present study investigated the role of rhizobacterial inoculation to enhance the heavy metals accumulation in *Canna indica* and *Zea mays* L. plant. Generally, in the soil, metals are present in insoluble form due to different environmental factors and rhizobacteria acts as bioinoculants that decrease the soil-water solution pH and helps in nutrient and metal uptake by converting them into readily available form (Kamran et al., 2015). As in present study, observed result revealed that the bioinoculation of indigenous species and consortium of three strains (SA, PC1 and PC3) consistently increased the Cd and Pb accumulation in experimental plant (*Zea mays* L. and *Canna indica*). The result are attributed to the hypothesis that the production of different degrading enzymes, organic acids, iron chelating agents by inoculated bacterial strains inhibits the phytotoxic effects of heavy metals (Cd and Pb) as well as activate the ATPases in the root plasma membrane that change the transport of ions through

membrane and enhance metal uptake through symplastic or apoplastic pathways. Result was also in accordance with the findings of Kamran et al., (2015) for Cd uptake by *Eruca sativa* inoculated with PGPR. The enhancement of metal uptake of plants by PGPR inoculation has been reported in several studies (Ghosh et al., 2011; Ma et al., 2013; Prapagdee et al., 2013; Liu et al., 2015) However, in contrary of this study Krishna et al., (2012) suggested that the cadmium level can be minimized in plants by using bacteria having the ability to produce plant growth promoting traits.

The endodermis with its Casparian bands represents a barrier to metal movement through the apoplasm, thus avoiding the transport to the shoot tissues (Ranathunge et al., 2005; Bose et al., 2008; El Faiz et al., 2015). Therefore, root metal accumulation is always higher than the shoot. As in present study, the concentrations of Cd and Pb in both the plant roots and shoots showed that roots accumulate greater concentrations than shoots, indicating high plant availability of the substrate metals as well as their limited mobility once inside the plant. However, in the case of Cd, root accumulation was always significantly higher than in the shoots, while for Pb exposed plants root accumulation was generally similar to shoot accumulation in present study. These results are also supported by the similar findings of Khatun et al., (2016). Tolerant species try to restrict the transfer of metals during soil to root and root to shoot transfer (Ahmad et al., 2011). These results show, that in general, the accumulation of metals was higher in root than shoot, and also revealed that the capacity for metal accumulation for *Zea mays* L. and *Canna Indica* plant was very closer. These results are in accordance with the work of Wuana and Okieimen (2010), who reported that *Zea mays* L. plants have high biomass and are tolerant to heavy metals, including Cd, Zn, and Pb. Some of previous reports have classified *Zea mays* L. as a root accumulator (Mench and Martin 1991; Li et al., 2009), which was also observed in

the present study. El Faiz et al., (2015) also reported the phytoaccumulation capability of Cd, Cu and Zn in root>shoot of *Canna indica* inoculated with Arbuscular Mycorrhizal Fungi (AMF). However, no reports are available for the phytoremediation strategy of *Canna indica* in terrestrial environment inoculated with PGPR.

Soil to plant translocation factor is one of the key components of phytoextraction and phytoremediation. The Bio-concentration factor (BCF) of a plant indicates it stability to accumulate metal from the sediment in its shoots, whereas the ability of a plant to translocate it from root to shoot is expressed by the Translocation factor (TF). These two factors are used to estimate the phytoremediation potential of a plant species (Marques et al., 2013; Khatun et al., 2016). Translocation factors (the ratio between metal accumulation in the shoots and roots), lower than 1 in most cases, were obtained with both metals (cadmium and lead) in both plants. This leads to conclusion that both *Zea mays* L. as well as *Canna indica* plant have adopted a tolerance/immobilization strategy, as suggested earlier by Madejon et al., (2003) and Marques et al., (2013) for *Helianthus annuus* exposed with Cd and Zn. The similar pattern of TF for Pb translocation from root to shoot in *Canna indica* were reported by Bose et al., (2008) under the soil amended with industrial waste, while no report are available for the presence of PGPR or PGPR assisted phytoremediation of heavy metals by *Canna indica*. As in present study, inoculation of PGPR consequently increases the TF and BCF of both metals (Cd and Pb). The result was in accordance with the study of Moreira et al., (2014) in *Zea mays* inoculated with PGPR exposed with Cd. BCF of Cd was much higher than those shown for Pb in both the plants species. This is coherent with the generally accepted pattern of high Cd transfer coefficient between soil and plant (Kloeke et al., 1984).

Principal Component Analysis (PCA) of the different sampling times provided important information used to define the co-operative analysis of growth variable as well as phytoremediation of heavy metals under different treatments (Bessa et al., 2016). Negative PC1 score with loading vectors explained the non-significant effects of treatment on plant parameters. The bi-plot of principle component analysis (PCA) provides a comprehensive result of the interrelationship between all studied variables. Angles for total dry biomass of *Zea mays* L. are very close to the axis suggesting its higher response under all the treatment of metals and rhizobacterial strain as compared to plant, *Canna indica*. All the other parameters, related to heavy metals accumulation by both the plants, show a close association in between, suggests their equal capability for cadmium (Cd) and lead (Pb) remediation by *Zea mays* and *Canna indica*. Differences in the angles and eigen value for TF and BCF of Cd and Pb suggests higher TF and BCF value for Cd and Pb respectively in both the plants.

At the end of this study, experimental results concludes that both the plants show close potential for their phyto-remediation ability by accumulating Cd and Pb wherein *Zea mays* L., which has a well developed root system as compared to *Canna indica*, is a better phyto-remediator in this case. Further, inoculation of plant growth promotory bacterial strains SA, PC1 and PC3 and their consortium helped plants in remediation of cadmium and lead from the contaminated soil. In this case also, *Zea mays* L. was better for extraction of cadmium and lead from the soil with the developed consortium as compared to individual strains.

Although, Zea mays L. is a well reported hyperaccumulator crop and was expected to perform better but still, *Canna indica* although had lower plant biomass but is a good accumulator of cadmium and lead as found in the present study. Bose et al., 2008 have also reported the similar findings with *Canna indica* but no findings are

available till date using PGPR strains which has been done in the present study and needs further advanced studies work for metal free sustainable environment and agricultural development.

Chapter 6

Conclusion

CHAPTER - 6

CONCLUSION

Heavy metal contamination of soils is one of the world's major environmental problems, posing significant risks to public and ecosystem health. Phytoremediation is a widely accepted and ecologically sound technique which uses plants to remediate metal contamination from soils, but this technology fails when contamination reaches beyond the threshold limit and under such conditions, Plant Growth Promoting Rhizobacteria (PGPR), can be considered as an important tool to mitigate the inherent demerits of Phytoremediation technology by inducing biomass production as well as metal tolerance of plants. Previous reports showed many species of bacteria which are found in soil, promote plant growth by producing plant hormones, stimulates root exudation and enhance nutrient availability to plant, besides controlling soil-borne plant pathogens.

Based on the findings obtained from experimental results, this study concluded that rhizobacterial strains isolated from different contaminated sites (Lucknow, Kanpur and Kanpur dehat) of Uttar Pradesh were showing multiple PGP traits and Cd and Pb resistance (upto 1200 of Cd and 1500 ppm of Pb). Three rhizobacterial strains, *Pseudomonas aeruginosa* designated as SA (Accession No. KX668495), *Pantoea agglomerans* designated as PC1 (Accession No. KX668493) and *Enterobacter cloacae*, designated as PC3 (Accession No. KX668494) were selected, based on their multiple PGP traits and maximum heavy metal resistance potential. Further, these isolates were characterized quantitatively for their PGP traits, results concluded that all the strains were capable to release a good quantity of organic phosphate, IAA, ACC deaminase, Siderophore, HCN, Ammonia and EPS in their respective liquid

medium containing 500 ppm of Cd and Pb. A rhizobacterial consortium was developed by these three isolates after checking their compatibility to grow simultaneously. Further, seed germination of *Canna indica* and *Zea mays* L. enhanced significantly when treated with developed consortium as well as each individual strains under 500 ppm of Cd and Pb. The consortium was better as compared to individual strains in the traits studied. Root/shoot length, dry plant biomass chlorophyll, carotenoid contents proline and metals accumulation in *Canna indica* and *Zea mays* L. plants were significantly improved by the inoculated rhizobacterial consortium as well as individual strains over control in a pot experiment carried out in the semi-controlled condition in greenhouse. The accumulation pattern of Cd and Pb in both the plants i.e. *Canna indica* and *Zea mays* L. during the experimental treatment varied and more cadmium was localized in root region while in case of Pb, translocation from root to shoot was greater in both the plants. Furthermore, the uptake of Cd and Pb in both plants of the present study increased and the removal was in the order of Pb>Cd. Moreover, comparative study with the help of principle component analysis for Cd and Pb phytoextraction strategy of *Canna indica* and *Zea mays* L. plants treated with rhizobacterial consortium as well as individual strains suggested that *Zea mays* L. plant accumulated high amount of Cd and Pb as compared to *Canna indica* with higher biomass production while in contrary *Canna indica* also accumulates good amount of Cd and Pb with lower biomass. With these results, study concluded that the heavy metal resistant rhizobacterial bioinoculants and its multifarious plant growth promoting properties such as ACC deaminase, IAA, siderophore, phosphate solubilisation, HCN, Ammonia and EPS production alleviated the toxic effects of Cd and Pb. Consequently, rhizobacterial consortium, as well as individual isolates, could be exploited for remediation of metal contaminated sites by

functioning as a phyto-stimulant for plants. Further studies are needed to evaluate the on-site/field application (natural environment) of this potential PGPR assisted phytoremediation technique and may assure their full utilization in this context for cleaning agricultural soil under sustainable development.

Chapter 7

Summary

CHAPTER -7

Summary

Soil's heavy metal contamination due to various natural and anthropogenic activities has been recognized as a serious environmental hazard and considered a major barrier to sustainable development, particularly in developing or underdeveloped nations. Amongst all the heavy metals, cadmium (Cd) and lead (Pb) have emerged as serious toxic environmental pollutants in the past few years because of their excessive use in manufacturing and agricultural industries. Their elevated concentrations in the soil, enormously pollute the natural ecosystem as well as alter or destruct the soil texture by reducing its fertility and nutrient availability. With the growing concern and more industrialization, there is an urgent need to address this severe problem because of their continuous accumulation in agricultural soils and water resources that pose a great threat to contaminate the food chain and cause potential risk to human health and ecosystem. Different conventional approaches such as physical, chemical, thermal and electrokinetic techniques have been developed by the researchers in recent years to mitigate the contamination of heavy metals from soil but unfortunately they all have failed with several demerits such as cost, non-eco-friendly nature, labour intensive, generate secondary pollutants that are more complex than previous contaminants. Taking into consideration all these problems, a plant based biological technique "Phytoremediation" has been identified around five decades as a low cost emerging, eco-friendly and sustainable solution for the remediation of heavy metal contaminated soils. Phytoremediation is a widely accepted technique in which plants are used as a remediator or accumulating agents for removal of toxic heavy metals from contaminated soils. Plants used in this technique have an increased rate of heavy

metal uptake, rapid translocation from root-to-shoot and excellent ability to detoxify and sequester heavy metals in their aerial parts. Because of sustainable features, phytoremediation has been considered as a best alternate for removal of heavy metals from contaminated soil without affecting the biological activity, structure and fertility.

Although phytoremediation is easily applicable and cost-effective technique, but it does have some inherent technical constraints like, it is restricted to the site with low pollutant concentration; the higher concentration of contaminant may check the plant growth. Moreover, hyperaccumulator plants are usually limited to their slow growth and low biomass. Limited bioavailability of tightly bound fraction of metal ions from the soil is another demerit of phytoremediation technology. Slow transfer rate of metal from soil to root and root to shoot makes this technique inefficient. To mitigate these drawbacks use of beneficial soil microbiota colonizing the rhizosphere, often known as Plant Growth Promoting Rhizobacteria (PGPR), endowed with the unique property of heavy metal resistant and plant growth promotion, have been considered as an important tool to promote phytoremediation technology by increasing plant biomass. PGPR's increase plants biomass by various mechanisms such as fixation of atmospheric nitrogen, mitigate stress by utilization of 1 aminocyclopropane-1-carboxylic acid (ACC) as a sole N source, production of siderophores and anti-pathogenic substances, production of plant growth regulators (phytohormones, such as auxins), and also through the transformation of nutrient elements like phosphorous and potassium.

The present study entitled "**Development of Plant Growth Promoting Rhizobacterial Consortium for Remediation of Cadmium (Cd) and Lead (Pb) contaminated soil by *Canna indica* and *Zea mays* L."** was carried out with the aim of isolation and characterization of Cd and Pb resistant plant growth promoting

rhizobacterial strains and their exploitation in the phytoremediation of Cd and Pb contaminated soil using *Canna indica* and *Zea mays* L.

Total thirty morphologically distinct bacteria were isolated initially from industrially contaminated sites based on primary screening which involved the notable growth in Cd and Pb supplemented media at 100 ppm. Further, out of thirty isolates, 94% isolates were growing at 100 to 200 ppm of Cd concentration, and 4% were growing at 200 to 600ppm Cd concentration, while only 1% isolates were growing up to 1200 ppm of Cd concentration. However, only 1% isolates were growing at 100 to 1500 ppm Pb and 2% isolates were growing at 100 to 750 ppm of Pb concentration, rest isolates were growing at ≤500 ppm of Pb concentration. Moreover, at the last of this experiment, it was screened-out that out of the total thirty isolates, three isolates (SA, PC1 and PC3) were growing at higher concentration (1200 ppm Cd and 1500 ppm Pb concentration) of both cadmium and lead. On the basis of maximum tolerance concentration of Cd and Pb, three isolates *viz.*, SA, PC1 and PC3 were finally selected for further study.

Further, these three selected rhizobacterial isolates (SA, PC1 and PC3) were characterized for their plant growth promoting attributes *viz.* ACC deaminase activity, IAA production, phosphate solubilization, siderophore, extracellular polysaccharide production, ammonia and HCN production. Results revealed that the selected bacterial isolates (SA, PC1 and PC3) were showing multiple plant growth promoting activities in the absence and presence of heavy metal (Cd and Pb) ions till 500 ppm concentration. Beyond this concentration, all the strains lost their PGP properties. Hence, 500 ppm concentration of both (Cd and Pb) metal ions were selected for further experiments. Some of the plant growth promotory traits (ACC deaminase and siderophore) enhanced in the presence of heavy metals while some traits (IAA, EPS

and phosphate solubilization) decreased, while some (Ammonia and HCN) remained unaffected.

All three test isolates depicted capability to cleave ACC as a sole source of nitrogen by producing ACC deaminase enzyme in DF salt media which is essential to balance the ethylene level in cells. Further, quantitative assessment of ACC-deaminase activity was determined and it was observed that isolate PC1 and PC3 enhanced the activity by 19.28% and 23.44%, respectively in the presence of Cd. While, in the presence of Pb ions, isolate PC1 increased the activity by 21.42%, strain PC3 by 27.70% and strain SA by 42.32%. On the other hand, it was observed the activity was unaffected by strain SA in the presence of Cd metal ions. Siderophore production by the selected isolates revealed that strains SA and PC3 were able to produce siderophore with and without Cd and Pb ions amended media. While strain PC1 was found negative for siderophore production in both conditions i.e. in absence and presence of heavy metal ions. The quantitative assessment with strains SA and PC3 revealed that 99.01 and 54.74 µM of siderophore were released in the culture supernatant in control conditions while, siderophore production increased by 6.056% in case of isolate SA in the presence of Pb metal ions. Moreover, it was observed that the selected isolates were able to produce IAA by utilizing L-tryptophan as a precursor compound in the presence of Cd and Pb metal ions. Quantitative estimation showed that IAA production increased as the concentration of tryptophan increased but at a certain concentration, it decreased gradually. Further, the highest amount of IAA was produced by strain PC1 (38.737 µg mL^{-1}) followed by strain PC3 (35.556 µg mL^{-1}) and SA (31.48 µg mL^{-1}) at 500µgml^{-1} tryptophan concentration. Phosphate solubilization test showed that all the selected heavy metal resistant rhizobacterial strains were able to solubilize inorganic phosphate which was confirmed by brown

colour halo zone on NBRIP media agar plate. Further quantitative assessment at different time intervals showed that solubilization was increased as the incubation period increases but after a certain time period, it decreases gradually. Further, selected rhizobacterial isolates produced HCN and Ammonia in the presence and absence of Cd and Pb amended media. Observed results revealed that HCN production by isolates, SA, PC1 and PC3 were positive in the absence of Cd and Pb heavy metals. However, in the presence of Cd, strain PC1 was negative for HCN production. For the production of ammonia, selected isolates showed positive results in the absence and presence of Cd and Pb. Extracellular polysaccharide production revealed that all the three heavy metal resistant rhizobacterial isolates were able to produce extracellular polysaccharide (EPS) in the absence and presence of Cd and Pb metal on LB agar plates. The mucoid gummy colonies on LB agar plate and increased viscosity in LB broth indicated the ability of strains to produce EPS. Further, EPS produced by bacterial isolates, SA, PC1 and PC3 characterized by FTIR depicted different characteristic peaks between 3401-805cm^{-1}. The stretching vibration at 3402, 3072 and 3040 cm^{-1} indicating O-H (hydroxyl) group and hydrogen bonding, a characteristic of polysaccharide ring.

Further, morphological characterization of selected isolates showed that strain SA possessed large hairy colony while PC1 and PC3 test isolates possessed tiny round-shaped colony, with convex elevation and margins were entire for one while undulate for other test isolate i.e. they depicted different morphological features. All test isolates were Gram negative, rod shaped and motile. Scanning electron microscopic (SEM) study of the selected isolates confirmed that all isolates were rod shaped. Further, biochemical characterization tests showed that all the three test isolates were negative for indole and methyl-red test; PC1 and PC3 were positive for voges-

proskauer test, while SA was negative for it. All the three isolates were positive for catalase, cellulase and citrate utilization while negative for urease production test. However, in case of oxidase test, isolate SA was positive and isolates PC1 and PC3 were negative. Isolate SA (*Pseudomonas aeruginosa*) was positive for lipase production while other two (*Pantoea agglomerans, Enterobacter cloacae*) failed to produce the same. By 16S rRNA partial gene sequencing and similarity index it was observed that strain PC1, PC3 and SA were closest to *Pantoea agglomerans, Enterobacter cloacae* and *Pseudomonas aeruginosa* respectively, showing 98-100% homology. The obtained sequences were submitted to Gene Bank (NCBI) under the accession number KX668493 (PC1), KX668494 (PC3) KX668495 (SA). For the evolutionary relationship, a phylogenetic tree was constructed using neighbor joining (NJ) method for the strains PC1, PC3 and SA. Antibiotic sensitivity pattern of selected isolates were showing resistant towards several antibiotics such as Piperacillin, Gentamycin and Chloramphenicol, while showing intermediate response for Cefoxitin antibiotic. Among the species, *Pantoea agglomerance* (PC1) and *Enterobacter cloacea* (PC3) were showing resistance pattern for most of the antibiotics.

To prepare successful microbial consortium compatibility test is an important test and was performed by agar diffusion method. Three rhizobacterial strains, PC1, PC3, and SA showed compatibility with each other.

Further, each individual strain and their consortium were tested for seed germination of *Canna indica* and *Zea mays* L. plant under Cd and Pb stress with suitable control. The results of seed germination tests revealed that each individual strain and their consortium increased germination percentage as well as seed vigour index of *Canna indica* and *Zea mays* L. under heavy metal (Cd and Pb) stress. Moreover, findings of

seed germination tests depicted that consortium gave better results than individual strains.

The study was undertaken to investigate the interactive effects of Cd and Pb on the growth and development of *Zea mays* L. and *Canna indica* plant species treated with consortium as well as individual strains, SA, PC1, PC3 at different time interval using pot experiments. Findings depicted that as the concentration of heavy metal (Cd and Pb) increases, the physiological growth parameters of plants decrease linearly. However, inoculation of rhizobacterial strains as well as their consortium increases the plant growth in each concentration of Pb and Cd contaminated soil. The root-shoot length and biomasses were directly related to plant growth which was observed to decrease consequently under Cd and Pb stress, while in presence of three test isolates and consortium, there was an increasing trend in all the three parameters where consortium was better than individual strains. Enhancement in plant length and biomass under metal stress condition, inoculated with PGPR strains and consortium depicted reduction in toxicity through diverse mechanisms such as ACC deaminase, IAA synthesis, siderophore and phosphate solubilisation.

Chlorophyll 'a' and 'b' content in leaves of both the plants was found to be higher at low concentration of cadmium and lead. However, with increase in concentration of Cd and Pb in soil, chlorophyll content declined. Similar to the chlorophyll inhibition pattern, present study also investigated the inhibition of carotenoid content in both the plant leaves under the toxic effects of Cd and Pb. Marked distortion of chloroplast ultrastructure leads to disturbed shape and inflated thylakoids and this decreases carotenoid content severely affecting photosynthetic performance and ultimately plant growth reduction. However, due to the inoculation of PGPR strains (SA, PC1 and PC3) and consortium, carotenoid content enhanced that helped plant overcome the

toxic effects of Cd and Pb. Proline is well known for osmolytic activity which protects the cells from the deleterious effects of ROS generated by cells under the influence of environmental stresses such as heavy metals, drought, and salinity. Observations of present study showed that the foliage proline content progressively increased with increasing concentration of Cd and Pb. However, after inoculation of rhizobacterial strains as well as their consortium, the proline content decreased successively in each treatment.

Uptake and accumulation of Cd and Pb in shoot and roots of *Canna indica* and *Zea mays* L. plants were directly correlated with the exposure time and concentration of heavy metals in the soil. It was observed that there was a linear increase of Cd and Pb concentration in response to increased exposure time as well as concentration (100-500 mg Kg^{-1}) of heavy metals in the soil by *Canna indica* and *Zea mays* L. The maximum Root Metal Content (RMC), shoot metal contents (SMC) and total metal accumulation (TMA) in *Canna indica* and *Zea mays* L. plants were observed at 500 ppm concentration of Cd and Pb at 90 DAS. Interestingly, inoculation of rhizobacterial strains as well as their consortium further increased the RMC, SMC and TMA as compared to their respective control. Bioconcentration factor (BCF) describes the ability of plant to uptake metal ions from the soil. It is an important factor required to estimate the accumulation potential of a particular plant species for the remediation of heavy metals. BCF in this study depicted that the value for *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were >1 at the concentration range from 100 ppm to 500 ppm while at 0 ppm concentration, the values were below one (<1) at all exposure time (30, 60, and 90 DAS) under four different treatments (T1, T2, T3, and T4). The value of BCF >1 indicated that the plants have good potential for remediation of Cd and Pb contaminated soil. Further, the translocation

factor (TF) of the plant describes the ratio of heavy metal (Cd and Pb) concentration in shoot tissues over heavy metal concentration in root tissues. Results from this study revealed that the TF value of *Canna indica* and *Zea mays* L. plants under Cd and Pb stress were <1 at all the concentration and all exposure times under four different treatments i.e. T1, T2, T3, and T4. Further, it was observed when rhizobacterial consortium as well as individual strains were applied, slight increase in TF values were obtained. The increase in TF value in the presence of rhizobacterial consortium indicated that rhizobacterial consortium help the plant in phytoextraction process of heavy metal contaminated soil. Moreover, a comparative phytoextraction study between *Canna indica* and *Zea mays* L. was carried out with the help of principle component analysis; results yielded twelve components, that explained 100% of the total variance, the loading factor with score plot indicate that the component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under higher concentration of Cd concentration at 90 DAS. While, the second component showed positive association with TDB and SMC under controlled condition as well as lower concentration of Cd concentration (upto 200) at all the sampling periods. However, in the presence of Pb metal ion, the loading factor with score plot indicated that the component-1 is associated with RMC, SMC, TMA, BCF and TF of both the plants under higher concentration of Cd concentration at 60 and 90 DAS. While the second component (Principal Component 2; PC2) represents the positive association with TDB and BCF under control condition as well as lower concentrations of Pb (upto 200) at all the sampling periods.

With these findings, study concluded that isolated rhizobacterial strains and their consortium have the ability to detoxify heavy metals as well as promote growth of *Canna indica* and *Zea mays* L. by enhancing nutrient availability, efficiently

diminishing the high level of ethylene, proline and other oxidative stress caused by heavy metals. Out of *Zea mays* L. and *Canna indica*, *Zea mays* L. is better for remediation with rhizobacterial strains but *Canna indica* holds promise owing to more accumulation of cadmium and lead in the tissues as compared to *Zea mays* L. Further, these rhizobacterial candidates and their consortium could be targeted for environmental management studies wherein they could efficiently cleanup toxic metal contaminated sites and concurrently improve the plant growth even in metal polluted soils. Moreover, there are lot of hidden mechanisms that naturally occur in heavy metal contaminated soils that are still to be understood, but the findings of this study will form a strong foundation and may serve as a baseline data for increasing the growth of plants in heavy metal contaminated soils, for bio-management of such soils using heavy metal resistant plant growth promoting rhizobacteria under sustainable environmental and agricultural development and reclamation of sites contaminated with heavy metals.

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Appendix

Compositions of Different media used in this study

Media ingredients	**gL^{-1} MQ water**
1. Luria Bertani medium	
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
pH	7.3±0.2
2. Luria Bertani agar medium	
Casein enzymic hydrolysate	10.000
Yeast extract	5.000
Sodium chloride	10.000
Agar	15.000
pH	7.3±0.2
3. Nutrient broth medium	
Peptone	5.000
Beef Extract	3.000
Sodium Chloride	5.000
pH	7.0±0.2
4. Nutrient Agar medium	
Peptone	5.000
Beef Extract	3.000
Sodium Chloride	5.000
Agar	15.000
pH	7.0±0.2
5. DF minimal salt medium (Dworkin and Foster 1958)	
Ammonium Sulphate	2.000
Potassium dihydrogen phosphate	4.000
Disodium phosphate	6.000

Magnesium sulphate	0.200
glucose	2.000
gluconic acid	2.000
citric acid	2.000
$FeSO_4 \cdot 7H_2O$	0.001
H_3BO_3	0.010
$MnSO_4 \cdot H_2O$	0.01119
$ZnSO_4 \cdot 7H_2O$	0.1246
$CuSO_4 \cdot 5H_2O$	0.07822
MoO_3	0.01
ACC solution	3.0 mM

6. Chrome azurol S assay solution

Chrome azurol S	0.0605
hexadecyltrimetyl ammonium bromide (HDTMA)	0.0729
Piperazine-1,4-bis (2-ethanesulfonic acid) (PIPES)	0.03024
$FeCl_3 \cdot 6H_2O$ 1 mM	10.000 ml

7. Tryptone Soya Broth

Pancreatic digest of casein	17.000
Papaic digest of soyabean meal	3.000
Sodium chloride	5.000
Dextrose	2.500
Dibasic potassium phosphate	2.500
pH	7.3±0.2

8. Peptone water broth

Peptone	10.000
Sodium chloride	5.000
pH	7.0±0.2

9. SIM Agar

Peptone	3.000
Beef Extract	3.000
$(NH_4)_2.SO_4$	0.020
$FeSO_4$	0. 250
Agar	15.000
pH	7.3±0.2

10. Pseudomonas Agar Base

Tryptone	10.000
Gelatin peptone	16.000
Potassium sulphate	10.000
Magnesium chloride, anhydrous	1.400
Agar	15.000
pH	7.1±0.2
Supplemented with cetrimide sodium fusidate cephalothin sodium (CFC)	

11. Kings agar medium A Base

Proteose peptone	20.000
Potassium sulphate	10.000
Magnesium chloride, anhydrous	1.640
Agar	15.000
pH	7.2±0.2

12. Kings agar medium B Base

Peptone	16.000
Magnesium sulphate	1.600
Dipotassium hydrogen phosphate	1.600
Glycerol	10.000
Agar	15.000
pH	7.2±0.2

13. Czapek Dox Agar

Sucrose	30.000
Sodium nitrate	2.000
Dipotassium phosphate	1.000
Magnesium sulphate	0.500
Potassium chloride	0.500
Ferrous sulphate	0.010
Agar	15.000
pH	7.3±0.2

14. NBRIP Medium

Glucose	10.000
Tricalcium Phosphate	5.000
Magnesium Chloride	5.000
Magnesium Sulphate	0.250
Potassium Chloride	0.200
Ammonium Sulphate	0.100
Agar	15.000
pH	7.1±0.2

15. Starch Agar medium

Starch (soluble)	10.000
Peptone	5.000
Beef Extract	3.000
Agar	15.000
pH	7.0±0.2

16. Muller Hinton Agar medium

Beef infusion	2.000
Acid hydrolysate of casein	17.500
Starch	1.500
Agar	17.250
pH	7.4±0.2

17. Simmons citrate Agar medium

Ammonium dihydrogen phosphate	1.000
Dipotassium Hydrogen phosphate	1.000
Sodium Chloride	5.000
Sodium Citrate	2.000
Magnesium sulphate	0.200
Bromothymol blue	0.080
Agar	17.250
pH	6.9±0.1

APPENDIX - II

S.No. **16S rRNA partial Sequences**

1. **>PC1 KX668493 (*Pantoea agglomerans*)**

GAAACGGTAGCTAATACCGCATAACGTCGCAAGACCAAAGAGGGGGACCTTCGG
GCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTAACGGC
TCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATG
GGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAA
AGTACTTTCAGCGGGGAGGAAGGTGTTGAGGTTAATAACCTCAGCAATTGACGTT
ACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTCTGTCA
AGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACTGCAGGC
TAGAGTCTTGTAGAGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGA
TCTGGAGGAATACCGGTGGCGAAGGCGGCCCCTTGGACAAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTA
AACGATGTCGATTTGGAGGTTGTGCCCCTTGAGGAGTGGCTTCCGGAGCTAACGC
GTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTG
ACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAG
AACCTTACCTACTCTTGACATCCAGAGAACTTAGCAGAGATGGATTGGTGCCTTC
GGGAACTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTCCGGCC
GGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTC
AAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGCTACAATGGCGCATACA
AAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTAGTCCG
GATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATC
AGAATG

2. **>PC3 KX668494 (*Enterobacter cloacae*)**

TACTGGAAACGGTAGCTAATACCGCATAATGTCGCAAGACCAAAGAGGGGGACC
TTCGGGCCTCTTGCCATCAGATGTGCCCAGATGGGATTAGCTAGTAGGTGGGGTA
ACGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACACT
GGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCA
CAATGGGCGCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGT
TGTAAAGTACTTTCAGCGGGGAGGAAGGTGTTGTGGTTAATAACCGCAGCAATTG
ACGTTACCCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATA
CGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGT
CTGTCAAGTCGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCGAAACT
GGCAGGCTGGAGTCTTGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATG
CGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAAAGACTG
ACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCC
ACGCCGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTCCGGAG
CTAACGCGTTAAATCGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAAT
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC
GCGAAGAACCTTACCTGGTCTTGACATCCACAGAACTTTCCAGAGATGGATTGGT
GCCTTCGGGAACTGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGA
AATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGGTT
AGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATG
ACGTCAAGTCATCATGGCCCTTACGACCAGGGCTACACACGTGCTACAATGGCGC
ATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAAGTGCGTCGTA
GTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTA

3. **> SA KX668495 (*Pseudomonas aeruginosa*)**

GGATAACCGTCCGGAAACGGGCGACTAATACCGCATACGTCCTGAGGGAGAAAG
TGGGGGATCTTCGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTT
GGTGGGGTAAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGAT
CAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGG
GAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA
GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACC
TTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC
CGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCG
CGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGC
ATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGC
GGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGA
CTGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTGGGATCCTTGAGATCTTA
GTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGAGTACGGCCGCAAGGTTA
AAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATT
CGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGA
TGGATTGGTGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTA
CCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGCTA
CAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAA
CCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCT
AGTAATCGTGAATCAGAATGTCACGGTGAATACGTTCCCG

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Exploring the survival tactics and plant growth promising traits of root-associated bacterial strains under Cd and Pb stress: A modelling based approach

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ABSTRACT

The study represents a microbial method for reducing heavy metal stress in terrestrial environment. Two rhizobacterial strains *Pantoea agglomerance* (PC1) and *Pseudomonas aeruginosa* (SA) having the ability to tolerate Cd^{2+} and Pb^{2+} ions stress, were employed in this study. The growth promotion and survival tactics of the strains under metal stress were explored through kinetic growth model using logistic equation, Luedeking-Piret model and Box Behnken design. Study also involves the interaction of strains with *Zea mays* L. under Cd^{2+} and Pb^{2+} ions stress. Results revealed that both strains have the potential to tolerate 500 mg L^{-1} of Cd^{2+} and Pb^{2+}, ions and maintained the plant growth promoting traits. The Luedeking-Piret model estimated the maximum value of IAA on biomass growth ($Y_{P/X}$) 5.377 µg g^{-1} and 10.3 µg g^{-1} under Cd^{2+} ions, while 7.742 µg g^{-1} and 18.071 µg g^{-1} under Pb^{2+} ions stress for strains SA and PC1, respectively. Further, phosphate solubilization activity was optimized with the help of response surface methodology using Box Behnken Design. The optimum solubilization by strain PC1 and SA was achieved at 100 and 150 mg L^{-1} of Cd^{2+}, and 150 and 200 mg L^{-1} of Pb^{2+} ion concentration at the pH range 6.75 and 7.5 respectively. The interactive study with *Zea mays* L. showed significant increase in seed germination in the presence of Cd^{2+} and Pb^{2+} ions thereby proving them as potent plant growth promoters and metal stress reducing biological agents. Hence, the findings of the study suggest that rhizobacterial strains could be a sustainable tool for restoration of metal contaminated sites.

1. Introduction

Heavy metals are recognized as serious environmental health hazard and considered as major barrier to sustainable development, particularly in developing or under developed nations. Their continuously increasing concentration ruins the balance of ecosystem and increases the economic loss along with human health impairments (Lal et al., 2018). In this context, Cadmium (Cd) and Lead (Pb) have emerged as serious toxic environmental pollutants in the past few decades because of their excessive use in manufacturing and agricultural industries (Chen et al., 2016). Elevated concentrations of Cd and Pb enormously pollute the natural soil ecosystem as well as alter or destruct the soil texture by reducing its fertility and nutrient availability, which ultimately affects plant growth severely (Shaheen et al., 2016). The major sources of Cd and Pb pollution into the environment includes mining and smelting of metalliferous ores, burning of fossils fuels, municipal wastes, fertilizers, and pesticide applications etc. (Lal et al., 2018). In the last few decades, enhanced concentration of heavy metals has attracted attention of environmentalists due to various toxic effects exerted by these substances especially, cadmium and lead. Remediation of cadmium and lead polluted soils often involves excavation and removal of soils to secured landfills, a technology that is expensive and requires site restoration (Hölzle, 2018). Alternatively, phytoremediation is a promising and eco-friendly approach for the remediation of toxic metal pollutants from the soil (Cunningham and Berti, 1993). It is a plant based technology that deploys hyper-accumulator plants to remove or reduce toxic metal contents from the soil. However, this technology fails when the concentration of available metal in the soil is beyond the

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Biosurfactant and exopolysaccharide-assisted rhizobacterial technique for the remediation of heavy metal contaminated soil: An advancement in metal phytoremediation technology

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ABSTRACT

Biosurfactant and exopolysaccharide producing plant growth promoting rhizobacteria can be a best tool for increasing the efficiency of plant assisted remediation of heavy metal contaminated soil; because they can thrive in the stressful environment in a luxuriant way, support plant growth and also contribute to the remediation process. Heavy metals are a noteworthy environmental pollutant and are regarded as biosphere hazard. Numerous chemically based techniques are used to enhance the efficacy of phytoremediation; however, most of them are ecotoxic, highly expensive and lead to incomplete removal of pollutants. To mitigate these technical inherent and to ensure complete removal of toxic heavy metals from soil, an advanced biological tool is the use of biosurfactant and exopolysaccharide-producing rhizobacteria. This can be a promising technique, that has been operative in nature and is cost effective, eco-friendly, efficient and having socio-economic importance over other conventional remediation techniques as well as sustainable for the environment. The present article critically reviews the potential role of root-associated metal resistant, exopolysaccharide and biosurfactant producing rhizospheric bacteria to remediate heavy metal contaminated soil and highlight some insight mechanisms for exploitation of plants and associated rhizobacterial interactions for enhancing heavy metal remediation.

Contents

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REVIEW

Bacterial biosurfactants can be an ecofriendly and advanced technology for remediation of heavy metals and co-contaminated soil

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Abstract Environmental pollution due to heavy metals has become a significant drawback as a result of their ecotoxicity. Hence, their remediation is of pressing concern. Many technologies are planned for their remediation; however, most of them are highly expensive and result in incomplete removal of contaminants. So, massive attention has paid to the event and application of the latest biologically techniques, that is effective in remedy and cost, not harming the prevailing surroundings. Hence, application of biosurfactant in heavy metal remediation is one among the recent ecofriendly technique. The present review critically highlights bacterial biosurfactants as a best alternative technique for heavy metals remediation. The review also emphasizes that bacterial biosurfactants can open up a new vista in remediation of metal-contaminated soil.

Keywords Heavy metal · Biosurfactant · Remediation · Soil washing · Soil flushing · Co-contaminated soil

Introduction

The fate of heavy metals is of immense environmental concern due to their persistent occurrence in nature and toxic properties. Heavy metals are electronegative elements with a density greater than 5 g/cm^3 (Duffus 2002). They are non-biodegradable in nature which is the main reason responsible for their prolonged persistent in the environment, and as a result, they pass from one level to another in the food chain causing many diseases and blocking the biological pathways (Tangahu et al. 2011). Comparably, accumulations of toxic heavy metals in soil and water bodies also have a detrimental effect on the ecosystem (Baeicii and Stotzky 1983; Sobolev and Begonia 2008). Hence, the presence of trace amount of heavy metals in the soils has been found to have serious hazardous effect. There are various techniques for remediation of heavy metal such as physical, chemical, biological and phytoremediation, but most of them are quite expensive and risky. So, large amount of attention has been paid on the development and implementation of new biologically techniques, which should be effective in remediation, easily available, not harm the existing environment, ecofriendliness and cost-effectiveness an alternative of conventional techniques, which are efficient at lower levels of contamination. Hence, application of biosurfactant in remediation of heavy metals is one of the recent ecofriendly techniques. Biosurfactants are diverse group of surface-active compound produced by microorganisms, which possess both hydrophilic and hydrophobic moieties. Structurally, they possess a hydrophobic moiety comprising of saturated or unsaturated fatty acids or hydrocarbon chains and a hydrophilic moiety of peptide cations or anions, mono-, di- or polysaccharides acid (Kiran et al. 2010; Muthusamy et al. 2008). Biosurfactants are potential compounds use in environment management, food industry, petroleum industry, pharmaceutical industry and other industries as these are environment friendly, easily degradable, economical and stable at elevated pH, temperatures and salt concentrations as compared to their

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Review Article

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Synthesis of Organic Nanoparticles and their Applications in Drug Delivery and Food Nanotechnology: A Review

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Abstract

Organic nanoparticles, nanocrystals and nanobeads are of major interest in material and life sciences. Biopolymer nanoparticles are offering numerous advantages that embrace the simplicity of their preparation from well-understood biodegradable, biocompatible polymers and their high stability in biological fluids during storage. Several types of polymers have been tested as potential drug delivery systems; including nanoparticles, dendrimers, capsosomes and micelles. In the present review, synthetic methods for the preparation of organic nanoparticles, types and significant applications of organic nanoparticles have been reviewed with suitable examples.

Keywords

Organic nanoparticles; Biopolymer; Drug delivery; Nanotechnology; Food nanotechnology

Introduction

Nanoscience and its applied aspect Nanotechnology is the science of the new era which is finding application in many areas including electronics, agriculture, defence, drug designing and drug delivery, fermentation technologies, food and food processing industries, chemical industries etc. It is about manipulating matter at the atomic or molecular scale, defined as: "*The study of process and fine-tuning of requisites at atomic, molecular and macromolecular scales, where premises differ profoundly from those at a larger proportion*" [1]. Drug delivery and food industry is one of the major fields where the scope of this branch is enormous.

Nanoparticles: A nanoparticle (NP), a solid colloidal particle is defined as "*a discrete entity with at least one dimension being 100 nm or less*" [2]. However, most of the particles utilized in drug delivery are in the size of 100-200 nm [3]. Because of their small size, NPs have different surface to volume ratios and surface properties become more important. These properties give NPs their unique and potentially toxic features compared to the bulk material or their separate molecules. Among these features, increased electrical conductivity, and improved hardness and strength are very interesting for the electronic, medicine, textile, defense, food, agriculture, cosmetics and aerospace industries and these are presently being applied widely throughout these industries. In food and agriculture systems, nanotechnology covers many aspects, such as food safety, packaging materials, disease treatment and new tools for molecular and cellular biology [4].

Organic Nanoparticles: Thousands of organic chemicals are present in various pharmaceuticals to consumer products being used like inks, dyes, flavouring agents and household cleaning products. In many of these products, the organic chemicals are dissolved to aid formulation or are chemically modified to improve their performance. If a chemical is insoluble in a liquid that is required for formulation, its activity and applicability is significantly limited. For instance, pharmaceutical products often have restricted bioavailability and efficacy due to their insolubility in water. This may either restrict the development of new drugs or the scope of current medicine. The same is true for nutraceuticals, biocides and a range of other potentially useful compounds. By forming very small dispersions of organic compounds, insoluble materials can be made to behave more like truly dissolved molecules without the need to produce new chemicals or use flammable, toxic or volatile solvents. This option is a valuable tool in the manufacturing and development of new products because the scope of chemical ingredients that becomes available offers huge potential for innovative and competitive product design.

Organic nanoparticles can be explained as solid particles composed of organic compounds (mainly lipids or polymeric) ranging in diameter from 10 nm to 1 μm [5]. They have received relatively little attention as compared to inorganic materials where enormous research and commercial investment has been made. The future benefits of inorganic nanoparticles, such as quantum dots, silicas, gold nanoparticle, titania and various catalysts, are not in question but grain nano solutions see a much larger commercial opportunity in the thousands of insoluble or poorly-soluble organic compounds that are used across many high- technology and commodity product areas. The pharmaceutical industry has led the research into organic nanoparticles over recent years. The search for nano-medicine has driven the development of new materials and the refining of well-established techniques. The "Bottom-up" synthesis of nanoparticles capable of encapsulating or carrying active molecule has seen the production of dendrimer technologies, protein conjugates, DNA delivery vehicles, liposomes and shell-cross-linked block co-polymer micelles, to name just a few. 'Top-down' techniques have focused mainly on attrition approaches such as wet nano-milling to grind large particles and achieve particle distributions with sub-micron average particle diameters. Many 'wet' approaches to form colloidal dispersions from liquid emulsion exist which typically rely upon the selective removal of the oil phase of an emulsion and subsequent solidification via precipitation, crystallization or encapsulation of any organic material that were dissolved within the solvent droplets. So far, none of these techniques has proved truly generic and they have been limited to specific classes of materials, classified either by their chemical reactivity or by their physical properties. Organic

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Petroleum Hydrocarbon Stress Management in Soil Using Microorganisms and Their Products

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CONTENTS

Chapter 3

Bioremediation of Petroleum Hydrocarbons and Heavy Metal Contaminated Sites by Biosurfactants: An Eco-friendly and Sustainable Technology

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SUMMARY

With the rapid industrialization and increasing demand for petroleum hydrocarbons as a source of energy, has resulted in its increased extraction, refinement and use. But, this increase in extraction and refinement has led to the contamination of soil and groundwater and needs utmost attention as it finds an easy entry through the food chain. Besides this, heavy metal contamination is another problem, which has aggravated due to industrialization. Various remediation technologies have been proposed for management of petroleum hydrocarbons and heavy metal contaminated soil but most of them are expensive and lead to incomplete decomposition of contaminants. So, large attention has been paid on the development and implementation of new

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