Plant-Microbe Interactions for Phytoremediation of Multi-Metal Contaminated Soil With Two Brassica Cultivars and Bacterial Strains

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This work is a dissertation in partial fulfilment of the award for the degree of

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in

Environmental Sciences

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NOOR U SEHAR

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Abstract

Contamination of the soil and environment with the toxic heavy metals and their metabolites is a serious environmental problem. In terms of contaminating agricultural soil and water, heavy metals lead the list of environmental pollutants. Our hypothesis is that bacteria that can mobilize biologically inaccessible heavy-metal fractions, cause alterations in root exudation, and stimulation of plant development, as well as are responsible for the increased accumulation of heavy metals by plants. Heavy metals may be extensively mobilized by rhizosphere bacteria and root exudates, increasing their bioavailability. The study focused on integration of bioaugmentation and phytoextraction for Cu, Cu, Cd and Pb (multi-metal) contaminated soil remediation in rhizosphere of Brassica juncea and NARC sarsoon in combination with *Bacillus tequilensis, Serratia marcescens* and *Bacillus safensis* strains with the focus on root exudates production and role in aiding the process. The results revealed that the (T12) with the *Brassica juncea* cultivar and consortia of strains demonstrated the highest extraction for Pb (80.79%), Cu (69.00%) and Cd (63.96%) metal extraction as compared to control T2 which extracted metal Pb (17.51%), Cu (20.98%) and Cd (17.69%). The best treatment for *NARC sarsoon* variety was T13 with bacterial consortium and extracted metal Pb (46.60%), Cu (46.94%) and Cd (37.96%) as compared to control T2 which extracted metal Pb (8.66%), Cu (19.52%) and Cd (11.48%). Variations in biochemical stress indicators showed significantly lower levels of MDA and H2O2, as well as higher levels of chlorophyll a (0.591 mg/g FW), Chl b (0.363 mg/g FW), total chlorophyll (0.404 mg/g FW), and carotenoids (151 mg/g FW) for T12 and comparable level of chlorophyll a (0.353 mg/g FW), Chl b (0.226 mg/g FW), total chlorophyll (0.580 mg/g FW), and carotenoids (114.8 mg/g FW) for T13. Integrated bio-augmentation and phytoextraction approach has a great potential for remediation of heavy metals rather than a single biological method.

Key Words: Phytoremediation, Bioaugmentation, *Brassica,* Cadmium, Lead, Copper, Root Exudates

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List of Abbreviations

AC	Abiotic control
APX	Ascorbate peroxidase
B	Bioaugmentation
B+P	Bioaugmentation + phytoremediation
CAT	Catalase
Car	Carotenoid
CFUs	Colony forming units
Chl a	Chlorophyll a
Chl b	Chlorophyll b
EC	Electrical conductivity
EDTA	Ethylenediamine tetraacetic acid
FW	Fresh weight
GPX	Guaiacol peroxidase
MDA	Malondialdehyde
NPs	Nanoparticles
OM	Organic matter
OOC	Oxidizable organic carbon
PC	Plant control
ROS	Reactive oxygen species
SOD	Superoxide dismutase
T chl	Total chlorophyll
TDS	Total dissolved solids
TOC	Total organic carbon
UV	Ultraviolet

Chapter 1

Introduction

The initiative focuses on identifying the optimal mixture of *Brassica* cultivars and bacterial strains known to facilitate the phytoremediation of heavy metals, specifically Cd, Cu, and Pb. *Brassica* plant variants have been found to hyper-accumulate metals like copper, lead, cadmium, and zinc, according to numerous reports. The growing industrialisation, agrochemical use, and irrigation with polluted waters are to blame for the increased metal ratio in agricultural soil. The widespread and ongoing use of copper as fertilizers and fungicides in agriculture and horticulture raises serious concerns about copper pollution of agricultural soils. Other significant sources of copper pollution abound. These include the emission of copper caused by the combustion of fossil fuels, especially coal. Additionally, Cd, the sixth most hazardous heavy metal, disrupts ecosystems and physiological processes in organisms when it leaches out of phosphate fertilizers or enters soil through industrial effluent. Furthermore, Pb from the petroleum sector, coal, and fertilizers has a major negative impact on soil, plants, and animals' health.

According to our hypothesis, increased plant accumulation of heavy metals results from bacterial mobilization of biologically inaccessible heavy metal fractions in soils, changes in root exudation or plant gene expression, and stimulation of plant development(I. Ali et al., 2022). In this investigation, we employed two types of *Brassica* cultivars, inoculated with three strains, *Bacillus tequilensis, Serratia marcescens* and *Bacillus safensis,* separately and in consortia to remove Cu, Pb, and Cd-contaminated soil. (Li et al., 2019). Each variant's control pots won't receive an immunization. In order to determine the optimal plant-microbe combination based on plant yield and metal concentration in the roots and shoots of both kinds, plant and soil studies were carried out after harvest.

1.1. Impacts of Heavy metals

An element must have an atomic number greater than 20, an atomic density greater than 5 g/cm3, and metal-like properties in order to be classified as a heavy metal (HM). Heavy metals (HMs) can be broadly categorized as essential and non-essential. Living things

need essential HMs to carry out their basic functions, including growth, metabolism, and the formation of various organs(Koller & Saleh, 2018). Cu, Fe, Mn, Co, Zn, and Ni are only a few of the essential heavy metals that plants require because they work together to create cofactors that are essential for the structural and functional integrity of enzymes and other proteins. Micronutrients are necessary substances that are usually required in trace amounts (between 10-15 ppm). Plants don't need heavy metals like Cd, Pb, Hg, Cr, or Al for any metabolic action, not even in trace amounts. shows how lead, cadmium, zinc, and copper poisoning manifests in diverse plant species(Singh et al., 2016). Exposure to heavy metals, which are detrimental to human health, has increased as a result of anthropogenic and industrial activity as well as modern industrialization. A significant environmental hazard that affects hundreds of millions of people globally is toxic metal poisoning of water and air.

Table 1.1 Maximum permissible limits for toxic heavy metals concentration in irrigation water, soil, and plants

Source: (European Union, 2002; WHO/FAO, 2007).

Metal (µg/g)	Irrigation Water (µg/mL)	Solid (µg/g)	Plant
Lead (Pb)	0.015	300	0.30
Cadmium (cd)	0.01	3	0.2
Chromium (Cr)	0.10	150	5
Arsenic (As)	0.01	20	0.1
Nickel (Ni)	1.40	50	67
Mercury (Hg)	0.01	30	0.03
Copper (Cu)	0.20	140	40
Iron (Fe)	0.50	50,000	450
Zinc (Zn)	2.0	300	60
Manganese (Mn)	0.20	80	500

Heavy metal pollution in food is another issue that is problematic for both human and animal health. In this regard, heavy metal concentrations in water, air, and food are measured(Tchounwou et al., 2012) Metals can exist naturally and last a long time in the environment, just like other types of contaminants. Thus, it is inevitable for humans to come into contact with metals, and some studies have revealed gender-specific

differences in metal toxicity. They regularly interact with biological systems, losing one or more electrons and creating metal cations that have an affinity for key macromolecules' nucleophilic sites. On different human organs, heavy metals produce a variety of acute and long-term harmful consequences. Heavy metal toxicity can result in immune system dysfunction, cancer, birth defects, gastrointestinal and kidney dysfunction, nervous system disorders, skin lesions, vascular damage, and skin lesions.

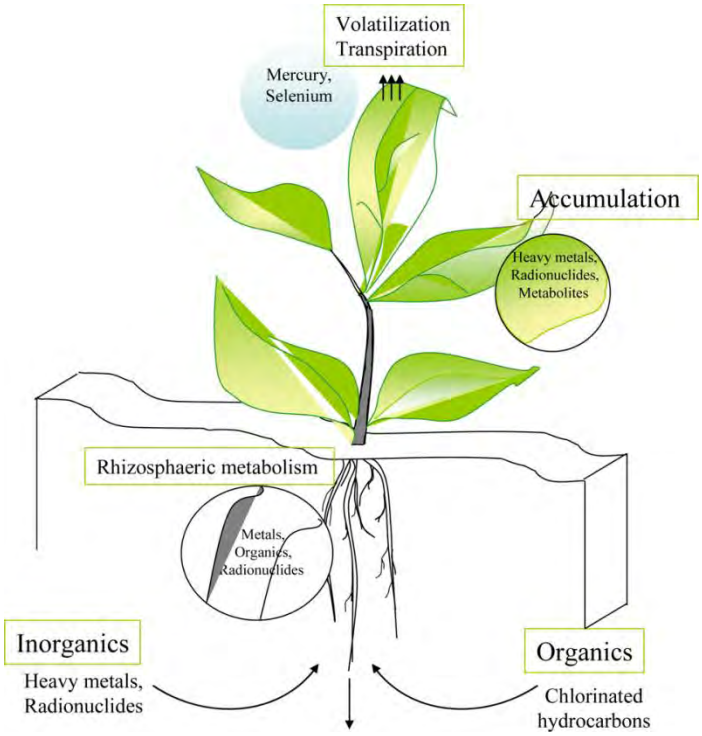

Figure 1: Phytoremediation of Heavy Metal

1.2. Copper

With an atomic mass of 63.5 g mol1, an atomic number of 29, and a density of 8.96 g cm3, copper (Cu) is a transition metal. It is the third most 3ignalin substance on the globe and the 25th most prevalent chemical component in the Earth's crust7.

Cu contents naturally range from 60 to 125 mg kg1 in soils. It appears in the physiological forms Cu+ and Cu2+ and is a crucial micronutrient for plant growth. Cu is a structural protein that takes part in hormone signaling, oxidative metabolism, cell wall metabolism, and the electron transport cycle of photosynthesis and respiration. The proteins with more than one Cu atom are ascorbate oxidase, Zn/Cu superoxide dismutase, and cu amino oxidase (8, 2, and 2 Cu atoms, respectively). Despite being inefficiently

mobile in plants, Cu2+ or Cu chelate is absorbed and can move from old to new leaves. It has a modest concentration in plant dry mass, typically between 2 and 20 mg kg1. However, most plants are poisonous at concentrations between 20 and 100 mg kg1 in the dry mass of plants. Copper soil pollution is largely caused by industrial, mining, and agricultural operations. Cu enters agricultural soils mostly through the intense application of copper-containing agrochemicals or swine manure.

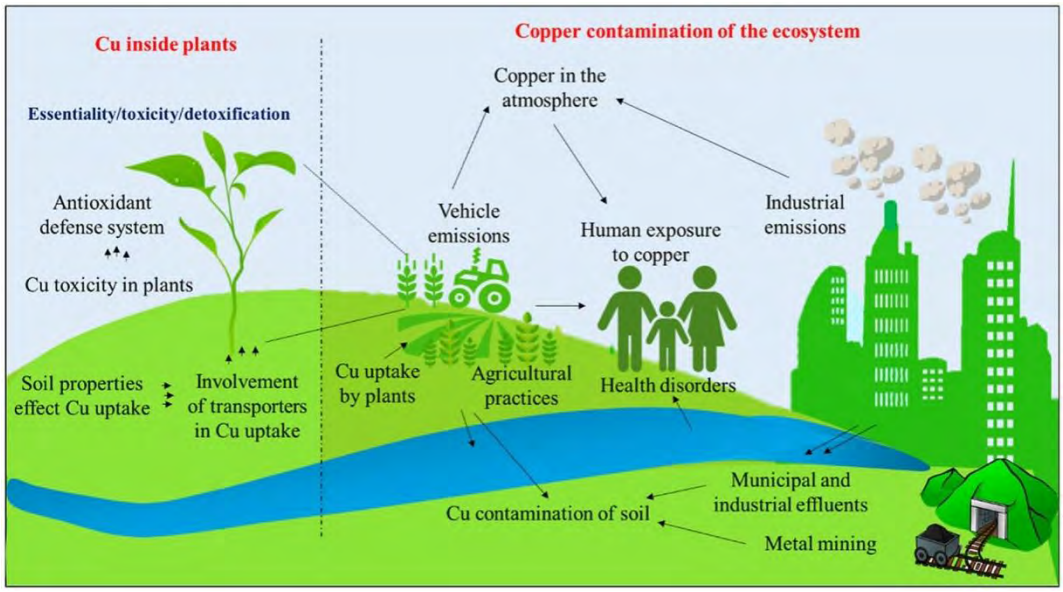

Figure 2: Copper Contamination

This scenario raises concerns since it suggests that in order to supply the demand for plant and animal feeds on a worldwide scale, crop acreage in agriculture would need to be increased. As a result, there will be an increase in the use of Cu-containing agrochemicals for phytosanitary purposes9. In 2050, nine billion people are expected to live on the planet. Through the cells of the root epidermis, plants absorb mineral nutrients from the soil matrix, and they subsequently transport those nutrients to the centre of the plant through the parenchyma, endoderm, and xylem. To support this unidirectional channel of transition-metal absorption, several metal transporters collaborate with other metal transport molecules that sequester or chelate ions. This ensures that enough ion absorption and transport happens in all plant tissues during the metagenesis of plants. Copper (Cu) is an essential element for plants due to its role in a number of redox

processes and the structure of the Fe-Cu cluster10. Despite being necessary in minute amounts at dangerous levels, copper (Cu) causes physiological and biochemical anomalies that hinder plant growth.

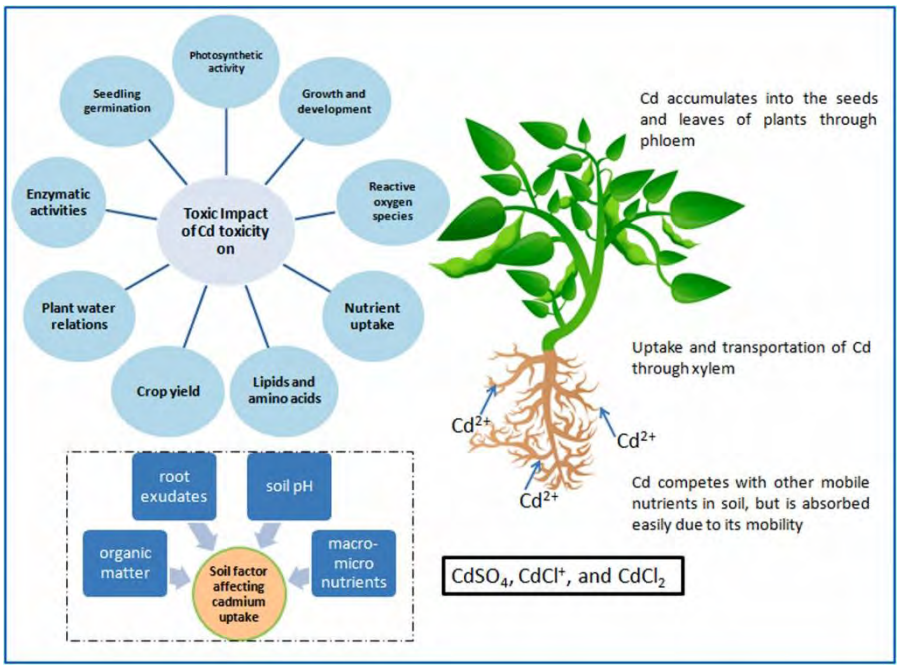

Figure 3: Metabolites of Copper

In higher plants, the average range of Cu content is 2 to 20 mg Cu kg1 DW. Cu toxicity may develop at the upper limit of this range if the plants are unable to withstand the stress brought on by hazardous levels of Cu. Understanding the physiological and biochemical mechanisms of Cu toxicity in plants is crucial for the selection of more tolerable genotypes based on biochemical and physiological indicators to heavy metal stresses given the growing agricultural and industrial activity, which are the main sources of Cu addition in nature. We suggest conducting a thorough analysis of plants raised in environments with hazardous levels of Cu based on their physiological, biochemical, and anatomical responses. Understanding these reactions will assist to better understand the fundamental processes of stress tolerance by toxic amounts of copper in higher plants, offering helpful knowledge for the development of genotypes resistant to toxic levels of copper in plant culture medium(Angulo-Bejarano et al., 2021).

1.3. Cadmium

From the standpoint of toxicity, cadmium (Cd) is a substance that should be avoided

because exposure to it can have both long-term and short-term consequences on the health of living things. Ocean water and the crust of the planet both naturally contain cd. Cd generally ranges in concentration in terrestrial habitats between 0.1 and 0.2 mg/kg, whereas the average concentration in ocean waters is 5 to 110 ng/L. Cadmium and its compounds are classified as Group 1 carcinogens by the International Agency for Research on Cancer because they cause lung cancer and have been positively associated to kidney and prostate cancer. Additionally, cd toxicity can result in kidney, bone, lung, and itai-itai problems. The WHO-established acceptable value for Cd in drinking water is 0.003 mg/L (Jaishankar et al., 2014). High Cd levels in Pakistan's drinking water may result from the metal plating, mining, and the effluent discharges of the marble, steel, and aluminium sectors. Cd levels in ground water samples collected from various areas in Pakistan ranged from 0.001 to 0.21 mg/L.

The samples from the tube well water in the Khyber Pakhtunkhwa (KPK) province's Hayatabad Industrial Estate ranged from 0.02 mg/L to 0.21 mg/L13. Similar to this, there are wide regional variations in the amount of Cd found in surface water samples, which can range from less than the limit of detection to 0.2 mg/L. Additionally, Cd was undetectable from March to April in a surface water sample from the Kalar Kahar lake in Chakwal, although it fluctuated seasonally from 0.01 to 0.05 mg/L in the other months of the year. S Surface water samples collected in the NWFP province (formerly known as KPK) showed Cd values ranging from 0.002 to 0.09 mg/L (mean 0.02 mg/L)14, with the highest result coming from the Kalpani drain. Similar variations were seen in the Malir River in Karachi (Sindh province), ranging between 0.002 and 0.07 mg/L Cd (mean 0.04 mg/L) from the same study.

Numerous studies show that wastewater samples collected from various areas in Pakistan contain significant amounts of Cd. The highest concentration of Cd in wastewater ever discovered was 5.35 mg/L, above the NEQS-Pak-set permissible limit of 0.10 mg/L for industrial and sewage effluent. This quantity was found in the Karachi neighborhood of Korangi. Additionally, the permitted limit imposed by NEQS was exceeded in the north and east of Lahore, Punjab province, where the concentration of Cd in wastewater ranged from 0.18 to 0.37 mg/L. Cd fluctuated between 0.19 and 0.62 mg/L, according to another

study on the efficacy of wetlands for eliminating heavy metals from industrial wastewater in Swabi (KPK province).

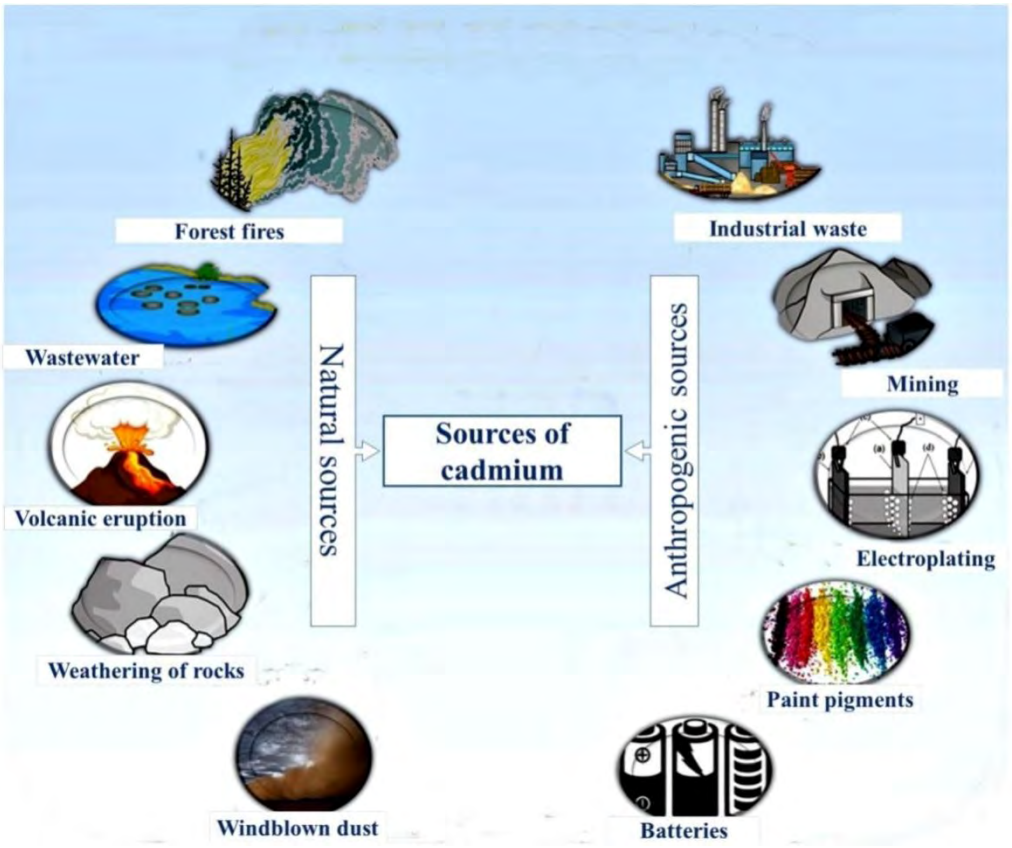

Figure 4: Sources of Cadmium

Both natural and man-made sources, such as mine/smelter wastes, phosphate fertilizers, sewage sludge, and municipal waste landfills, contribute to the amounts of Cd found in soil and sediments. On a global basis, Cd concentrations in sediments have been shown to range from 0.03 to 1 mg/kg in marine sediments to 5 mg/kg in river and lake sediments and references therein.

The levels of Cd in the soil of the various regions of Pakistan vary noticeably between the selected areas, ranging from 0.02 to 184 mg/kg in healthy soil to polluted soil from mining or other activities. In a different investigation from the district of Sargodha, the highest levels of Cd in the soil were discovered to be 6.74 mg/kg. The forage's Cd accumulation in the range of 1.14 to 4.20 mg/kg indicated that there may be a risk of Cd entering higher food chains given the greater amounts of Cd in the soil(Wuana &

Okieimen, 2011).

The soil of Pakistan's capital city, Islamabad Territory, and the dust road that runs alongside the Islamabad Expressway both contained Cd amounts of 5.8–6.1 and 4.5–6.8 mg/kg, respectively. Similar to the Aqaba-Shuna Highway (Jordan) and the Istanbul Highway, their values are higher than those of many other cities throughout the world (Turkey).

Siddique et al. found that the greatest concentration of Cd in sediments was 24.34 mg/kg at the Gizri Creek site in the Malir River's most downstream portion in Karachi. The Lyari area in Karachi, where the Lyari River drains into the Arabian Sea to transport garbage away from the city, got the second-highest value. In addition, the mean Cd concentrations in the River Ravi sediments in the Punjab province ranged from a maximum of 3.17 mg/kg (at Shahdera Bridge) to a minimum of 0.99 mg/kg at Lahore Siphon. Toxic metals can enter the human body by the ingestion of contaminated food crops, water, or dust. Numerous researches from Pakistan have raised the possibility that heavy metals can spread to vegetables or food crops. In Quetta, Balochistan region, lettuce irrigated with varied wastewater concentrations had a critical dangerous level of Cd of 5.63 mg/kg (on average), with a reference/control value of 2.498 mg/kg. The accumulation of elevated Cd concentrations was attributed to the cultivation of lettuce using wastewater effluents.

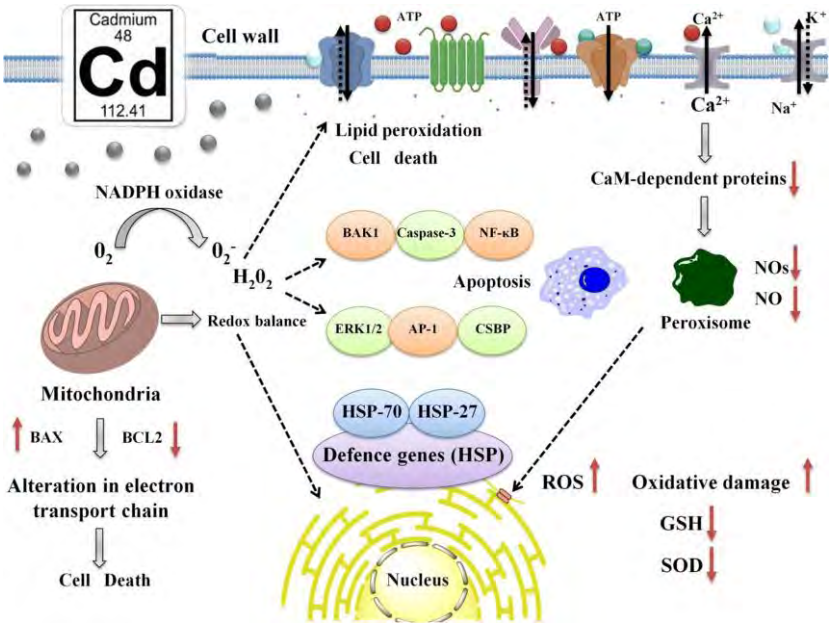

Figure 5: Fate of Cd in Cell

The crucial hazardous level of Cd uptake has been clearly identified by Cd concentration data collected by several researchers in numerous regions of the country for a variety of plants, including lettuce S. oleracea had the greatest concentration of Cd and M. sylvestris had the lowest in Gilgit, Northern Pakistan, where the mean Cd levels in all vegetable samples ranged from 0.24 to 2.1 mg/kg(S. Khan et al., 2010).

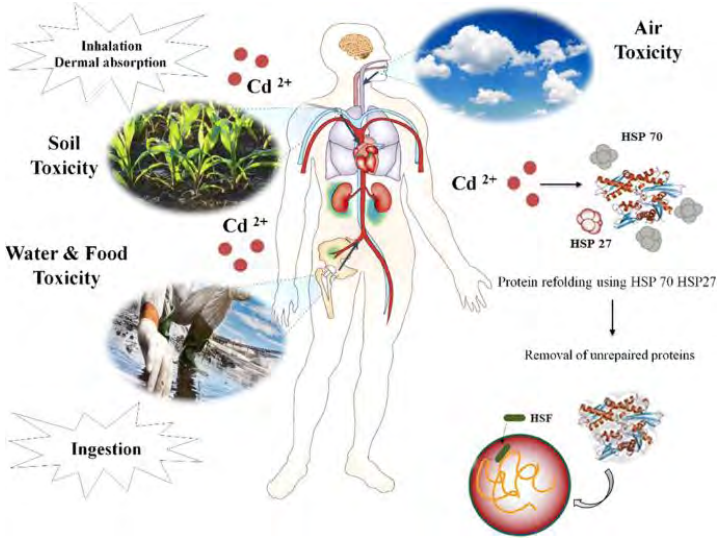

Figure 6 Cadmium Adverse Impacts on Environment

Many metals can enter humans through one of two different routes; the other is through

food ingestion. Heavy metal poisoning in the air is a serious health concern since it enters our lungs as soon as we breathe it in. The WHO suggested a recommended threshold for air, of 5 ng/m3, in consideration of the effects of Cd on health. In the atmosphere, heavy metals are typically found as part of tiny particles known as particulate matter (PM10 or PM2.5)(López et al., 2005). Outdoor air pollution and its particle matter have just been labelled as human carcinogenic by the IARC Working Group (IARC Group 1) The bulk of Pakistani studies report an average airborne Cd content in suspended particulate matter of less than 5 ng/m3. However, a study from Lahore found that PM2.5 has an annual mean Cd concentration of 69 ng/m320(Rasheed et al., 2015).

1.4. Lead

Lead (Pb) exposure can cause a range of health problems in both children and adults, from IQ and metabolic alterations to convulsions, renal failure, and even death. The International Agency for Research on Cancer states that while inorganic lead compounds are possibly carcinogenic to humans, organic lead compounds cannot be categorized as such (Group 2A) (Group 3)(Sanders et al., 2009)

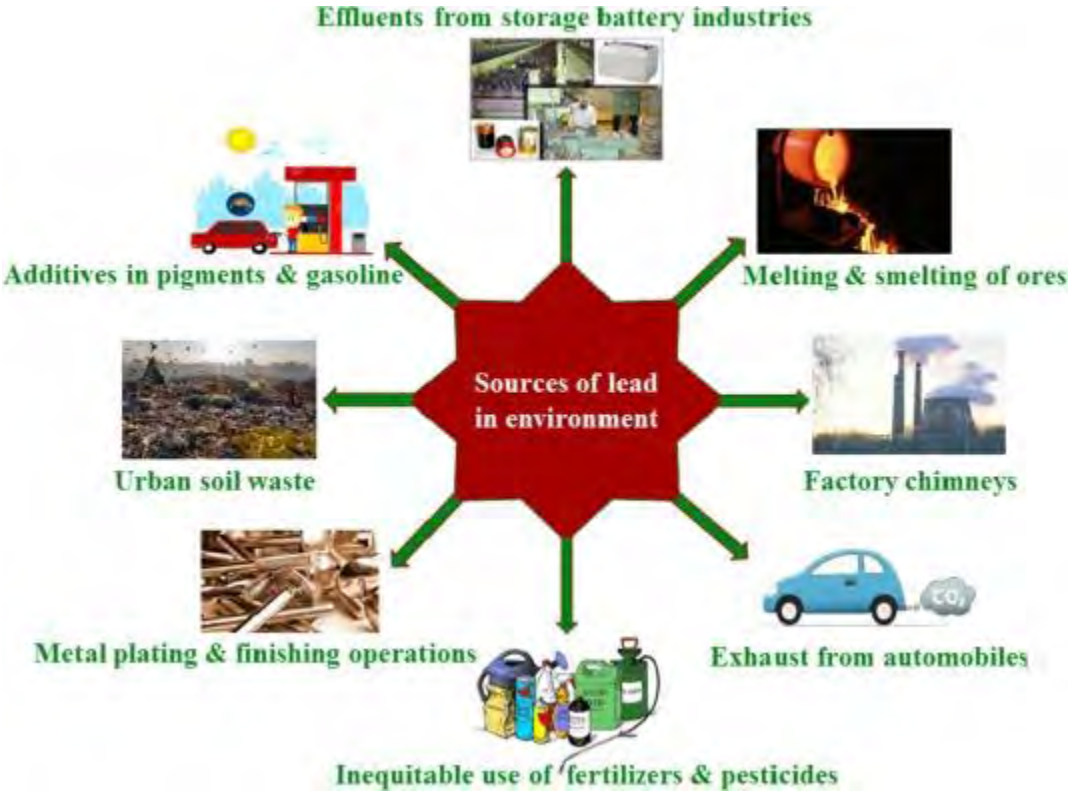

Figure 7: Sources of Lead

Lead is found in small amounts as lead sulphide (galena) in the earth's crust, but lead is widely distributed throughout the environment as a result of human activity. At any stage of the process, from mine to final usage, pb contaminates crops, land, water, food, air, and dust. The majority of Pakistan's groundwater samples came back above the 0.01 mg/L WHO permissible level for drinking water, and Pb contents vary from 0.001 to 4.7 mg/L depending on the location. The sample from the Pearl valley in Azad Jammu and Kashmir has dissolved levels of Pb between 1.8 and 4.7 mg/L. (AJ&K)(Javaid et al., 2008). A comparison of water samples collected from the Kharick II well with WHO guideline values revealed Pb concentrations that were 466 higher (South, AJ&K) The majority of the ground water samples in Hattar Industrial Estate (KPK) had concentrations above the limit of 0.01 mg/L, with an average of 0.26 mg/L .

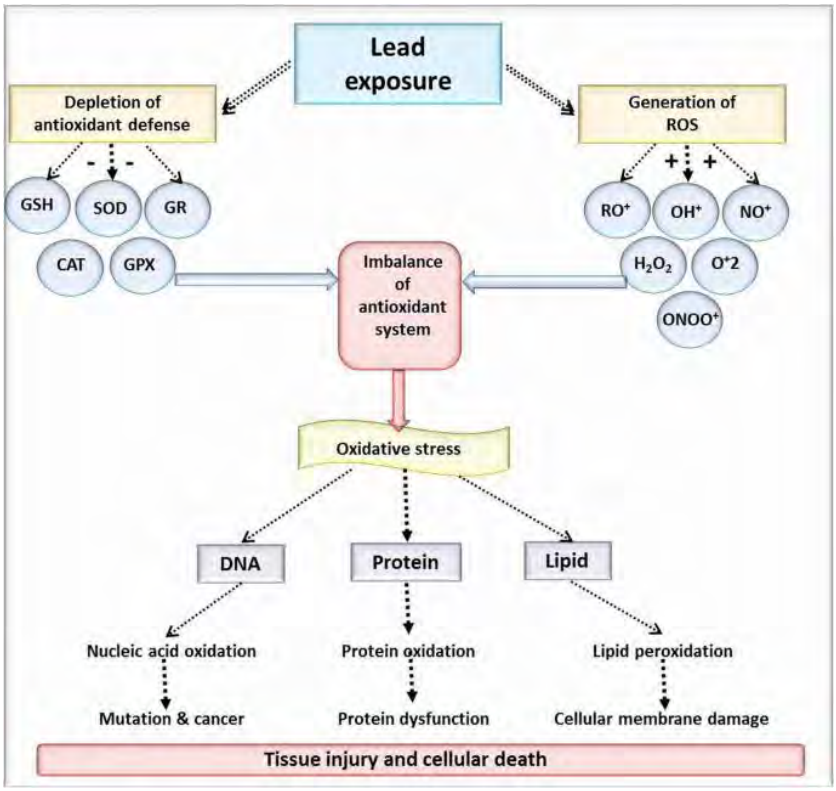

Figure 8: Lead Exposure to Cell

In a similar vein, 100% of the samples tested in Sialkot, Punjab province, had lead levels in drinking water that were higher than the cutoff point (0.01 mg/L). Individual investigations showed that Pb levels in both surface and ground waters were higher than the permissible limits in a greater percentage of the nation's water sources. The Bara River water in the Akbarpura area in the district of Nowshera, KPK, had the highest result of 0.62 mg/L24, with the average Pb concentration in surface waters thought to be substantially higher. More than 50% of the examinations found that Pb levels in wastewater samples were higher than the permitted limit of 0.50 mg/L set by Pakistan's National Environmental Quality Standard. The highest Pb contamination (2.34 mg/L) was discovered in samples taken from three textile industries in Hattar Industrial Estate, KPK. Wastewater channels are therefore the most dangerous for soil, plants, and other species, including people, due to their high Pb level(Waseem et al., 2014). The lead (Pb) level of ordinary soil that the European Union applies sewage sludge to is far lower than the permitted lead threshold (50-300 mg/kg). The one location in Kohistan region, Gilgit

Baltistan province, where the maximum Pb concentration of 103000 mg/kg (mean 1753 mg/kg) was discovered in contaminated soil during mining operations with mean reference soil value of 70 mg/kg, is the only exception to the aforementioned statement. Additionally, the pollution of heavy metals, particularly 26 Pb, in roadside soil is connected with the volume of traffic on the roadways. The fifth-largest industrial city in Pakistan, Hyderabad Metropolis, has a mean Pb concentration of 36.45 mg/kg along National Highway 5, whereas Sindh Province has the highest concentration, 176 mg/kg, near to the city's bypass route(M. N. Khan et al., 2011).

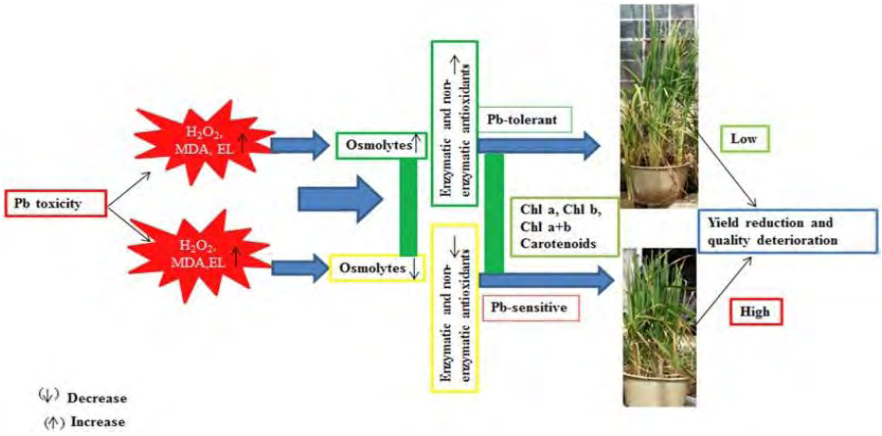

Figure 9: Lead Toxicity

The highest lead concentrations were found in the coastal sediments of the Arabian Sea along the urban Karachi coast in Pakistan's varied coastal districts, at 121 mg/kg, followed by 49.5 mg/kg in the surficial sediments of the Lyari River28.

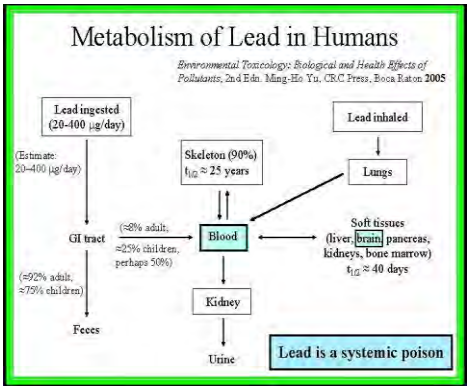

Figure 10: Lead in Humans

With annual emissions from industrial coal and oil combustion estimated at 450 million kg and from natural sources at 30 million kg, lead has been identified as one of the

hazardous components of airborne PM. It's likely that increases in Pb level in certain regions are the result of heavy traffic, brick kilns, and the usage of leaded gasoline. Pb concentration in the urban air of Islamabad has decreased as a result of the use of Pb-free gasoline in recent years, but the Pb content is still high, ranging from 0.002 to 4.7 g/m3. Lead pollution appears to be a serious problem when compared to Islamabad's local environment and the WHO air quality criteria for Europe (0.5 g/m3, annual average). In nonurban locations, average lead levels are often less than 0.15 g/m3, while in most European cities, urban Pb levels typically range between 0.15 and 0.5 g/m3(*Basic Information about Lead Air Pollution | US EPA*, n.d.).

Vegetables may contain up to 0.3 mg/kg of lead, according to the European Union. In Pakistan, pb levels in several vegetable species range from 0.03 to 44 mg/kg, with Sylvester's from Gilgit having the highest level (Northern Pakistan). In another study, the average Pb concentration in the edible and leafy sections of vegetables was found to be 15.58 mg/kg and 27.49 mg/kg, respectively. It was discovered that 83% of the vegetable samples had levels of the edible component that were much higher than the EU permitted limit. The bulk of studies focused on vegetables grown on contaminated soils brought on by human activities like mining, such as sludge- or wastewater-treated soil(Hamid et al., 2017).

1.5. Exposure routes

To depict exposure routes, toxicologists use pharmacological drug disposition models. A distinct organ is ultimately harmed by contaminants that enter the human body. The physiologically effective dosage, which is the contaminant concentration or dose at the target organ, has the strongest correlation with the severity of the adverse effect. The exposure route is the internal pathway taken by the poison as it travels from the exposure point to the target organ.

The contaminant must first pass an intake barrier, such as the lining of the nasal passages or lungs, at different points throughout the gastrointestinal system, or the skin, in order to begin the exposure pathway, which can be oral or dermal and provides a potential dose to the person. The potential dose is generally lower than the concentration at the exposure point because intake is frequently less efficient than 100% of the time. Following

ingestion, the contaminant is dispersed throughout a section of the human body. Due to the distribution volume and clearance capacity, the applied dose that protects the organ from potentially harmful substances and passes through the biological barrier (vascular, renal, placental, blood-brain, etc.) is decreased (i.e., elimination). The process of contaminant absorption involves a chemical penetrating a biological barrier and, because of the biological barrier's capacity for transport, producing an internal dose that is lower than the given dose. Finally, the internal dose is further decreased to the previously reported biologically effective dose by metabolic and evaporative mechanisms (reverse transport across the biological barrier).

Selective ion transporters are necessary for eukaryotes, creatures whose cells contain a nucleus, to control absorption over the cell membrane and into the cytoplasm. The biological barrier to the ingestion of harmful inorganic compounds is provided by the selectivity of these ion transporters. There is no evidence that any of the known ZnT zinc efflux transporters export cadmium ions from mammalian cells; rather, zinc homeostasis is maintained in mammalian cells through a combination of import and export transporters. Nevertheless, there are still alternative ways for cadmium to escape the body, such as by the efflux of Mn2+ or cadmium complexes with ligands like cysteine or glutathione. the cytoplasmic processes that initiate the elimination of arsenate by mammalian cells. These processes include intracellular decrease of As(IV), followed by methylation and export of As (III)(*Metal Transport across Cell Membranes Occurs by Three General Mechanisms*, n.d.).

1.6. Conventional physiochemical remediation methods

Since soil contamination by heavy metals has captured our attention for some time, it is clear that innovative reclamation strategies are urgently required. Although traditional techniques have been in use for a long time and have produced excellent results in the rehabilitation of heavy metal-contaminated soil, they have drawbacks. The chemical and physical procedures usually result in by-products (toxic sludge or contaminants), whilst the biological process is extraordinarily sluggish and time-consuming. They are not cost-effective. They are conquered using a mix of two or more strategies. These facts have led to the development of innovative biosorption, nanoremediation, and microbial fuel cell

technologies that make use of the metabolic functions of microorganisms for bioremediation. These remediation techniques are effective and affordable, and their use in bioresource and environmental technologies is expanding. Additionally, we have talked about combining the aforementioned procedures with more recent research on physiochemical and physiobiological methods to remove heavy metals from contaminated soils. These mixtures have shown synergistic effects that significantly increase the effectiveness of removing heavy metals while being commercially feasible(Kirpichtchikova et al., 2006).

Since the beginning of industrial modernization, human activity has been the main cause of the atmospheric release of heavy metals and organic pollutants. The ecological system's foundational component, soils, are severely polluted The physical, chemical, and biological characteristics of soil are being weakened by processes like pollution, salinization, and erosion. Numerous harmful substances have been released and deposited in the soils as a result of pollution. On the social, economic, and environmental fronts, contaminated soils clearly have negative effects (Karimyan et al., 2020).

Heavy metals are described as metallic elements that are toxic even at very low concentrations (less than 1 ppb) and have a high density and atomic weight. These metals are necessary for all biological systems, and they must be present in the right concentrations. They impede the metabolic activities of other metal ions and chemical functional groups that are present in our body when present in higher amounts. They have a tendency to lessen the body's metabolic processes at incredibly low quantities. These heavy metals can occasionally alter the active sites of biological molecules, making them dangerous to both microorganisms and large animals. In the worst-case circumstances, some metals might even reappear in humans after climbing the food chain (a process known as bioaccumulation)(Borrill et al., 2019).

Co-contamination is the term for the presence of heavy metals along with various other industrial pollutants in polluted soils, and it has grown significantly over the past ten years. Heavy metals are more difficult to bioremediate than organic pollutants since they cannot be metabolised and can either be changed into less hazardous forms or immobilized to reduce their bioavailability[41]. Mineralization of organic pollutants can

produce carbon dioxide and water. Furthermore, heavy metals hinder the biodegradation of both organic and inorganic contaminants, making it challenging to clean up co-contaminated soils.Due to widespread industrial activities and the untreated distribution of wastes containing these metals, pollution caused by heavy metals has elevated to the top of the list of problems. Its toxicity to humans, plants, and animals is also becoming a major medical/health concern. Agricultural practices and industrial effluents both cause significant heavy metal contamination(H. Ali et al., 2019).

1.7. Physicochemical methods

Because they work better together than alone, physical separation and chemical extraction techniques for soil restoration are combined into physiochemical treatments. The following discussion covers various physiochemical techniques for cleaning up polluted soils(I. Sharma, 2020).

1.7.1 Soil washing

In soil washing, aqueous chemical extraction on a solid substrate is used to remove the majority of pollutants from the bulk soil fraction. Heavy metal contaminants are aggressively combined and scraped with a washing liquid from the polluted soils during this ex situ cleanup method. Recently, the use of low-frequency ultrasonic waves helped contaminants desorb due to macroscale mixing and microscale sonophysical effects(S. Sharma et al., 2018) Working in low-acid environments and requiring less washing liquids are two benefits of this approach. Because of a variety of factors, soil washing is no longer a viable option.(Ahmed et al., 2022)

1.7.2. Chemically activated adsorption

Adsorption is improved by chemically modifying the adsorbate to raise its adsorptive capacity, such as with activated carbon. After 150 minutes of contact, the greatest potential was discovered in a microporous activated carbon that was created from sawdust that had been treated with citric acid and impregnated with ZnCl2 (Nayak et al., 2017).

1.7.3. Ultrasonic leaching

This technique uses an ultrasonic treatment to hasten the extraction of heavy metal from

soils while employing a highly acidic solution. Heavy metals can permeate into the acidic solvent thanks to the breakdown of soil particles caused by sonication, which speeds up the extraction procedure. Zn and Pb were both dissolved to 95%, 92%, and 87.3%, respectively(Park & Son, 2017).

1.8. Physical methods

There is a wide range of physical methods for heavy metal removal from contaminated soils for different types of waste. Almost all impurities can be removed physically using certain approaches. There are negative aspects as well, though. In contrast to other strategies, the pollutants eliminated by physical methods often require additional processing and have a high application cost. The distribution of pollutant particle sizes serves as the foundation for the majority of physical separation methods. Here are a few instances of such physical remedies for soil with heavy metal contamination(S. Khan et al., 2008):

1.8.1. Heat treatment

Using this method, the substrate (such as soil or sludge) is heated to 300–400 °C. During this process, heavy metals and hydrocarbons are heated to extremely high temperatures, which causes them to evaporate. This technique has the advantages of shorter treatment times and complete removal of metals like Cd and Cu (94 and 97%, respectively). The heavy metals can also be removed from the produced ash using thermochemical methods, such as heating the ash to high temperatures of 900–1000 °C while treating it with KCl and MgCl2 (Tomczyk et al., 2020).

1.8.2. Electroremediation

The electrokinesis principle underlies electroremediation. In order to restrict the pollutants close to the vicinity of electrodes, where they can later be retrieved, this procedure applies a low electric current to the contaminated substrate. Basically, electrokinetic remediation operates according to three principles:

- It includes ionizing metals in an electric field that is being applied, which causes heavy metals that are positively charged (cations) to migrate toward the cathode.
- As a result of viscous drag caused by the electromigration of charged ions,

electro-osmosis is the movement of electrolytic ions.

- Colloidal charged ion particle migration is a component of electrophoresis.

When heavy metals migrate to the electrodes, ion exchange, electro deposition, or precipitation can be utilized to remove them(Vidu et al., 2020).

The biggest drawback of this method is the ineffective extraction of heavy metals from soil. According to recent findings, the introduction of boosting agents helped the cations become soluble and move toward the cathode. As novel boosting agents, methylglycinediacetic acid (MGDA) and non-ionic surfactant were employed (EA). The outcomes showed that EK and EA worked together synergistically to remove a significant amount of Hg and PAH (more than 60% of the metals were mobilized). As a result, the employment of boosting agents makes the removal of impurities more practical and economical(Jachuła et al., 2012).

1.8.3. Soil replacement method

The principle behind the soil replacement approach is to replace polluted soils whole or partially, in order to reduce the concentration of pollutants in the soils. This method completely isolates the polluted soil biome and its surrounds to stop it from adversely affecting the nearby natural and typical environment. Three basic operating strategies can be used to summarize the soil replacement process: The first involves simply replacing the soil; the polluted old soil is entirely replaced by the fresh dirt. The key challenge with this approach is the need to inexpensively clean the removed soils in order to stop any potential secondary pollution. The second procedure entails spading or deep diving the soils out of the contaminated area. By using this procedure, the concentrations of the pollutants in the contaminated soils are reduced, resulting in their degradation. The third method, known as soil importing, involves bringing clean material from other locations and combining it with contaminated soil. The aforementioned soil restoration methods are effective but are frequently used when a small area of soil is seriously contaminated due to their higher cost(Azubuike et al., 2016).

1.8.4. Vitrification technology

The process of vitrification involves fast freezing contaminated soils (substrate) after they

have melted at extremely high temperatures, creating solids through the glass transition. This solid creation that resembles glass, also known as a vitrified product, captures and immobilizes the pollutant, keeping them separate from the environment. Leaching activity and low porosity are seen. Therefore, co-contaminated soils can be treated through vitrification.

In this technique, polluted soil is heated to extremely high temperatures (between 1700 and 2000 °C), melting the metals and turning them into vitrified form. This method was used in Japan both before and after the nuclear attack on Hiroshima and Nagasaki to reduce the radioactive waste produced by its nuclear reactors.

1.9. Applied methods for H.M. remediation

Heavy metals and metalloids are capable of remaining in soils as a result of emissions from rapidly growing industrial areas, mine tailings, the disposal of high metal wastes, the use of leaded fuel and paint, the application of fertilizer to land, animal manures, and atmospheric deposition. They are prevented from decomposing biochemically by the fact that the majority of metals are organic pollutants that are transformed into carbon (IV) oxide by biological activity and that their overall presence in soils lasts a long time. Some of the most tried-and-true techniques are bioremediation, immobilisation, nanoremediation, soil cleansing, and phytoremediation.

1.9.1. Bioremediation

As a method of decontaminating soil and other habitats, bioremediation mostly makes use of microbes, plants, or microbial or plant enzymes.

Utilizing fungi to release amino acids, organic acids, and other metabolites to adsorb heavy metals and the minerals that contain heavy metals is known as "microbial remediation," which involves using microbes to absorb, precipitate, oxidise, and reduce soil-borne heavy metals (Siegel et al., 1986). Ex-situ or in situ bioremediation includes the processes of biosorption, bioaccumulation, biotransformation, and biomineralization. For microorganisms to live in environments contaminated with heavy metals, a range of coping methods have emerged (Wasiullah et al., 2015). Pollutants are converted into non-hazardous substances as part of the bioremediation process through biotransformation and biodegradation. Accelerating the breakdown of dangerous organic pollutants in

ground water, soil, chemicals, materials, and sediments to levels that are tolerable for the environment is a step in the bioremediation process (Arpita et al., 2014). Bioremediation can be used to clean up a polluted area.

Cleaning up a contaminated region with biological agents, primarily microorganisms like bacteria, fungus, and yeast, is known as bioremediation. Microorganisms eat pollutants during bioremediation processes as food or as a source of energy. Biotechnology includes both basic research and field applications, with a focus on bioremediation and natural attention (Kumar et al., 2009).

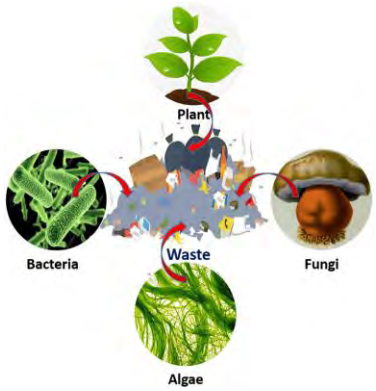

Figure 11: Bioremediation

1.9.2. Bioremediation of Copper, Cadmium and Lead Contaminated Soil

Heavy metals are absorbed by microorganisms both passively (adsorption) and actively (uptake) (bioaccumulation). The majority of microbial cell walls are composed of polysaccharides, lipids, and proteins, all of which include carboxylate, hydroxyl, amino, and phosphate groups and are capable of binding heavy metal ions. The biosorption method has more promise as a workable solution for larger-scale use since bacteria would need more resources for their active absorption of metals, which raises the BOD or COD in the waste. The maintenance of a healthy microbial population is particularly difficult because of external variables like heavy metals, a dangerous agent. The fungus Penicillium, Aspergillus, and Rhizopus have been the subject of numerous research as potential heavy metal removal microbial agents in aqueous solutions.

A number of metals must be present at specific concentrations for biological systems to function. Low levels lead to reduced metabolic activity. Certain metals are hazardous in large quantities (Diels et al., 1999). Numerous studies have been done on how heavy

metal contamination affects the make-up of microbial populations. Heavy metal contamination can be eliminated by microbes in a practical and inexpensive manner (Mishra, 2017). To lessen ambient metal pollution, several bacteria have evolved heavy metal resistance mechanisms (Hesse et al., 2019). Kelly et al. discovered that although the soil's pre-existing microbial community could eliminate the heavy metals, an increase in microbial population was linked to a drop in heavy metal pollution.

Because of its relative safety and ability to expedite the cleanup process, bioremediation has experienced a considerable rise in interest in recent years. By means of metabolic absorption, biomineralization, microbial oxidation/reduction of metal species, and cell surface adsorption, bacteria and other microbes have the power to alter the fate of metals in the environment (Rajesh et al., 2019). Because they are planktonic cells that create little to no extracellular polymeric substances (EPS), which include functional groups that serve as metal-binding sites, bacteria have been used in a range of studies. However, the bulk of bacteria are found in the environment as biofilms that are attached to mineral surfaces. The majority of microbial cells produce EPS to create the structured biofilm, which may have an impact on metal absorption and transport.

All living things require copper (Cu), a micronutrient, yet even in small levels, it can be detrimental. Because of this, only a narrow range of concentrations exhibit its advantageous effects. Anthropogenic activities including mining and fungicide spraying have occasionally caused Cu contamination of environmental compartments to surpass the toxicity threshold. The primary focus of this review is the bioremediation of soils with copper pollution. There is discussion of the mechanisms by which bacteria, in particular, can mobilize or immobilize copper in soils, as well as the associated bioremediation methods, of varying degrees of maturity, as follow: Bioimmobilization, bioleaching, and phytoextraction with bioaugmentation-assisted bioleaching have all been developed to lessen the in situ leaching of copper into groundwater. Using a unique method called bioaugmentation-assisted phytoextraction, copper can be removed from soil more effectively in situ. They laid out the precise requirements for each application as well as practical answers for regulating microbial activities.

1.9.3. Phytoremediation

A procedure known as phytoremediation is used to lower the concentrations of pollutants in the environment or their negative effects. Among other things, it can be used to eliminate radionuclides, heavy metals, and organic contaminants (such as, polynuclear aromatic hydrocarbons, polychlorinated biphenyls, and pesticides). It is an in-situ, cost-efficient, environmentally responsible, and sustainable solar-driven remediation method (Vithanage et al., 2012). Plants tolerate toxins without endangering topsoil, preserving its worth and fertility. They could be able to increase the fertility of the soil by integrating organic materials (Mench et al., 2009). "Phytoremediation" is the combination of two ideas. In-situ cleanup is referred to as "green remediation," sometimes known as "botano-remediation," "agro-remediation," or "vegetative remediation."

Environmental toxins can be transferred, contained, or neutralised by using plants, related microbes, improved soil, and agronomic practises. Although metal-accumulating plants have been employed on wastewater discharges for more than 300 years, the idea to utilise them to filter out heavy metals and other contaminants was first put forth in 1983. Plants can decompose organic pollutants, but metal contaminants can be removed and stabilised (Wuana and Okieimen., 2011).

Plants may suffer if the soil has high levels of metals. Poor plant and soil growth brought on by metal toxicity can result in metals being mobilized in water runoff and subsequently deposited in nearby water bodies. Additionally, exposed soil is more prone to dust contamination in the air and soil erosion. In these situations, remediation's primary objective is to reclaim the ground in order to lessen soil erosion and pollution dispersion.

Some plants can grow in heavy metal-contaminated soil. These plants, in particular, have a propensity to hyper-accumulate pollutants in their roots or shoots. When the plants are grown, fully formed, or have reached a certain level of heavy metal enrichment, they are harvested, burned, and cured. The polluted soil layer will be completely free of heavy metals as a result. Utilizing plants and the microbial population that coexists with them is a novel method for eliminating heavy metals. The procedure depends on finding plants with a high capability for high metal tolerance and accumulation. Intracellular ion

extrusion back into the external solution and ion influx control, which increases transporter function at low intracellular ion supply and inhibits at high levels, are two examples of such systems (increasing transporter function at low intracellular ion supply and inhibiting at high levels). There are additional detoxifying procedures in species that collect metal in thousands of parts per million (Lasat, 1999). When the idea of phytoextraction was reintroduced, engineering simulations showed that crops able to concentrate metals more than 1-2 percent were required for a successful plant-based decontamination of even weakly contaminated soils. A non-accumulator facility would undoubtedly go out of business due to the extremely harmful accumulation of such significant amounts of heavy metals. Hyperaccumulator species, on the other hand, might be able to reach such levels. However, because plants can only absorb and withstand a specific amount of metals, the amount of metal that may be removed is constrained (Lasat, 1999). Phytoextraction (phytoaccumulation), Phytofiltration, Phytostabilization, Phytovolatilization, and Phytodegradation are a few of the phytoremediation techniques.

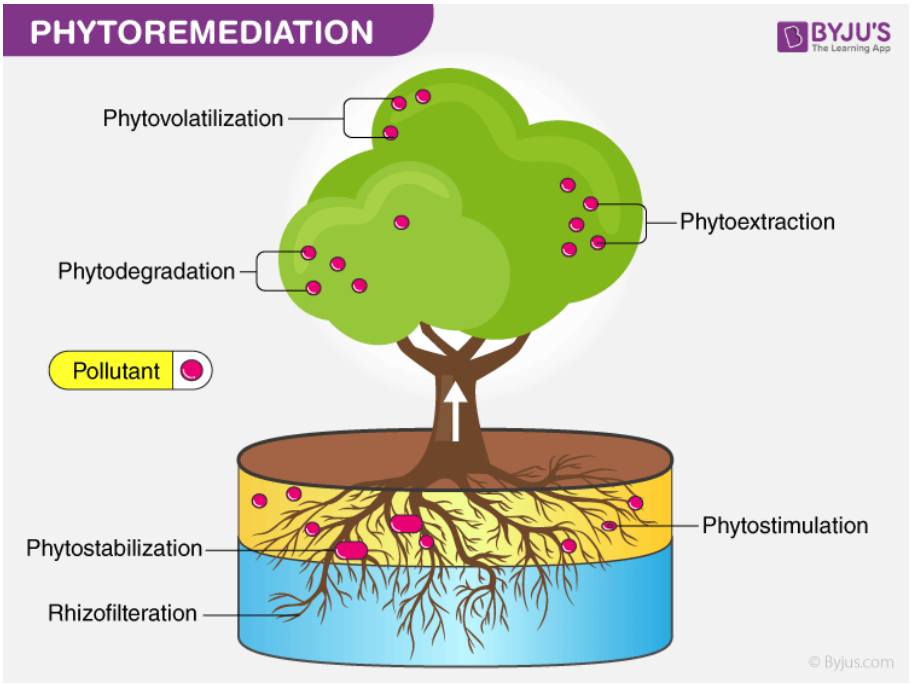

Figure 12: Phytoremediation Types

1.10. Integrated approach

For the treatment of multi-metal polluted soil in this experiment, bioaugmentation and phytoextraction were used as an integrated method. The method turned out to be somewhat more successful. Two plant varieties were chosen for the purpose. Treatments were designed for both plant cultivars were to be inoculated with each of three selected bacterial strains as well as their consortium.

1.11. Root Exudates

To explore the potential of different metal stresses for root exudation and assessment of antioxidant potential of root exudates, Brassicaceae cultivars were chosen. Mustard and canola both have strong phytochemical profiles and are said to provide health advantages. These plants can withstand external stresses, and as a result, they exude phytochemicals through their roots. There are around 3,500 species in the 350 genera that make up the Brassicaceae family. This family of vegetables plays a significant role in the global human diet. The phenolic components of Brassicaceae are what give them their nutritional value. Therefore, the goal of the current experiment was to investigate how the abiotic stress affected the exudate profiles of the aforementioned plants. It is well established that different plant components, such as the flower, leaves, stem, and root, naturally release beneficial bioactives into the environment for a variety of functions, including plant defence and pollination. The majority of photosynthetically fixed carbon is released by plant roots as root exudates.

By inducing chemotactic responses in rhizospheric bacteria, root exudates seem to play a part in early colonisation. They are a key source of nutrients for the rhizosphere's microorganisms. The root exudates were gathered over a period of time.

1.12. Problem statement

Heavy metals in soil have a sufficiently long half-life and their concentration remains in the soil environment for a longer period of time than organic pollutants do. Most heavy metals do not degrade biochemically. To any microorganism or plant, however, they may change their speciation and bioavailability. The biodegradation of organic pollutants may be slowed down by heavy metals in the soil. Polluted soil impairs soil physiochemical qualities, plant physiochemical properties, and human physiological health through direct

or indirect consumption of or contact with HMs. Additionally, it pollutes groundwater, diminishes agricultural output, lowers food hygiene standards (safety and marketability) due to phytotoxicity, and contributes to food insecurity and environmental problems.

1.13. Objectives

- The plant varieties *Brassica juncea* and *NARC sarsoon* will be compared and variety with better metal uptake ability will be identified. The most promising metal resistant bacterial strains *Bacillus tequilensis, Serratia marcescens* and *Bacillus safensis*, will be introduced onto copper, cadmium and lead contaminated soil systems and get tested for their effect on heavy metal accumulation in plant.
- Our work objective to evaluate the effect of inoculation on production of root exudates and establish role of root exudates in metal uptake by plant.
- To explore the potential of metal stress for root exudation and assessment of antioxidant potential of root exudates

Chapter 2

Materials and Methods

2.1. Collection and Processing of Soil

Fresh soil was obtained from the Nursery Bara kahu, Islamabad. After collection, soil was air dried and sieved using 2mm sieve to remove debris and obtain homogenized soil (pH 7 ± 0.1). Soil was then autoclaved at 121 degrees centigrade and 15 psi pressure.

Figure 13: Dried Sieved Soil Prior To Autoclaving

2.2. Soil Spiking

The salts required for spiking of the soil were identified and their molar weight was used to calculate their concentration. Then the soil was spiked with 120 mg/kg cadmium, 300 mg/kg copper and 600 mg/kg lead in the form of salt solution of Cadmium Sulphate (0.371 g/kg), Copper nitrate trihydrate (1.14 g/kg) and Lead nitrate (0.63 g/kg) manually. The soil was allowed to stabilize for approximately 1 month. Physicochemical parameters (EC, pH, OM, NO_3, PO_4) of soil were analyzed before and after the spiking. The soil was then used for the cultivation of plants to be grown in multi-metal contaminated soil.

Figure 14: Spiked Soil undergoing stabilization

2.3. Bacterial Strains

Three pre-isolated bacterial strains *Baccilus tequilensis,* NCCP 1031 (Pb19), *Serratia marcescens* NCCP 2268 (LW1) and *Bacillus safensis* NCCP 2261 (NS5) were used because of their metal resistant potential. All strains were sourced from Environmental Microbiology and Bioremediation Lab, Quaid-i-Azam University, Islamabad.

2.4. Plant Materials

NARC Sarsoon and *Brassica juncea*, two Brassica species that have been identified as hyperaccumulators of heavy metals, were chosen. All seeds were sourced from National Agriculture Research Center (NARC), Islamabad. Healthy seeds were rinsed and washed with distilled water.

2.5. Experiment Design

Experiment was conducted in a greenhouse on the botanical garden (QAU, Islamabad). In the greenhouse, pots with the aforementioned dimensions (15×7×7 cm) were filled with 400 g pot-1 of spiking soil. In experimental pots, 12 seeds of each cultivar were directly sowed before being housed in a greenhouse for a further three months (from December 2021 to March 2022 with prevailing seasonal growth conditions). The greenhouse conditions of 16 hours of light and 8 hours of darkness, at 30-33 ºC, and soil moisture in the pots at around 60% of their water-holding capacity, were maintained throughout the experiment.

On December 14, 2021, I took 400g of soil, (fresh as well as spiked) in each of 39 pots

according to the labeled treatment. Two a-biotic control treatments were also used, one containing fresh soil with plant while second pot containing only spiked soil to check effect of environmental conditions. Each treatment had three replicates. The pots were closely monitored for seed germination. Upon germination, the seedlings were thinned to five in number. To prevent contamination from leaching, each pot was set on a saucer. For the arrangement of the pots in the greenhouse, complete randomized block design (CRBD) was used.

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1. Control 1: Spiked soil
2. Control 2: Fresh Soil+ *Brassica juncea*
3. Control 3: Fresh Soil+ *NARC Sarsoon*
4. T4 Spiked Soil+ *Brassica juncea*
5. T5 Spiked Soil+ *NARC Sarsoon*
6. T6 SS + *Brassica juncea* + *Bacillus tequilensis*
7. T7 SS + *NARC Sarsoon*+ *Bacillus tequilensis*
8. T8 SS + *Brassica juncea* + *Serratia marcescens*
9. T9 SS + *NARC Sarsoon* + *Serratia marcescens*
10. T10 SS + *Brassica juncea* + *Bacillus safensis*
11. T12 SS + *NARC Sarsoon*+ *Bacillus safensis*
12. T13 SS + *Brassica juncea* + *Bacillus tequilensis* + *Serratia marcescens* + *Bacillus safensis*
13. T14 SS + *NARC Sarsoon* + *Bacillus tequilensis* + *Serratia marcescens* + *Bacillus safensis*

2.6. Inoculum preparation

The soil was inoculated with two selected bacterial strains. Nutrient broth was prepared for both strains. Single colonies were picked with a loop and then dipped into the broth and the flasks were placed on a shaker at 30 ºC for 24 hours. At 30 ºC, bacterial suspensions were nurtured in nutrient broth before being centrifuged and resuspended in 0.9% (w/v) NaCl. After seedlings stage the inoculation of 15ml bacterial suspension were

applied at each pot of bacterial treatment containing 1.2 *10^8 bacterial cells/ml. As a control, 0.9% NaCl was used to treat spiking soil in place of the inoculum suspension (Ren et al., 2019). After 60 days experimental plants were harvested for further analysis.

2.7. Analytical Procedures

Prior to experimentation, a subsample of sieved soil (2 mm) was used to examine physicochemical characteristics, heavy metals contents and nutrient analyses (phosphorous, nitrates, and organic matter). Soil pH, TDS and EC was measured by the EUTECH instrument pc 510. 10g of soil was taken using top balance machine in a glass beaker. Then 50ml of deionized water was poured into it for making (1:5 w/v) soil-water suspension. The suspension was mixed using orbital shaker and allowed to stand for 30 min. Using standard buffer solution, the pH meter was first calibrated at 6.86 and room temperature was also adjusted. The electrode was carefully rinsed with distilled water and with the use of tissue paper, drops of water were cleaned from the tip of electrode. The probe was put in the sample solution for at least 1 min and the reading was noted. The same procedure was applied for determination of total dissolved solids and electric conductivity (Zhang et al., 2020).

2.7.1. Heavy Metal Analysis of Collected Samples

For elemental evaluation of soil samples, they were oven dried in a single day at 80 ºC. After drying, samples were crushed manually and sieved by using 0.59 mm ASTM sieve to obtain homogeneous soil sample and used for further evaluation. For this test, aqua regia (containing 1:3 ratio of HNO_3 and HCL) was made. After preparing the aqua regia, 1 g of the sample was added in 15 ml aqua regia and boiled till the volume reduces to 3 to 5 ml. Then on the next day 5 ml of perchloric acid ($HClO_4$) was added into the leftover and boiled again till the volume of 3 to 5 ml was left. The leftover was cooled down and filtered using the Whatman filter paper (Number 42). Deionized water was used to raise the volume up to 15 ml. A blank sample was also analyzed in the same way but without the soil sample addition to remove any error during the procedure. A spectrophotometer for atomic absorption was used to analyze each sample in triplicate (Charles, 1991).

2.7.2. Determination of Nitrates

Soil nitrates were quantified by the chromotropic acid method (Estefan et al., 2013). In a nutshell, 1 g of sieved and dried soil was combined with 5 ml of 0.02 N CuSO4.5H2O and shaken for 15 min at 100 rpm on an orbital shaker. Following mixing, each sample was filtered using Whatman No. 42 filter paper, and 3 ml of the resulting filtrate was combined with 1 ml of 0.1% chromotropic acid before being placed in an ice bath. After this 6 ml of sulfuric acid (concentrated) was added in the solution and swirled. To prevent excessive heat formation, prepared mixture was left on shaking to cool down at room temperature. After 45 min yellow color was formed, to which absorbance of the mixture were taken on 430 nm, using spectrophotometer. A blank control was also prepared, containing all ingredients except soil, further standards of NO_3, using KNO_3 dissolved in 0.02 N $CuSO_4.5H_2O$, were also prepared. The concentration of NO_3 in ppm was quantified using values derived from the calibration curve.

2.7.3. Determination of Extractable Phosphorous

The standard Olsen sodium bicarbonate procedure was used to determine the amount of extractable phosphorus in soil samples (Estefan et al., 2013). For 30 minutes, samples were shaken at 150 rpm using an orbital shaker, then filtered through filter paper (Whatman no. 40). 3-5 drops of 0.25% nitrophenol indicator were added in filtrate (5ml) and mixed with 5N H_2SO_4 drop wise until the solution changes from yellow to colorless. After acidification the volume of the acidified solution was increased to 20 ml by using distilled water and 4 ml of ascorbic acid solution. A blank control of all ingredients except soil was made, and a phosphate standard of 1 to 5 ppm was made. After 10 minutes, the Rayleigh spectrophotometer UV9200 / VIS7220G was used to measure the absorbance of the blank, standard, and sample at 882 nm. The amount of extractable P in mg/kg was calculated using the values derived from calibration curve

2.7.4. Total Organic Carbon, Oxidizable Organic Carbon, and Organic Matter Analysis

The Walkley Black technique was used to calculate the organic matter of the soil (Nelson and Sommers 1982). Take around 0.5 g of the dry soil and place it in a 500 ml beaker. By

using pipette took 5 ml solution of potassium dichromate (1N) and added approximately 10 ml of H_2SO4, after adding mixed the suspension by stirring. After leaving it for 30 minutes 100 ml of distilled water was added and after that 5ml of concentrated H_3PO_4 and let the mixture to cool. After adding almost 15 drops of the diphenylamine indicator in the beaker placed it on the magnetic stirrer. After that by applying the method of titration, titrated this solution with solution of ferrous ammonium sulfate (0.5 M) and noted when color changes from violet to green. Blanks with no soil were made and analyze in the same way.

2.7.5. Determination of Soil Texture

The texture of soil was assessed by hydrometer method. In this method 40 g of soil was taken in the glass beaker and mixed it with 60 ml of dispersion solution of sodium hexa meta phosphate. After covering the beaker with watch glass, it was left overnight. Then carefully transferred this mixture into the soil stirring cup on next day and filled the cup to three quarters with water. The suspension was kept on shaking overnight.

Figure 15: Using Hydrometer to Find Soil Texture

On next day the suspension was transferred to 1 liter cylinder or hydrometer jar and 1 liter volume was made using water. Then sand, silt and clay content were assessed by using hydrometer (ASTM 152H GILSON Comp Inc., USA) in the suspension (Strickland et al. 1988).

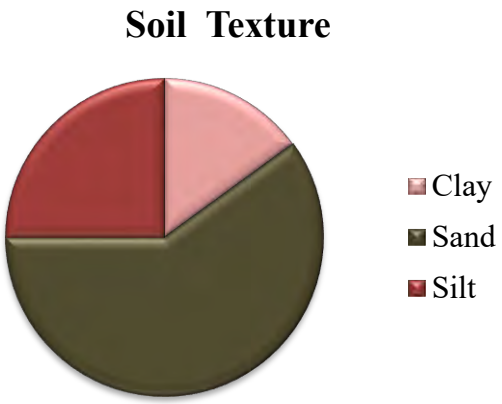

Figure 16: Constituents' Proportion in Soil

The same procedure was applied for blank but without soil. The texture of soil was sandy-loam. Soil brought from Bhara Kahu had 15% clay, 60% sand and 25% silt content that made it sandy loam in texture.

2.7.6. Soil Bacterial Count

For all treatments including bacterial inoculum, the soil was examined for bacterial colony forming units (CFU) and the survival of aggregated bacterial strains that can withstand heavy metals. Bacterial isolates were obtained by plate counting. The soil suspension was made with 0.9% normal saline solution of NaCl (10 grams of soil in 90 ml of normal saline), and serially diluted by mixing 9 ml sterile saline solution with 1 ml of the previous diluent. For each procedure, a nutrient agar plate with known concentrations of heavy metals was covered with 50 l of the diluent from 10-1 to 10-4. After 24 hours of incubation at 30°C, the plates were counted for the number of colonies on each plate (Liu et al., 2020).

2.8. Plant Analysis

2.8.1. Morphological Parameters

Harvested plants were subjected to physiological analysis. The final plant growth period was set at 60 days to investigate the effects of plant density on heavy metal uptake. The root and shott length were recorded. Gravimetric readings recorded on an electric weighing balance were used to measure the fresh and dried weight of the root, shoot, and

were expressed in g plant-1. Samples were dried in an oven at 70°C until their dry weight remained constant. A representative number of fresh leaves were also preserved at -20 °C for biochemical and enzymatic analysis.

2.8.2. Heavy Metal Quantification in Plants

Heavy metals concentration in plants was quantified through the method of wet oxidation (Estefan et al., 2013). It involves the digestion of plants by mixture of acids (HNO_3 and $HClO_4$). 1g of the plant sample was grinded and soaked with concentrated HNO_3. Samples were left for pre digestion for 6 to 8 hours. After that 10 ml of acid mixture (HNO_3 and $HClO_4$ in 9:4) were added and placed on the hot plate at the temperature of about 120 to 180 °C. When white fumes started to appear and white content was left then samples were placed at room temperature to cool down. After that samples were filtered using Whatman No. 42 filter paper. By adding distilled water, the capacity was increased to 15 ml. Blank sample was also prepared in the same way without plant content. Heavy metals were quantified using the atomic absorption spectrophotometer.

2.8.3. Chlorophyll A, Total Chlorophyll, Chlorophyll B, and Carotenoid Contents Assay

According to the Arnon (1949) methodology, 40 mg of fresh leaf samples were briefly immersed to generate a homogenous leaf extract in around 2 ml of 80% acetone solution (v/v). This extract was then used to assess the amount of chlorophyll and carotenoids in the sample. For five minutes, the extract was centrifuged at 5000 g. A fresh, clean falcon tube was used to properly store the supernatant. The pellets were vortexed for 1 minute at 5000 g with 1 cc of 80% (v/v) acetone in water. The previously harvested supernatant and the newly obtained supernatant were mixed for analysis. After obtaining absorbance (A) values at wavelengths of 663, 645, and 470 nm, the equations from Lichtenthaler (1987) were used to calculate photosynthetic pigments such as chlorophyll a, chlorophyll b, the total chlorophyll, and carotenoids.

2.8.4. Determination of Lipid Per-Oxidation

Method of Venkatachalam et al., 2017 was adopted for the analysis. In this procedure, 0.1 g of fresh leaf samples were obtained and macerated in cold 1 ml of TCA (5%) until they

became homogeneous before being centrifuged at 10,000 g for 10 min. Then, in a 1:1 ratio, TBA solution (0.67%) was added to the supernatant, and the combination was heated for almost 30 minutes at 95 °C. The mixture was heated, then chilled for about a minute before being centrifuged at 10,000 g for ten minutes. The absorbance was measured using a UV spectrophotometer at wavelengths of 450 nm, 532 nm, and 600 nm. Malondialdehyde g-1 of fresh weight was used to measure lipid peroxidation.

2.8.5. Hydrogen Peroxide Production

Yusuf et al., 2011 reported the method of H_2O_2 and this method was adopted to quantify the content of hydrogen peroxide. In this, 0.1 g of fresh leaf was mashed in 1 ml of pH 7.4 extraction buffer that also contained 50 mM potassium phosphate buffer and 0.5 mM EDTA (PPB). After that, this mixture was centrifuged for 15 minutes at 10,000 rpm. For the purpose of further estimating the H_2O_2 content, the supernatant was subsequently collected and taken as a leaf extract. To create the reaction mixture for measuring the amount of H_2O_2, 40 l of leaf extract, 1 ml of PPB with a pH of 6.5 (0.05 mM), and 352.8 l of 1% $Ti(SO_4)_2$ produced in 20% H_2SO_4 were all combined. The mixture was then centrifuged for roughly 15 minutes at 6000 g. The absorbance at 410 nm was measured using a UV spectrophotometer as part of an analysis of the supernatant to determine the strength of the yellow colour that was forming. By using the molar extinction coefficient (ε) of 0.28 M^{-1} cm^{-1}, H_2O_2 was expressed as M H_2O_2 contents g^{-1} of fresh weight..

2.8.6. Determination of Antioxidant Enzymes Activity

The following section introduces a method to quantify enzyme activity. The Venkatachalam et al. method was used to create leaf extract (2017). In a nutshell, leaf samples (0.1 g fresh samples) were macerated in 1 ml of pre-chilled extraction buffer (pH 7.4) containing roughly 50 mM potassium phosphate (PPB) and 0.5 mM EDTA, and then centrifuged at 10,000 g at 4 °C for 15 minutes. In order to measure the enzymatic activity of the homogenized sample, the obtained supernatant from the homogenized sample was collected, employed as a leaf extract, and kept at 4°C. For all of the enzyme activities in the sample, the values were given in units of g^{-1} of FW.

2.8.6.1. Assay for Catalyze (CAT) Activity

CAT behaviour was assessed by determining the rate of H_2O_2 evaporation using the method (Maehly, 1954). The reaction mixture included 50 µl of diluted enzyme extract and 2.5 ml of 50 mM phosphate buffer pH 7.4, as well as 0.1 ml of 1% H_2O_2 and 0.1 ml of H_2O_2. The drop in absorbance coincided with the decrease in H_2O_2 at 240 nm (ε = 39.4 mM^{-1} cm^{-1}). The values are given in units g^{-1} of the fresh weight of the sample.

2.8.6.2. Assay for Ascorbate Peroxidase (APX) Activity

Using a modified version of Chen and Asada's (1989) procedure, ascorbate peroxidase activity (APX) was measured. For this, a reaction mixture made by mixing 50µL of leaf extract with 1mL of reaction buffer made of 500µM ascorbate, 100µM EDTA, 1.54mM H_2O_2, and 50mM PPB, having pH at 7.0 was used to observe the absorbance at 240nm. To compute the APX activity ε of 2.8 mM^{-1} cm^{-1} was used.

2.8.6.3. Assay for Guaiacol Peroxidase (GPX)

The method of Upadhyay et al. (2019) to quantify the activity of guaiacol peroxidase (GPX) was applied. The reaction mixture was prepared by mixing 20µl of leaf extract with 2.5mL reaction buffer made by 50mM PPB at pH 6.1, 1mL 1% Guaiacol and 1mL 1% H_2O_2. A420 was examined after 1 minute to determine the changes. The activity was calculated, using ε equal to 26.6 mM^{-1} cm^{-1}.

2.8.6.4. Calculation for APX, CAT, and GPX

The concentrations of enzyme unit were calculated by using Beer's law, which is

C (Units ml^{-1}) = A / ε.L

Where, C= concentration, A= Absorbance, ε= Molar extinction coefficient, and L= Length of cuvette (1 cm)

Then for each expressing the values for gram of fresh weight C is multiplied with DF:

$$C \text{ (Units } g^{-1}) = (C) \times (W/1000) \times B$$

Where, C = Concentration derived from Beer's Law, V= ul of enzyme extract used for assay, and W= plant sample per ml of extraction buffer (0.1 g per ml of extraction buffer).

2.8.7. Collection of Root Exudates

Since salts may hinder their value in stimulating breakdown or desorption of pollutants and allelochemicals, collection of root exudates is frequently done in sterile distilled water or diluted nutrient solution. Since freeze-drying diminishes the activity, it is better to analyze the enzymatic activity of root exudates that have been collected in a liquid medium. The relevant collection of plant bioactives that spontaneously release is at the centre of all efforts to develop a sustainable extraction procedure in order to attain prospective results. Exudates are initially gathered and then screened. The exudate bioactives and possible biological applications of the filtrate are next chemically profiled. The production of plant metabolites is impacted by a plant's interaction with stress conditions. The results of the overall research project showed that exudates from the researched plants exhibit increased antioxidant potential because they are rich in polyphenolic components. Plants quickly activate a number of enzymatic and non-enzymatic defensive mechanisms in response to external stress, which promotes the generation of secondary metabolites. These stressors have no negative impact on plants, and by enhancing plant defences, they lower the chance of malady. The absence of a suitable collection mechanism poses a challenge to the effectiveness of this sustainable approach. Unfortunately, there is still no documented ideal technique for extracting root exudates.

Figure 17: Extraction Setup for Root Exudate Collection

The approach used in this study, is simple, gradual pouring of miliq water over intact plant-soil pot system and collect exudates under the pot. It is a remarkably eco-friendly way to get around the overpowering damaging impacts of other extraction methods.

2.8.8. In-vitro testing of Root exudates

Quantification of Phenolic compounds using HPLC

To measure the concentration of phenolic compounds and its major metabolites, samples of soil were analyzed. Phenolic compounds derivatives were extracted from soil samples by using an extraction procedure that Copaja et al. (2014) reported. Intact plant-soil pot system was taken and was poured over 10 times with 20mL of distilled water. Extracts were obtained and filtered (0.45µm) and evaporated under vacuum. The residues were dissolved in 1ml HPLC grade Methanol and analyzed by HPLC.

Using HPLC, the concentrations of phenolic compounds in the different treatments were measured in mg/ml of sample.

Total phenolic content (TPC)

The 96-well plate was used to conduct the assay. An aliquot of 20 l from the 4 mg/ml stock solution of each extract was added to the corresponding well of a 96-well plate, and

then 90 l of FC reagent was added. After 30 minutes of incubation at 37°C, 90 l of sodium bicarbonate were added to each well on the plate. Using a microplate reader, the sample extracts' absorbance was measured at 630 nm. Gallic acid was applied as a positive control in two-fold serial dilutions and DMSO as a negative control to generate a calibration curve.

Total flavonoid content (TFC)

A 96-well plate was employed for the assay's performance. A 20-l aliquot of the test extracts was put to each well, together with 10 l of potassium acetate, 10 l of aluminium chloride, and 160 l of distilled water. A microplate reader was used to measure the test extracts' absorbance at a wavelength of 415 nm after the plate had been incubated at room temperature for 30 minutes. Quercetin was used as the positive control and DMSO as the negative control in order to create the calibration curve for this experiment.

Total reducing power (TRP)

The estimation of the reducing power of extracts was done using a potassium ferricyanide colorimetric test. Following incubation at 50°C for 20 minutes in a water bath, an aliquot of 100 l of each test extract was mixed with 200 l of phosphate buffer and 250 l of potassium ferricyanide. Every test sample received 200 l of trichloroacetic acid before the mixture was centrifuged for 10 minutes at 3000 rpm at room temperature. The 150 l supernatant was then collected and combined in a 96-well plate with 50 l of FeCl3. At 630 nm, the absorbance was then measured. A positive control was ascorbic acid, and a negative control was DMSO.

Total antioxidant capacity (TAC)

Using a phosphomolybdenum-based assay, the test sample's overall antioxidant capacity was calculated. A mixture of 900 ml of TAC reagent and 100 l of test extract was used. DMSO served as the negative control. The reaction mixture was then incubated for 90 minutes at 95°C in the water bath. The absorbance of the test and standard solutions was assessed at 630 nm after cooling.

Free radical scavenging assay:

By using stable free radical DPPH, the compounds were examined for antioxidant activity. To get the final concentrations of 200 g/ml in the reaction mixture, 10 l of plant extract was combined with 190 l of DPPH solution. A microplate reader was used to measure the absorbance at 517 nm following 30 minutes of incubation at 37°C. The following formula was computed for determining %free radical scavenging activity:

$$\text{Free Radical Scavenging Activity} = 1 - \text{As}/\text{Ac} * 100$$

Where, As and Ac are the absorbance of sample and negative control respectively.

Chapter 3

Results

3.1. Physico-chemical Properties of Fresh and Contaminated Soil

The physicochemical characteristics of treated soils have a significant role in determining how well the soil may be recycled and how adaptable it is. The experimental soil's various physico-chemical properties, including pH, EC, OOC, TOC, OM, nitrates, and phosphates, are quantified in Table 3.1. The pH was in the range of 6.9-7.1. Electrical conductivity (μScm^{-1}) measured for all treatments showed statistically significant difference among treatments. Fresh soil showed least EC (μScm^{-1}) 164 and the highest EC (399 µS) was observed in abiotic control. The combined application of bacteria and *Brassica* lowered EC of the contaminated soil. The inoculation of bacterial strains individually and in combination showed significant reduction in EC in all the treatments. While for OOC, TOC, and OM significantly highest levels were noted for B+P treatment (T12) that were 3.35, 4.47, 5.78, respectively. Lowest OOC, TOC, and OM values were noted in abiotic control. Bacterial inoculation and plants improved the organic matter content in soil.

The available nitrates were highest (48.1 mg/kg) in treatment B+P (T12) followed by T10, T8 and T6. The bacterial inoculation improved nitrate content in the soil. Nitrates content was high in spiked treatment because of salts used for metal spiking had nitrate. Statistically significant difference was observed among treatments in nitrates quantity. The extractable phosphorous (mg/kg) was different in each experimental treatment. The combined treatments (B+P) showed highest extractable phosphorous. The peaked levels of extractable phosphorous (663 mg/kg) were seen in T12, whereas the lowest levels (253) were discovered in the abiotic control.

Table 3.1 Physico-chemical properties of fresh and spiked soil

Treatment	Description	pH	EC (μScm^{-1})	OOC %	TOC %	OM %	NO_3 $mgkg^{-1}$	PO_4 $mgkg^{-1}$
T1	Spiked soil	7.10±0.11^{a}	399±2.0^{a}	1.11±0.24^{g}	1.48±0.32g	1.91±0.41^{g}	9.56±0.014^{j}	253±0.6^{m}
T2	Fresh Soil+ *Brassica juncea*	6.91±0.05^{g}	104±0.7^{l}	1.29±0.13^{fg}	1.72±0.17^{fg}	1.76±0.19^{h}	7.84±0.014^{h}	264±0.4^{l}
T3	Fresh Soil+ *NARC Sarsoon*	6..91±0.03^{fg}	111±1.2^{k}	1.24±0.15^{fg}	1.65±0.21^{fg}	1.24±0.13^{i}	7.78±0.022^{l}	258±0.4^{l}
T4	Spiked Soil+*Brassica juncea*	7.06±0.08^{ab}	263±0.7^{c}	1.46±0.12^{efg}	1.95±0.16^{efg}	2.22±0.23^{fg}	7.92±0.008^{m}	289±0.5^{j}
T5	Spiked Soil+*NARC Sarsoon*	7.06±0.07^{ab}	268±0.7^{b}	1.33±0.11^{efg}	1.77±0.15^{efg}	2.14±0.27^{fg}	7.86±0.022^{n}	279±0.5^{k}
T6	SS + *Brassica juncea* + *Bacillus tequilensis*	6.91.±0.09^{cde}	213±0.9^{f}	3.29±19^{c}	4.39±0.26^{c}	3.02±0.44^{e}	12.23±0.014^{e}	414±0.4^{e}
T7	SS + *NARC Sarsoon*+ *Bacillus tequilensis*	7.01±0.11^{ab}	234±0.9^{9}	1.58±0.32^{ef}	2.11±0.43^{ef}	2.29±0.20^{efg}	8.92±0.022^{k}	318±0.5^{i}
T8	SS + *Brassica juncea* + *Serratia marcescens*	6.93±0.06^{de}	208±0.6^{g}	3.35±0.30^{c}	4.47±0.39^{ef}	4.62±0.65^{d}	8.11±0.014^{c}	488±0.5^{c}
T9	SS + *NARC Sarsoon* + *Serratia marcescens*	6.94±0.08^{bc}	234±0.6^{e}	1.66±0.27^{ef}	2.21±0.36^{ef}	2.52±0.21^{efg}	9.63±0.022^{i}	362±1.2^{h}
T10	SS + *Brassica juncea* + *Bacillus safensis*	6.91±0.08^{def}	201±1.3^{h}	3.82±0.08^{a}	4.39±0.10^{a}	5.67±0.33^{c}	14.47±0.014^{b}	670±0.4^{a}
T11	SS + *NARC Sarsoon*+ *Bacillus safensis*	6.90±0.03^{cd}	233±0.9^{d}	1.75±0.26^{e}	2.33±0.34^{e}	2.72±0.56^{ef}	10.90±0.022^{g}	364±0.4^{g}
T12	SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	6..91±0.06^{def}	199±0.8^{i}	3.94±0.11^{a}	4.62±0.14^{a}	5.78±0.51^{b}	15.11±0.014^{a}	663±0.5^{b}
T13	SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	6.90±0.03^{cde}	229±0.7^{e}	2.68±0.38^{d}	3.58±0.51^{d}	2.86±0.47^{ef}	11.64±0.022^{f}	387±0.2^{f}
T14	Fresh Soil	6.92±0.07^{efg}	164±0.8^{j}	3.96±0.30a	5.28±0.39^{b}	6.83±0.51^{a}	12.34±0.008^{d}	484±0.5^{d}

T= Treatments, C= Control,, FS + P= Fresh soil + Plant, AC= Abiotic control, P= Phytoremediation, B= Bioaugmentation, OOC= Oxidizable organic carbon, TOC= Total Organic Carbon, OM= Organic Matter, NO_3= Nitrates, PO_4= Extractable Phosphorous, EC= Electrical Conductivity, SS= spiked soil, The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in column are non-significant

3.2. Physiological Parameters of *Brassica juncea and NARC sarsoon*

The root length, fresh weight, and dried weight of the roots and shoot length, fresh weight, and dried weight of the shoots were examined, and the results are displayed in Table 3. Plants grown with different treatments showed statistically significant difference in all parameters. The plant from Treatment 12 had the longest shoots and the largest fresh and dry weights, measuring 40 cm and 12.10 g and 6.80 g, respectively. Trends followed by T10, T8, T6, T13, T11,T9, T7, T4 and T5. The treatments B+P showed significantly higher shoot length, fresh and dry weight. The lowest values for shoot parameters were observed in T4 and T5 without any amendment. Bacterial inoculation improved plant growth as well as metal uptake by plant (phytoextration).

Table 2: Impact of different treatments on the physiological parameters of Brassica Cultivars.

Description	Treatment	Shoots			Roots	
		Length(cm)	Fresh weight(g)	Dry weight(g)	Fresh weight(g)	Dry weight(g)
C	1 Fresh Soil+ *Brassica juncea*	46 ± 2^{a}	2.21 ± 0.09^{d}	1.58 ± 0.69^{b}	2.72 ± 0.20^{e}	1.50 ± 0.10^{b}
C	2 Fresh Soil+ *NARC Sarsoon*	35 ± 2^{cd}	1.14 ± 0.14^{e}	0.75 ± 0.43^{f}	2.73 ± 0.07^{e}	1.03 ± 0.01^{f}
P	3 Spiked Soil+*Brassica juncea*	9 ± 1^{g}	2.20 ± 0.18^{d}	1.28 ± 1.04^{fg}	0.67 ± 0.06^{gh}	0.45 ± 0.05^{hi}
P	4 Spiked Soil+*NARC Sarsoon*	8 ± 1^{g}	0.51 ± 0.08^{f}	0.38 ± 0.15^{g}	0.64 ± 0.04^{h}	0.42 ± 0.03^{i}
B + P	5 SS + *Brassica juncea* + *Bacillus tequilensis*	33 ± 0.76^{cd}	2.48 ± 0.07^{bc}	1.70 ± 0.79^{b}	3.93 ± 0.15^{d}	1.21 ± 0.04^{de}
B + P	6 SS + *NARC Sarsoon*+ *Bacillus tequilensis*	12 ± 1^{f}	2.23 ± 0.09^{d}	1.35 ± 0.89^{e}	0.73 ± 0.06^{gh}	$0.46\pm0.0.06^{hi}$
B + P	7 SS + *Brassica juncea* + *Serratia marcescens*	34.67 ± 2.52^{cd}	2.51 ± 0.10^{bc}	1.74 ± 1.17^{d}	5.47 ± 0.15^{c}	1.32 ± 0.04^{d}
B + P	8 SS + *NARC Sarsoon* + *Serratia marcescens*	12.67 ± 2.08^{f}	$2.34\pm0.04_{cd}$	1.51 ± 0.91^{d}	0.85 ± 0.05^{g}	0.55 ± 0.05^{h}
B + P	9 SS + *Brassica juncea* + *Bacillus safensis*	35 ± 1.86^{c}	2.66 ± 0.25^{b}	2.04 ± 0.56^{a}	5.83 ± 0.11^{b}	2.22 ± 0.08^{c}
B + P	10 SS + *NARC Sarsoon*+ *Bacillus safensis*	22.50 ± 2.29^{e}	2.44 ± 0.06^{c}	1.54 ± 0.97^{d}	1.77 ± 0.06^{f}	0.76 ± 0.04^{g}
B + P	11 SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	40 ± 2.00^{b}	3.61 ± 0.06^{a}	2.30 ± 1.43^{b}	8.50 ± 0.10^{a}	4.55 ± 0.13^{a}
B + P	12 SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	32.17 ± 1.04^{d}	2.45 ± 0.05^{bc}	1.60 ± 0.89^{c}	3.90 ± 0.01^{d}	1.14 ± 0.06^{e}

T= Treatments, C= Control, FS + P= Fresh soil + Plant, AC= Abiotic control, P= Phytoremediation, P+B= Phytoremediation + Bioaugmentation, SS= spiked soil, The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in column are non-significant.

3.3. Chlorophyll A, Chlorophyll B, Total Chlorophyll, and Carotenoid Contents

Chlorophyll a, chlorophyll b, total chlorophyll and carotenoids were assessed for each treatment after plant harvesting. The resulted chla, chlb and total chlorophyll showed significant variation (Fig.21) Treatment (T12) showed highest carotenoid concentration and was lowest for contaminated control plant (T5) (Fig. 22).

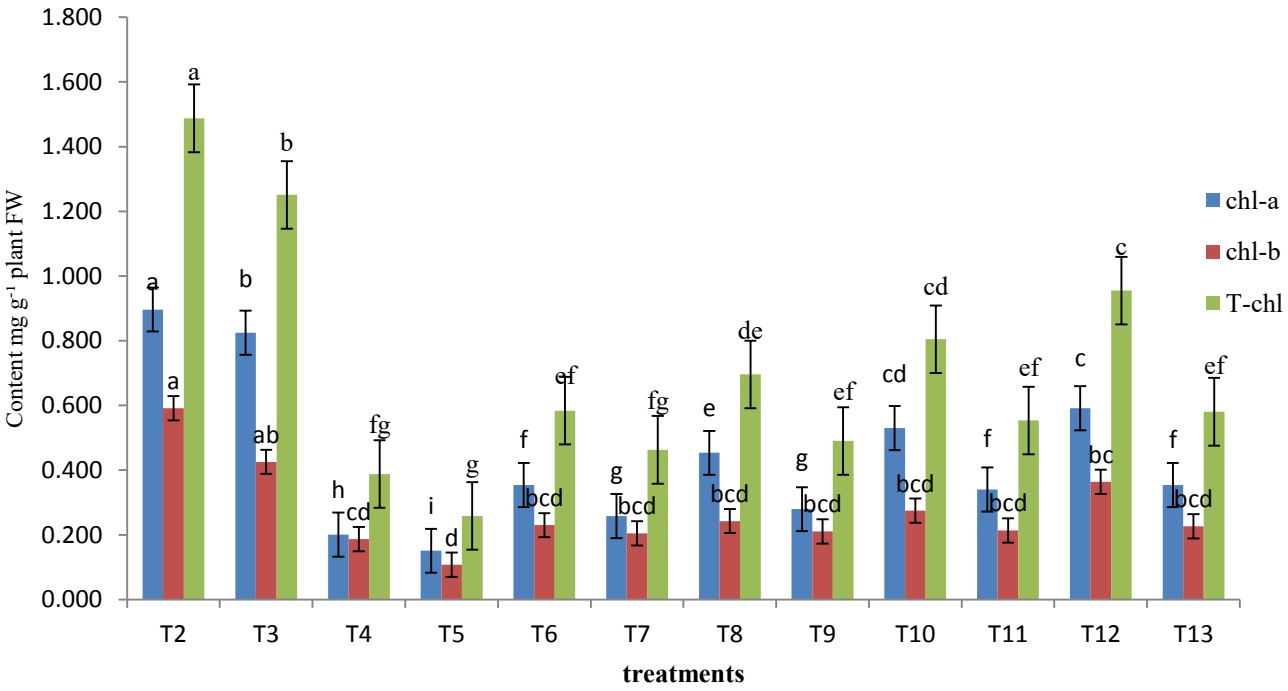

Figure 18: Chlorophyll a, Chlorophyll b and total Chlorophyll in plants

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

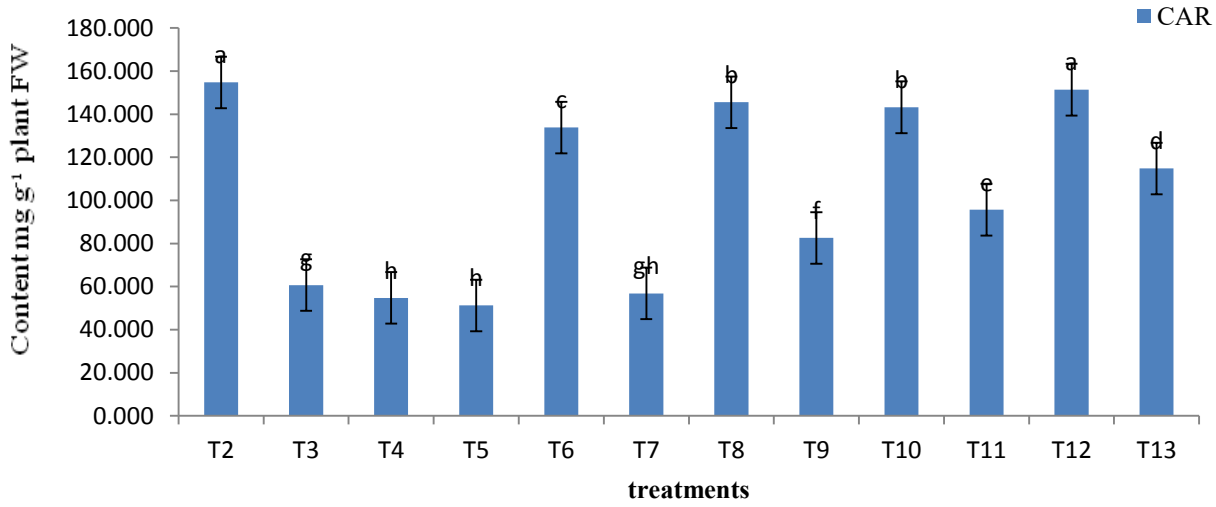

Figure 19: Carotenoid levels in different treatments

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.4. Effects of the Applied Treatments on Soil Enzymatic Profile in Soil

The impact of different adopted treatments on the studied soil enzymatic activities, including urease, phosphatase, and catalase for soil is presented in Table 4. Among all studied soil enzymatic activity statistically significant differences were noted, between the applied treatments. Soil catalase activity (0.02 M KMnO4 g^{-1} h^{-1}) was recorded in soil as as the highest in the treatment SS + Brassica juncea + B. tequilensis+S. marcescens+B. safensis: 0.019 ± 0.6^{a}, the significantly lowest soil catalase activity was noted for SS and SS+P treatments, 0.009 ± 0.3 and 0.012 ± 0.38, respectively. Phosphatase activity (µg p-nitrophenol g^{-1} h^{-1}) was the highest in the same treatment, i.e. T12 that was 97.701 ± 0.32^{a}. For the urease activity (µg urea hydrolysed g^{-1} h^{-1}) the significantly higher activity was noted for treatment 12, having value of 1596 ± 0.33^{a}.

Table 3: Impact of Different Treatments on Soil Enzymatic Activities in Soil

Treatments	Urease µg urea hydrolysed g^{-1} h^{-1}	Phosphatase µg p-nitrophenol g^{-1} h^{-1}	Catalase 0.02 M $KmnO_4$ g^{-1} h^{-1}
1Spiked soil	599 ± 0.38^{m}	56.99 ± 1.73^{j}	0.009 ± 0.3^{h}
2 Fresh Soil+ *Brassica juncea*	1530 ± 0.38^{d}	95.61 ± 0.69^{b}	0.015 ± 0.49^{c}
3 Fresh Soil+ *NARC Sarsoon*	785 ± 0.33^{g}	77.132 ± 0.64^{e}	0.012 ± 0.38^{f}
4 Spiked Soil+*Brassica juncea*	697 ± 0.66^{k}	64.154 ± 0.85^{h}	0.01 ± 0.33^{g}
5 Spiked Soil+*NARC Sarsoon*	690 ± 0.5^{l}	61.185 ± 0.9^{i}	0.01 ± 0.33^{g}
6 SS + *Brassica juncea* + *Bacillus tequilensis*	1091 ± 0.66^{e}	83.59 ± 1.33^{c}	0.012 ± 0.4^{e}
7 SS + *NARC Sarsoon*+ *Bacillus tequilensis*	728 ± 0.33^{j}	70.673 ± 0.58^{g}	0.012 ± 0.37^{f}
8 SS + *Brassica juncea* + *Serratia marcescens*	1566 ± 1^{c}	84.876 ± 0.21^{c}	0.013 ± 0.42^{d}
9 SS + *NARC Sarsoon* + *Serratia marcescens*	778 ± 1^{i}	74.343 ± 0.85^{f}	0.012 ± 0.38^{f}

10 SS + *Brassica juncea* + *Bacillus safensis*	1575 ± 0.38^{b}	96.017 ± 0.21^{b}	0.017 ± 0.54^{b}
11 SS + *NARC Sarsoon*+ *Bacillus safensis*	781 ± 0.87^{h}	73.707 ± 0.58^{f}	0.012 ± 0.38^{f}
12 SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	1596 ± 0.33^{a}	97.701 ± 0.32^{a}	0.019 ± 0.6^{a}
13 SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	1019 ± 0.66^{f}	81.111 ± 0.58^{d}	0.012 ± 0.38^{ef}

FS+P=Fresh soil + Plant, SS= spiked soil, SS+P= Spiked soil + Plant,
The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages.

3.5. Effects on enzymatic activities of *Brassica*

Enzyme activities such as H_2O_2, MDA, APX, GPX and CAT in *Brassica* on getting exposed to the selected HMs are presented in Figure 3.3, 3.4 and 3.5 respectively. Higher levels of H_2O_2, MDA, APX, GPX and CAT were noted in T4 and T5 (contaminated soil with *Brassica*), suggesting that the higher levels of HMs contributed stress for plant. The B + P treatments showed strong antioxidant defense for H_2O_2, MDA, APX, GPX and CAT. Fresh soil plant control antioxidant activities showed lowest results for H_2O_2, MDA, APX, GPX and CAT.

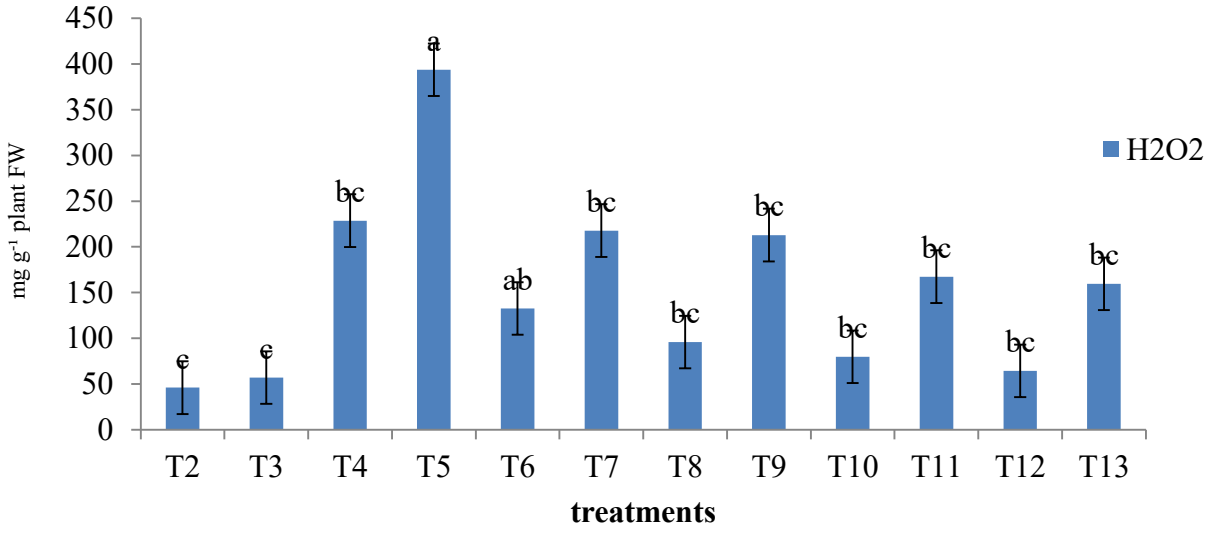

Figure 20: Stress injury due to HMs exposure to Brassica (H2O2 content µM of H2O2 g -1 of FW)

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

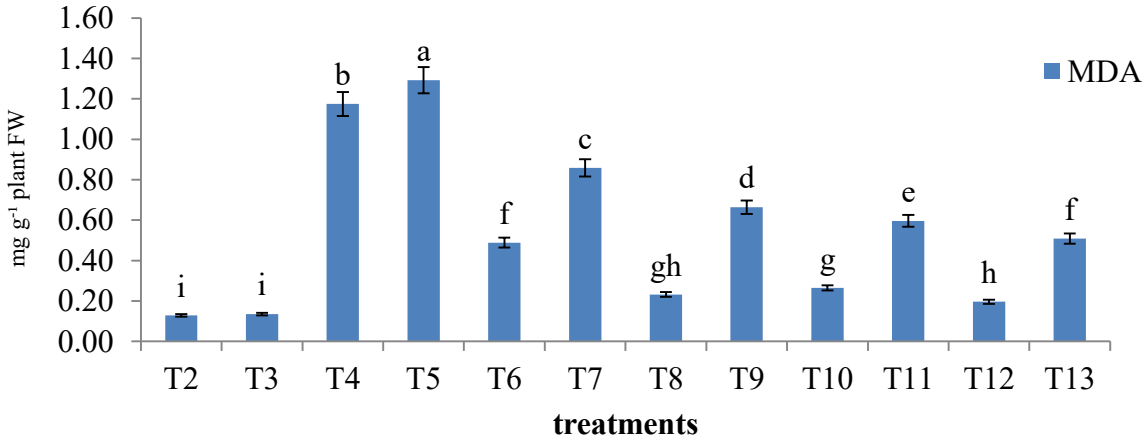

Figure 21: Stress injury due to HMs exposure to Brassica (MDA content µM g-1 of plant FW)

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

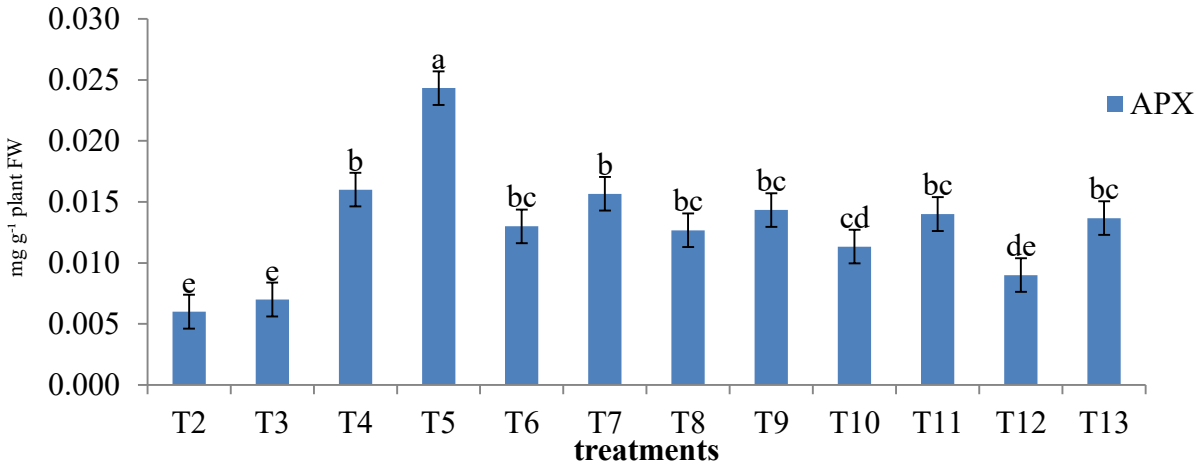

Figure 22: The enzymatic profile (APX) of Brassica with reference to different applied treatment

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant

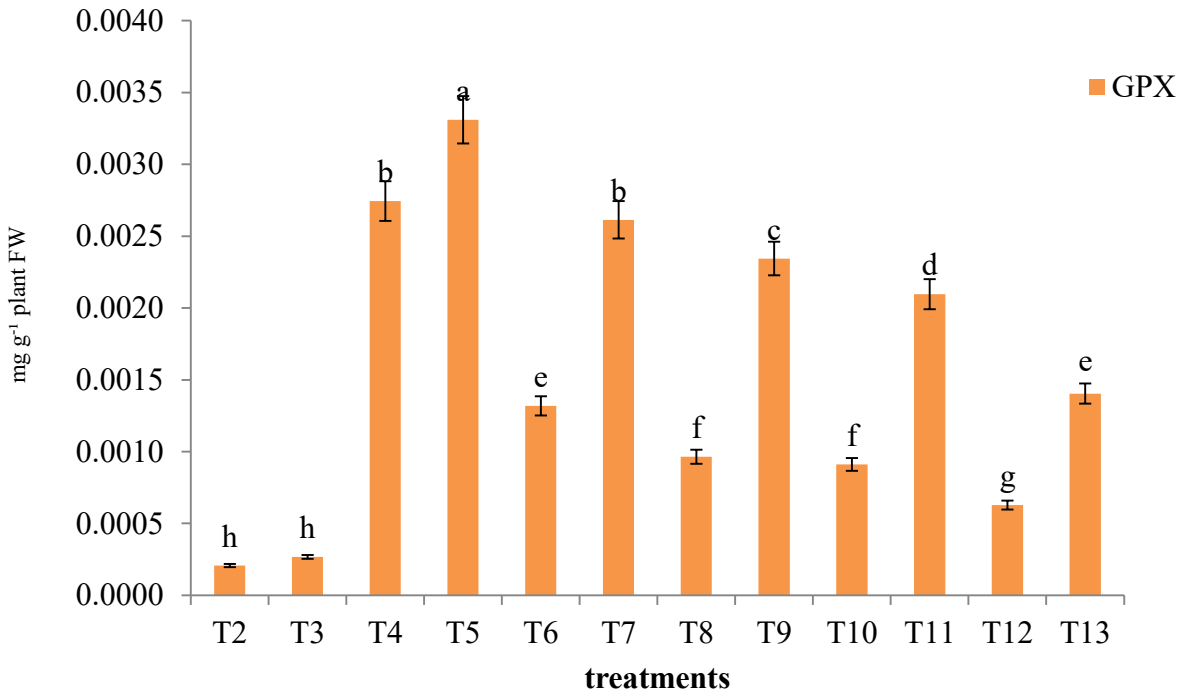

Figure 23: The enzymatic profile (GPX) of Brassica with reference to different applied treatment

Data are presented in means (n = 3 ± SD). Significantly highest mean was "a" followed by later alphabets for lower means. Similar small letters in are non-significant.

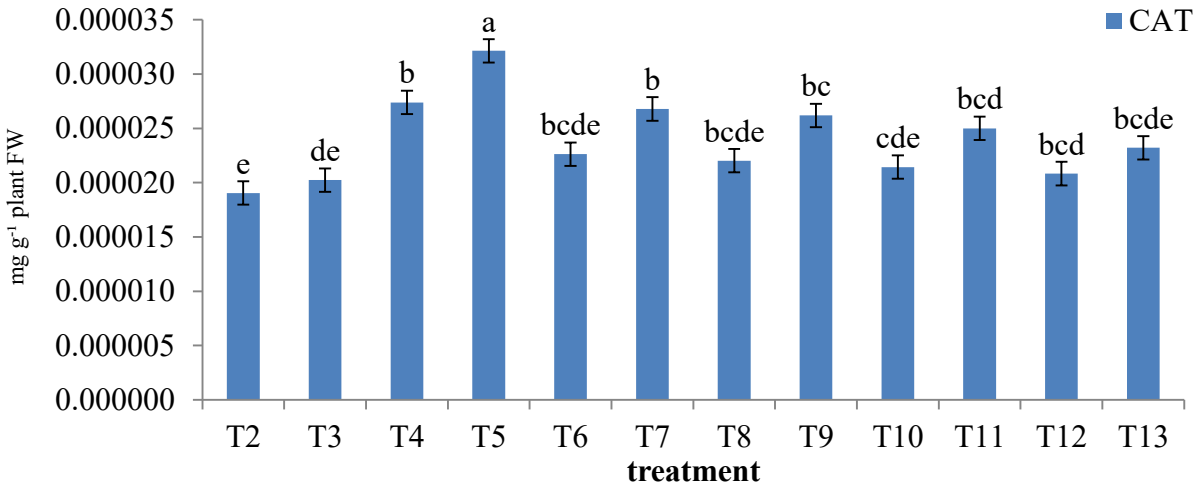

Figure 24: The enzymatic profile (CAT) of *Brassica* with reference to different applied treatment

*Values are expressed in Units g^{-1} of FW of plant leaf sample.

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.5. Cadmium content in soil

In this study metal concentrations in soil after harvesting were quantified. All applied treatment showed significant differences in cadmium contents in soil. The results showed that inoculation of bacterial strains enhanced it's uptake by plant. Maximum concentration of Cd (105 mg/kg) was noted in control soil with no amendment followed by T5 (*NARC Sarsoon* in Spiked Soil). The minimum concentration of Cd was observed in treatments where bacterial strains were applied alone or in consortium in combination with *Brassica juncea*. T12 in which consortium of bacterial strains in rhizosphere of *Brassica juncea* was used showed the minimum (7.02 mg/kg) of available Cd (Fig. 28).

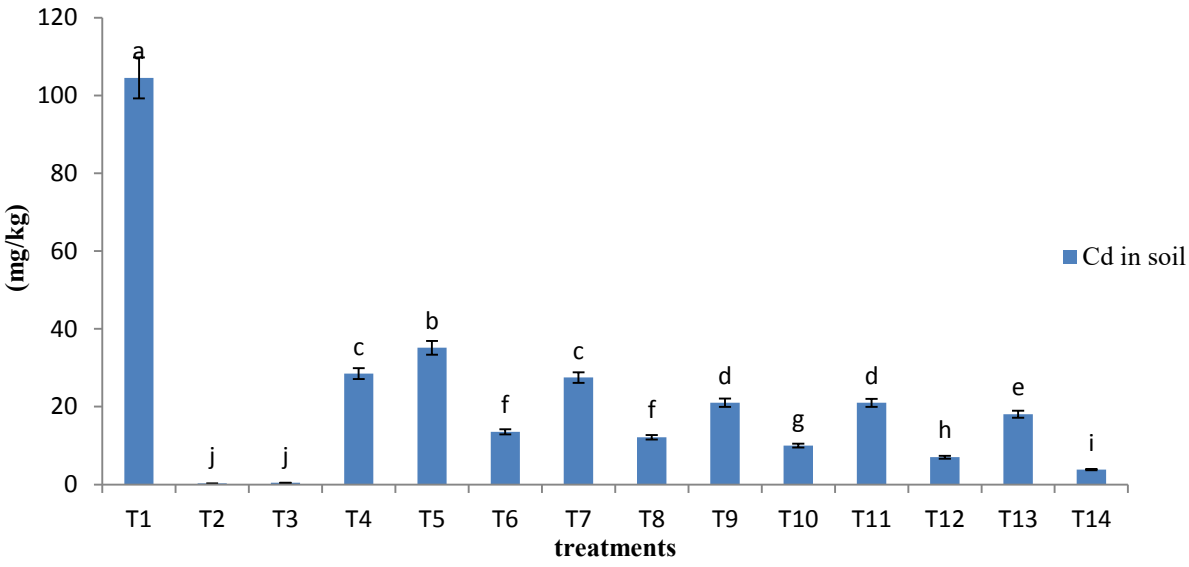

Figure 25: Cadmium content in soil after harvesting of plants

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.6. Cadmium content in roots and shoots of *Brassica* Cultivars

All the treatments showed significant differences in Cd uptake after harvesting in root and shoot of *Brassica* (Fig. 29). The B+P treatments showed highest Cd uptake in roots that were 26 mg/kg. Lowest Cd uptake (7.80 mg/kg) was observed in roots of T5, without

any amendment. Same trend was followed in plant shoots for Cd uptake. The treatment B+P showed highest Cd uptake that was 40 mg/kg. Lowest Cd uptake in shoots was observed in treatment T5, which is 5.5 mg/kg. The application of bacterial strains enhanced Cd uptake in plant.

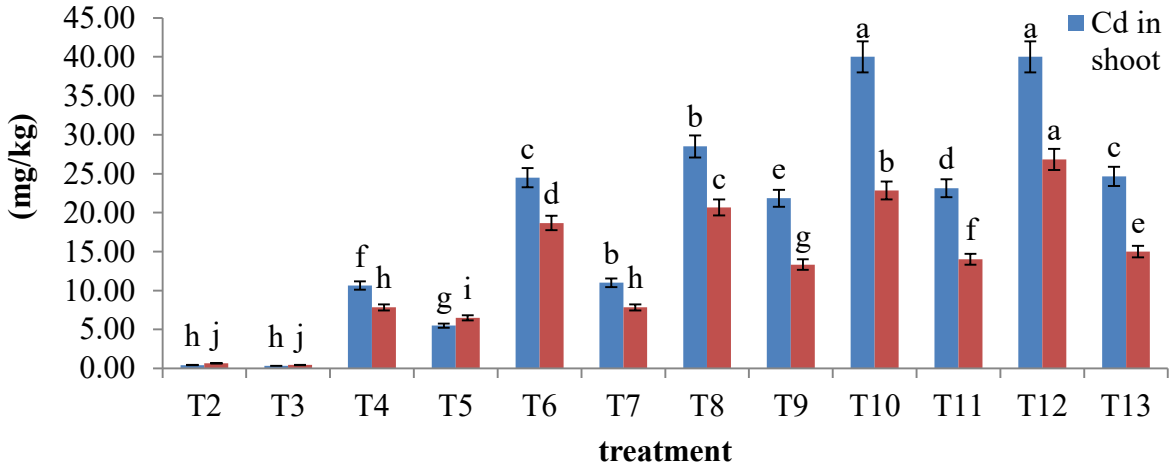

Figure 26: Cadmium content in roots and shoots of *Brassica* Cultivars

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.7. Lead content in soil

All the treatment showed significant differences in lead content in soil. The results showed that inoculation of bacterial strains enhanced Pb phytoextration in soil. Maximum available Pb (317.3 mg/kg) was noted in control soil with no amendment, followed by T5. The minimum available Pb was observed in treatments where bacterial strains and *Brassica juncea* were applied in combination. T12 in which combination of bacterial strains in rhizosphere of *Brassica* was used showed the minimum (107 mg/kg) of available Pb (Fig. 30).

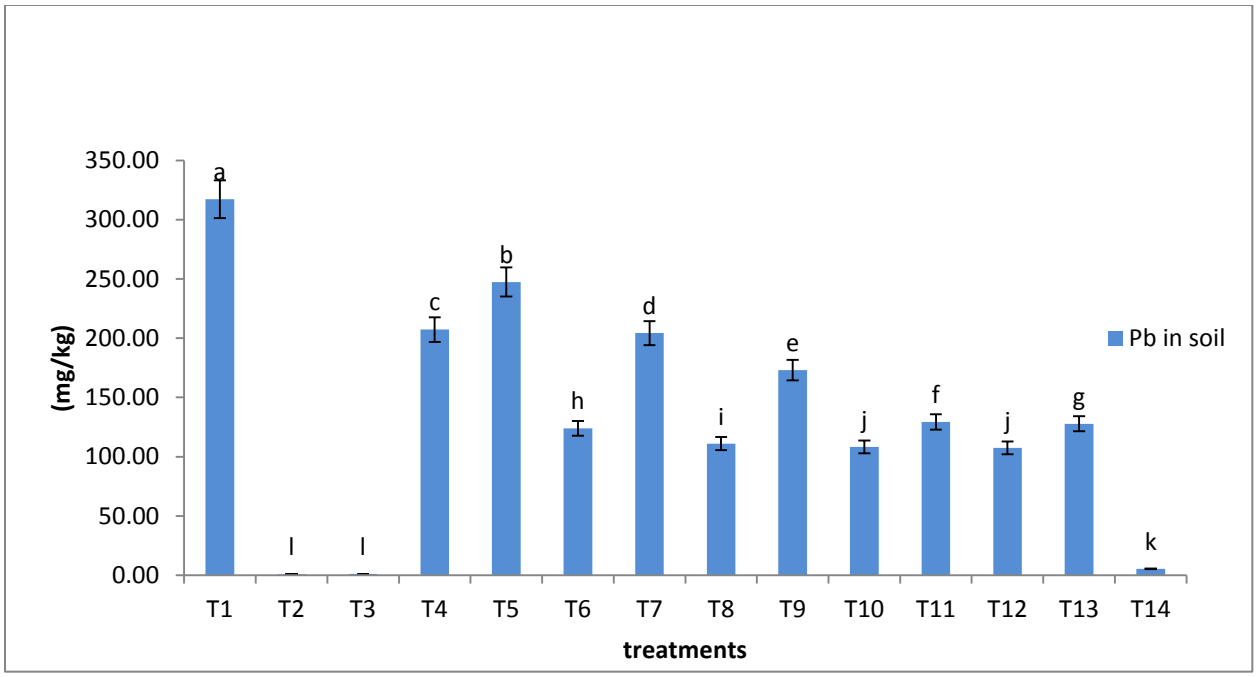

Figure 27: Lead content in soil after harvesting of plants

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.11. Lead content in roots and shoots of *Brassica* Cultivars

All the treatments showed significant differences in Pb uptake after harvesting in root and shoot of *Brassica* (Fig. 31). The B+P treatments showed highest Pb uptake in roots that were 210 mg/kg. Lowest Pb uptake (25.6 mg/kg) was observed in roots of T5, without any amendment. Same trend was followed in plant shoots for Pb uptake. The treatment B+P showed highest Pb uptake that was 46.3 mg/kg. Lowest Pb uptake in shoots was observed in treatment T5, that 1.67 mg/kg. The application of bacterial strains enhanced Pb uptake in plant.

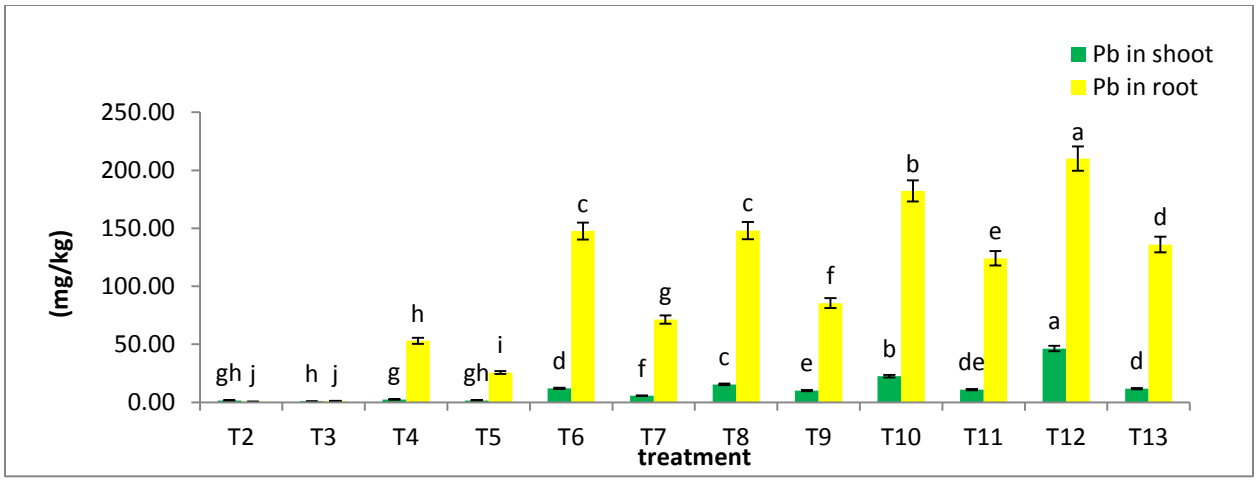

Figure 28: Lead concentration in roots and shoots of Brassica Cultivars

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.12. Copper content in soil

All the treatment showed significant differences in Copper content in soil. The results showed that inoculation of bacterial strains enhanced Cu extraction by plants from soil. The bacterial strains converted the non-available Pb to available fractions. Maximum Cu content (321 mg/kg) was noted in abiotic control soil with no amendment. The minimum Cu content was observed in treatments where bacterial strains were applied in combination with plants. T12 in which combination of bacterial strains in rhizosphere of *Brassica juncea* was used showed the minimum (97.30 mg/kg) of available Pb (Fig. 3.12).

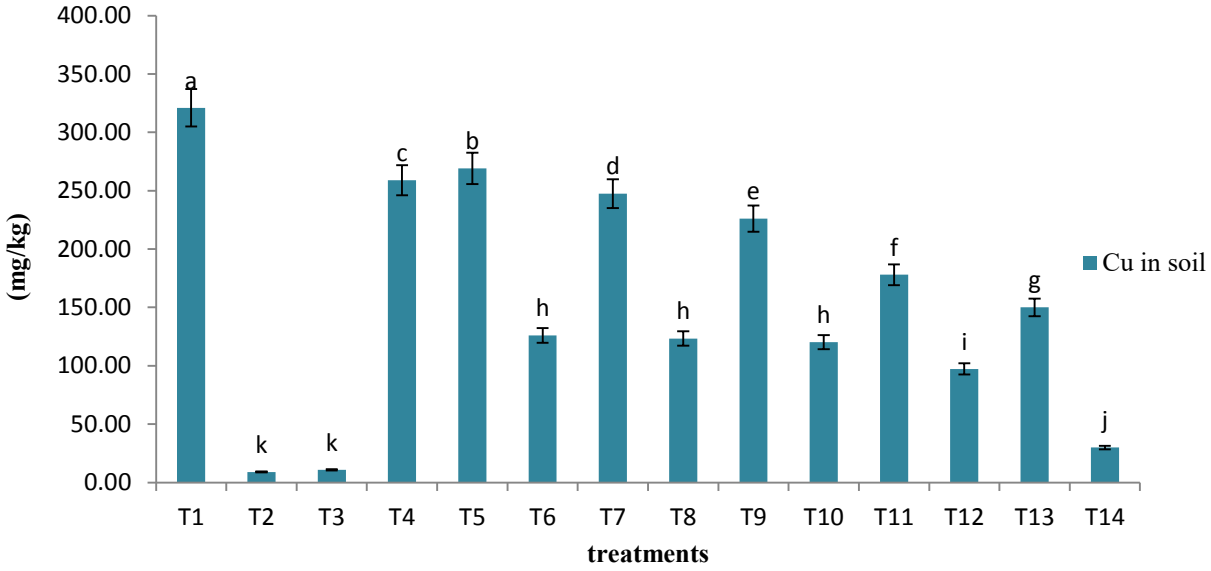

Figure 29: Copper content in soil after harvesting of plants

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

3.13. Copper content in roots and shoots of *Brassica* Cultivars

All the treatments showed significant differences in Cu uptake after harvesting in root and shoot of Brassica (Fig. 3.11). The B+P treatments showed highest Cu uptake in roots that were 172.17 mg/kg. Lowest Cu uptake (51.83 mg/kg) was observed in roots of T5, without any amendment. Same trend was followed in plant shoots for Cu uptake. The treatment 12 (Brassica juncea and bacterial strains) showed highest Cu uptake that was 49.03 mg/kg. Lowest Cu uptake in shoots was observed in treatment T5 (*NARC sarsoon)*, without any bacterial innoculation that was 11 mg/kg. The application of bacterial strains enhanced Cu uptake in plant.

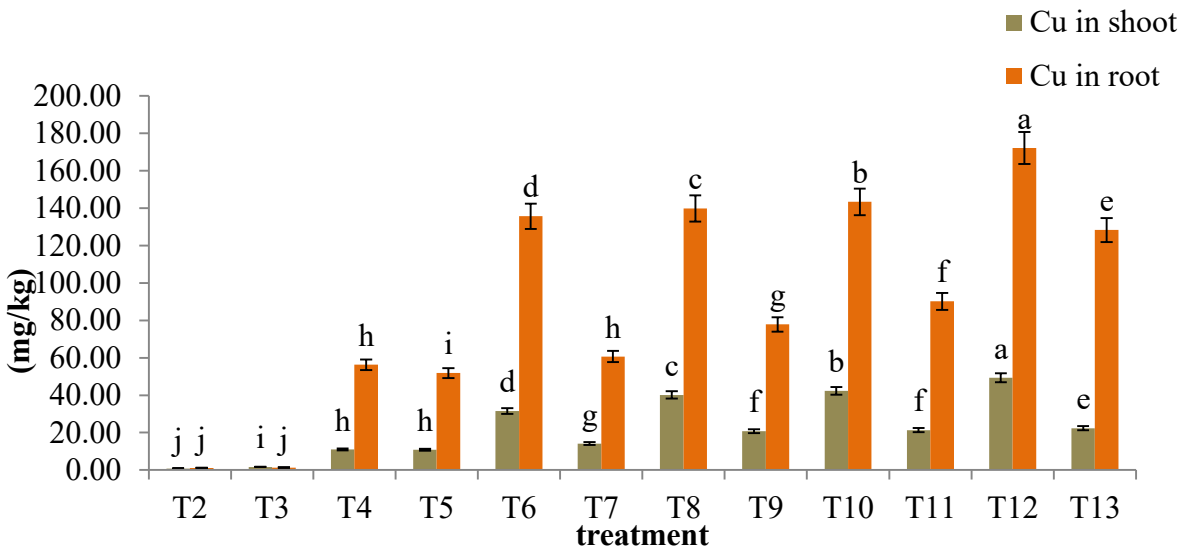

Figure 30: Copper concentration in roots and shoots of *Brassica*

The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in are non-significant.

Table 4: The average concentration, the accumulation coefficient and the translocation factor of cadmium, in the roots and shoots of *Brassica.*

Cadmium					
Treatments	**Concentration (mg kg^{1})**		**Accumulation Coefficient (AC)**		**Translocation Element Factor (TF)**
	Roots	**Shoots**	**Root/Soil**	**Shoot/Soil**	**Shoot/Root**
T2	0.67	0.42	0.56	0.20	0.36
T3	0.45	0.33	0.13	0.20	1.53
T4	7.83	10.65	0.16	0.17	1.03
T5	6.50	5.50	0.15	0.15	1.00
T6	18.67	24.50	0.15	0.15	0.97
T7	7.83	11.00	0.15	0.15	1.01

T8	20.67	28.50	0.13	0.12	0.96
T9	13.33	21.85	0.17	0.17	0.99
T10	22.83	40.00	0.11	0.11	1.06
T11	14.00	23.13	0.15	0.15	0.98
T12	26.83	40.00	0.08	0.09	1.10
T13	15.00	24.67	0.15	0.14	0.98

3.10. Accumulation coefficient and Translocation factor for Lead

The AC and TF of Pb within Brassica are shown in Table 3.4. For Pb, the accumulation coefficient was calculated to assess how differently Pb is absorbed by Brassica. For Pb, the metal concentration ratio between shoots and roots was estimated. The Pb TF from the roots to the shoots is represented by this fraction. For lead, the TF was less than 1. This finding showed that roots of *Brassica* cultivars accumulated Pb more than shoots did.

Table 5: The average concentration of Lead in the roots and shoots Brassica the accumulation coefficient and the translocation factor.

Lead					
Treatments	**Concentration (mg kg^{1})**		**Accumulation Coefficient (AC)**		**Translocation Element Factor (TF)**
	Roots	**Shoots**	**Root/Soil**	**Shoot/Soil**	**Shoot/Root**
T2	0.67	1.67	0.83	2.00	2.50
T3	1.17	1.02	1.00	1.02	0.87
T4	53.05	2.50	207.33	0.01	0.05
T5	25.68	1.67	247.50	0.01	0.06
T6	147.67	12.00	123.83	0.10	0.08
T7	71.48	5.67	204.33	0.03	0.08
T8	148.00	15.50	111.00	0.14	0.10

T9	85.48	10.17	173.00	0.06	0.12
T10	182.33	22.50	108.17	0.21	0.12
T11	124.15	11.00	129.33	0.09	0.09
T12	210.00	46.33	107.50	0.43	0.22
T13	136.00	11.85	127.83	0.09	0.09

Table 6: The average concentration of Copper in the roots and shoots Brassica the accumulation coefficient and the translocation factor.

Copper					
Treatments	**Concentration (mg kg^{1})**		**Accumulation Coefficient (AC)**		**Translocation Element Factor (TF)**
	Roots	**Shoots**	**Root/Soil**	**Shoot/Soil**	**Shoot/Root**
T2	1.17	0.83	0.13	0.09	0.71
T3	1.33	1.67	0.12	0.15	1.25
T4	56.33	11.00	0.22	0.04	0.20
T5	51.83	10.83	0.19	0.04	0.21
T6	135.67	31.50	1.08	0.25	0.23
T7	60.67	14.17	0.25	0.06	0.23
T8	139.83	40.17	1.13	0.33	0.29
T9	77.83	20.83	0.34	0.09	0.27
T10	143.33	42.33	1.19	0.35	0.30
T11	90.17	21.33	0.51	0.12	0.24
T12	172.17	49.33	1.77	0.51	0.29
T13	128.33	22.33	0.86	0.15	0.17

Table 7 Root Exudate Quantification

Treatment	Description	TPC	TAC	DPPH	TRP
		µg/ml	µg /ml	%Scavenging	µg /ml
T2	Fresh Soil+ *Brassica juncea*	155.25±1.5^{h}	6.64±0.02^{g}	9.55±0.02^{g}	1.24±0.02^{cde}
T3	Fresh Soil+ *NARC Sarsoon*	153.40±2^{h}	5.71±0.25^{h}	8.23±0.04^{h}	1.23±0.02^{cde}
T4	Spiked Soil+*Brassica juncea*	119.31±2^{i}	3.85±0.15^{i}	7.64±0.05^{h}	1.22±0.02^{de}
T5	Spiked Soil+*NARC Sarsoon*	85.65±1.5^{j}	3.77±0.19^{i}	5.58±0.02^{i}	1.20±0.02^{e}
T6	SS + *Brassica juncea* + *Bacillus tequilensis*	217.10±1.3^{c}	8.70±0.25^{d}	43.08±0.2^{c}	1.26±0.02^{bc}
T7	SS + *NARC Sarsoon*+ *Bacillus tequilensis*	164.77±1^{g}	6.75±0.02^{g}	9.70±0.2^{g}	1.24±0.02^{cde}
T8	SS + *Brassica juncea* + *Serratia marcescens*	220.69±1.1^{c}	9.52±0.02^{c}	57.90±0.35^{b}	1.26±0.02^{bc}
T9	SS + *NARC Sarsoon* + *Serratia marcescens*	175.37±2.1^{f}	7.47±0.02^{f}	12.50±0.49^{f}	1.24±0.02^{cd}
T10	SS + *Brassica juncea* + *Bacillus safensis*	226.32±0.8^{b}	13.00±0.02^{b}	58.51±0.81^{b}	1.30±0.02^{b}
T11	SS + *NARC Sarsoon*+ *Bacillus safensis*	202.65±2^{e}	8.41±0.01^{e}	35.46±0.42^{e}	1.25±0.02^{cd}
T12	SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	487.12±2^{a}	17.73±0.02^{a}	64.32±0.86^{a}	1.49±0.02^{a}
T13	SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	210.22±2^{d}	8.53±0.02^{de}	39.44±0.63^{d}	1.26±0.02^{cd}

Table 8 Root Exudate Quantification through HPLC

Treatment	Description	Vanilic Acid	Rutin	Galic Acid	Catechin	Syringic Acid	Coumaric Acid
		µg /ml	µg /ml	µg /ml	µg /ml	µg /ml	µg /ml
T2	Fresh Soil+ *Brassica juncea*	0.34±0.03^{h}	2.053±0.04^{h}	77.06±1.05^{i}	1.57±0.05^{h}	0.36±0.02^{h}	0.39±0.02^{h}
T3	Fresh Soil+ *NARC Sarsoon*	0.13±0.02^{h}	1.843±0.03^{i}	55.46±1.73^{j}	1.36±0.05^{h}	0.23±0.02^{i}	0.28±0.02^{i}
T4	Spiked Soil+*Brassica juncea*	0.05±0.02^{h}	1.226±0.03^{j}	6.98±0.79^{k}	0	0	0.24±0.02^{i}
T5	Spiked Soil+*NARC Sarsoon*	0.03±0.02^{h}	0.223±0.04^{k}	0.45±0.08^{l}	0	0	0
T6	SS + *Brassica juncea* + *Bacillus tequilensis*	3.14±0.03^{d}	5.026±0.04^{d}	121.86±1.55^{d}	51.91±0.12^{c}	5.50±0.04^{d}	3.01±0.04^{c}
T7	SS + *NARC Sarsoon*+ *Bacillus tequilensis*	0.76±0.05^{g}	2.926±0.04^{g}	92.15±1.57^{h}	18.42±0.09^{g}	0.79±0.03^{g}	1.18±0.03^{g}
T8	SS + *Brassica juncea* + *Serratia marcescens*	3.77±0.04^{c}	5.776±0.03^{c}	131.60±1.24^{c}	51.91±0.12^{c}	6.38±0.05^{c}	3.08±0.03^{c}
T9	SS + *NARC Sarsoon* + *Serratia marcescens*	1.01±0.01^{fg}	3.030±0.03^{f}	95.21±1.33^{g}	21.64±0.58^{f}	2.79±0.03^{f}	1.56±0.05^{f}
T10	SS + *Brassica juncea* + *Bacillus safensis*	4.49±0.6^{b}	5.943±0.03^{b}	136.76±1.32^{b}	69.63±0.4^{b}	10.14±0.06^{b}	4.63±0.04^{b}
T11	SS + *NARC Sarsoon*+ *Bacillus safensis*	1.24±0.05^{f}	3.053±0.06^{f}	101.36±1.25^{f}	24.40±0.55^{e}	2.95±0.08^{e}	1.66±0.04^{e}
T12	SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	4.83±0.03^{a}	7.276±0.03^{a}	316.35±1.53^{a}	90.60±0.84^{a}	10.22±0.04^{a}	7.64±0.05^{a}
T13	SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	1.54±0.06^{e}	3.923±0.08^{e}	107.31±1.11^{e}	44.64±0.33^{d}	2.99±0.03^{e}	2.03±0.05^{d}

Treatment	Description	Emodin	Gentisic Acid	Caffeic Acid	Ferrulic Acid	Cinnamic Acid	Apigenic	Quercitin
		µg /ml	µg /ml	µg /ml	µg /ml	µg /ml	µg /ml	µg /ml
T2	Fresh Soil+ *Brassica juncea*	1.74±0.04^{i}	0	1.47±0.05^{d}	0.33±0.04^{gh}	0.26±0.03^{h}	0.12±0.04^{h}	0
T3	Fresh Soil+ *NARC Sarsoon*	1.69±0.04^{i}	0	1.68±0.03^{c}	0.28±0.04^{hi}	0.10±0.03^{i}	0.08±0.02^{h}	0
T4	Spiked Soil+*Brassica juncea*	1.59±0.05^{j}	0	1.30±0.03^{e}	0.22±0.04^{i}	0.08±0.02^{ij}	0	0
T5	Spiked Soil+*NARC Sarsoon*	1.17±0.04^{k}	0	0	0.05±0.03^{j}	0.03±0.02^{j}	0	0
T6	SS + *Brassica juncea* + *Bacillus tequilensis*	19.56±0.05^{d}	3.71±0.04^{d}	0.32±0.03^{f}	1.17±0.05^{d}	3.73±0.03^{d}	0.77±0.04^{d}	0.02±0.05^{d}
T7	SS + *NARC Sarsoon*+ *Bacillus tequilensis*	3.09±0.04^{h}	0.08±0.03^{h}	2.48±0.05^{b}	0.37±0.04^{fg}	1.39±0.04^{g}	0.19±0.03^{g}	0
T8	SS + *Brassica juncea* + *Serratia marcescens*	21.14±0.05^{c}	4.26±0.03^{c}	0	1.33±0.05^{c}	6.08±0.04^{c}	1.17±0.03^{c}	0.06±0.02^{c}
T9	SS + *NARC Sarsoon* + *Serratia marcescens*	14.59±0.03^{g}	0.23±0.03^{g}	10.95±0.03^{a}	0.42±0.04^{f}	1.78±0.03^{f}	0.19±0.03^{g}	0
T10	SS + *Brassica juncea* + *Bacillus safensis*	22.92±0.08^{b}	6.11±0.03^{b}	0	2.08±0.02^{b}	6.68±0.03^{b}	1.61±0.04^{b}	0.45±0.04^{b}
T11	SS + *NARC Sarsoon*+ *Bacillus safensis*	17.22±0.04^{f}	1.45±0.03^{f}	0.18±0.03^{g}	0.54±0.04^{e}	1.82±0.04^{f}	0.42±0.04^{f}	0
T12	SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	30.15±0.05^{a}	7.21±0.04^{a}	0.17±0.02^{g}	5.88±0.06^{a}	11.93±0.05^{a}	2.87±0.03^{a}	1.53±0.02^{a}
T13	SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	18.31±0.03^{e}	3.21±0.03^{e}	0.12±0.03^{h}	0.58±0.04^{e}	2.24±0.03^{e}	0.57±0.03^{e}	0

3.13. Soil Bacterial Count

Three different heavy metal resistant bacterial strains *Bacillus safensis*, *Baccillus tequilensis* and *Serratia marcescens* were used for inoculation in this experiment. Table 8 shows the results of bacterial colonies survived in each treatment. The treatments (B+P) showed highest number of microbial colonies in soil. T12 had the highest CFU/g soil concentration, followed by T10 and T8 and T6. Treatment 7 showed the lowest bacterial count. The findings indicated that plants have a favorable impact on bacterial development in soil.

Table 9: Soil Bacteria Count for Different Treatments

Description	Treatment	CFU (Cells g^{-1} of soil)
T6	SS + *Brassica juncea* + *Bacillus tequilensis*	$1.90*10^{5} \pm 3.76*10^{3}$ d
T7	SS + *NARC Sarsoon*+ *Bacillus tequilensis*	$1.32*10^{5} \pm 2.74*10^{3}$ d
T8	SS + *Brassica juncea* + *Serratia marcescens*	$6.50*10^{7} \pm 2.74*10^{4}$ c
T9	SS + *NARC Sarsoon* + *Serratia marcescens*	$1.37*10^{5} \pm 2.74*10^{3}$ d
T10	SS + *Brassica juncea* + *Bacillus safensis*	$7.00*10^{7} \pm 2.74*10^{3}$ b
T11	SS + *NARC Sarsoon*+ *Bacillus safensis*	$1.40*10^{5} \pm 1.22*10^{3}$ d
T12	SS + *Brassica juncea* + *B. tequilensis*+*S. marcescens*+B. *safensis*	$9.80*10^{7} \pm 3.76*10^{4}$ a
T13	SS + *NARC Sarsoon* + *B. tequilensis*+*S. marcescens*+*B. safensis*	$1.70*10^{5} \pm 1.22*10^{3}$ d

T= Treatments, C= Control, FS + P= Fresh soil + Plant, AC= Abiotic control, P= Phytoremediation, P+B= Phytoremediation + Bioaugmentation, SS= spiked soil. The data are shown as means (n = 3 SD). Significantly, "a" was the column with the greatest mean, with succeeding alphabets having lower averages. Similar small letters in the same column have less significance.

Chapter 4

Discussion

The direction of current research was toward the use of integrated methods for heavy metal remediation. This experimental study used two Brassica cultivars to test the efficacy of bioaugmentation and phytoremediation, two independent remediation approaches used to treat soil contaminated with cadmium, copper, and lead. Along with phytoextraction, the effectiveness of bioaugmentation was also investigated.

In soils polluted with heavy metals, certain plants thrive. High potential exists for the soil to be cleaned up by hyperaccumulator plants (gathered mainly in the root or shoots). Heavy metals are removed from the contaminated soil layer by the plants once they reach the permitted standards level for heavy metals. Using plants with consortia of microbial system to remove heavy metals is a new technology (Su et al., 2014). In this study, *Brassica juncea* and *NARC sarsoon* was used with different combinations of *B. tequilensis, S. marcescens* and *B. safensis* for remediation of Cu, Cu, Cd and Pb with concentrations of 300mg kg^{-1}, 120mg kg^{-1} and 400 $mgkg^{-1}$ in spiked soil.

A sustainable remediation seeks to reduce pollutant concentrations to within regulatory limits while posing no risk to the environment or human health. Therefore, the goal of the current research was to determine whether bio-augmentation and phytoextraction could be used to restore multi-metal-contaminated soil.

Microorganisms both passively (adsorption) and actively (uptake) absorb heavy metals (bioaccumulation). Some bacteria have developed heavy metal resistance mechanisms that can be exploited to clean up heavy metal contamination in the environment. Additionally, biological methods for heavy metal removal, such as bioaugmentation or bioaccumulation, may offer an appealing alternative to physio-chemical techniques. Thus, using bio-phytoremediation for remediation objectives is a potential pollution-reduction strategy since it includes sustainable remediation to treat and restore the natural state of soil (Dixit et al., 2015). As a result, multiple findings demonstrated that the bioremediation methods rely on the microorganisms' active metabolizing and mobilizing capacities of harmful metals from the soil. Three different bacterial strains were used for

this study (i.e. *Serratia marcesens, Bacillus safensis* and *Bacillus tequilensis*) to measure the potential of bioaugmentation from soil environment with diverse combination of treatments. Bacterial count trend fluctuated for all applied treatment in which highest CFU was observed for treatments (B+P) and lowest was observed in treatments with no innoculation. Treatments in which microbial strains were added showed significant microbial count due to resistance to added heavy metals.

Soil quality measurement is complicated due to the numerous factors that contribute to good soil. The study of both pH and EC provides a more comprehensive picture of the soil's chemical properties. Changes in pH or EC can have an impact on certain microbe mediated processes, making these parameters indicative of effects on microbial activity (Smith et al., 1997). The EC and pH of the soil used with various treatments in this experiment were measured. For all applied treatments and fresh soil, the pH stayed within the range of 6.9 to 7.1. The small pH change shows that the HMs-contaminated soil did not impact the soil's natural balance. While the EC showed differences for different applied treatments i.e., 111 μScm^{-1} for FS + P while (T12) showed 199 μScm^{-1} and abiotic control showed highest EC that was 399 (μScm^{-1}).

Nitrates and phosphates, which are nutrients that are readily available, are regarded as the main contributors in crop yield. Nitrates and/or phosphates are present in nearly all of the biochemical components found in plants that stimulate plant growth. There is a quantifiable relationship between crop yield and the accumulation of each of these components by plants, with deficiencies in either element resulting in a loss of potential for plant growth (Sinclair & Vadez, 2005). The elevated soil's nutrient condition demonstrated better degradation's efficiency. The amount of available nitrates and extractable phosphorous both were observed higher in B + P (T12). While the lower amount of N and P were found in AC and slight changes were quantified in P and B treatments, which indicates that alone treatments did not show any difference in remediation of contaminated soil.

Soil organic matter contributes to increased soil fertility by increasing soil water retention, minimising nutrient leaching, and boosting soil porosity and aeration, so

enhancing plant development and microbial population (Macci et al., 2015). In order to improve the environment for plant growth, soil amendments are typically applied, and these contain organic matter (Weyers and Brockamp, 2020). The carbon contained in organic matter is known as TOC. Ordinarily, the amount of oxidizable organic carbon (OOC) in the soil is calculated, and the resulting TOC and OM are then converted using a constant factor. This study resulted significant differences in each treatment for OOC, TOC and OM. With treatment B+P (T12) found the highest concentration of organic matter. The lowest concentration was found in AC treatment due to heavy metal conditions in soil.

Contamination of agricultural soils with toxic heavy metals has become a severe global issue due to their toxic effects in plants. When these HMs enter plants, they can reduce plant growth and photosynthesis, negatively change plant morphology, and eventually cause food quality crisis (Nagajyoti et al., 2010; Ali et al., 2015). When too many HMs components reach the plant shoot, plant oxidative stress increases, and this stress can lower plants' ability to absorb minerals and nutrients (Ali et al., 2014; Murtaza et al., 2015). Remediation of metal-contaminated soils has therefore become crucial. According to the findings of the current study, contaminated soil showed a marked decrease in all plant physiological parameters of soil. Almost all parameters i.e., fresh weight, dry weight, and length of roots and shoots were observed highest for T12 followed by T10 > T8 > T6 > T13. The application of *Bacillus tequilensis, Serratia marcescens* and *Bacillus safensis* (heavy metal resistant bacterial strains) inhibited the biomass of *Brassica* by enhancing the HMs remediation.

Brassica roots and aerial parts had greater amounts of Cd, Cu, and Pb when the soil's Cd, Cu, and Pb content surpassed threshold values, according to research (Brun et al., 2000). The P treatment in this study had the highest levels of Cd, Cu, and Pb uptake in the plant roots and shoots, respectively. Brassica roots accumulate more Pb than shoots do, indicating that the roots of canola are more active than shoots in the phytoremediation of Lead. This is consistent with research by Cho-Ruk (2006) and Parsadoost et al. (2008). In addition to accumulating substantial concentrations of non-essential metals, like Cd,

hyperaccumulator plants can also absorb high levels of key micronutrients. According to standard definitions, hyperaccumulators are species that can accumulate metals 100 times more efficiently than regular non-accumulator plants. A hyperaccumulator will thereby concentrate more than 10 ppm Hg, 100 ppm Cd, and 1,000 ppm each of Co, Cr, Cu, and Pb (Lasat, 1999). Brassica has the potential to be a plant that can hyperaccumulate Cu, Cd, and Pb from contaminated soils. Because microbes alter soil chemistry, mobilize metal fraction, and make it available for plant uptake, the application of inoculated bacteria in polluted soil with Brassica improved heavy metal remediation in plant roots and aerial parts.

In soil metal availability remained higher in AC control followed by P treatment due to high availability of HMs in soil. The lower concentration of HMs is observed in B+P treatment. In the contaminated soil, bacterial inoculation lowered the accumulation of heavy metals, positively impacted soil and plant features, and encouraged plant development, demonstrating its viability for sustainable agricultural output.

Chlorophyll is a crucial chemical compound present in autotrophic organisms that is essential for carrying out photosynthesis, a process that sustains life. Heavy metals (Pb, Cr, Ni, Cd, and Zn) have very negative impacts and reduce the amount of photosynthetic pigments (chlorophyll a, chlorophyll b and carotenoids). Chlorophyll is vulnerable to any stress injury caused by any heavy metal and can have its content reduced as a result (Aldoobie et al., 2013). Measuring the chlorophyll content demonstrates that chlorophyll is a biomarker of environmental stress (i.e., high chlorophyll levels suggest high availability of nutrients, especially N and P, while low chlorophyll level indicates stress situation in plants) (Rastogi et al., 2017; Hussain et al., 2018). The highest chlorophyll levels in this study were noted for B+P (T12) which is indicator of high nutrient availability and thus for higher chlorophyll content, lowest was observed for P treatment due to heavy metal stress.

Carotenoids are also plant pigments which play role as quencher of the high ROS that can initiate a cascade of functions as chain breaking antioxidants (Young and Frank, 1996). The trend for carotenoid content was observed as Bacteria strain consortium + *Braasica*

juncea > Single Bacteria strain + *Braasica juncea* > Bacteria strain consortium + *NARC sarsoon* > Bacteria strain + *NARC sarsoon* > P. Abiotic stress causes organisms such as plants to produce ROS, such as hydrogen peroxide (H2O2), superoxide radical (O2) and hydroxyl radical (OH), as byproducts of normal metabolism at various subcellular levels. (Puertas et al., 2006). High production of MDA and H_2O_2 results in increased activities by SOD, APX, CAT and GPX.

While a rise in H2O2 content indicates the formation of ROS, the MDA content demonstrates damage from lipid peroxidation. Environments stressed by heavy metals led to a gradual rise in the amount of MDA and H2O2. While the P treatment without any soil additions resulted in a considerable decrease in MDA and H2O2 concentration, the soil amendments with various treatment combinations showed dramatically varied outcomes (Habiba et al., 2014). The lipid peroxidation caused by oxidative stress is measured using the environmental stress marker MDA, but inside plants, hydrogen peroxide produces ROS that serve as signalling molecules (Niu and Liao, 2016). PC showed lowest levels of MDA and H_2O_2 content among all treatments which indicates stress free environment. Highest level of MDA was observed for treatments without any bacterial inoculation followed by Treatments with *NARC sarsoon* and strains and same for H_2O_2 content. Among all the applied treatments B+P (T12) showed lower levels of MDA and H_2O_2 resulting reduction in stress.

Plants create ROS as a first line of defence against oxidative stress because it is essential for plant survival and it can halt the free radical specie chain reaction (Khan et al., 2019). Enzymatic antioxidants, such as guaiacol peroxidase (GPX), ascorbate peroxidase (APX), and catalase (CAT), were examined for this research. In T4 and T5 treatments, higher amounts of APX, CAT, and GPX were seen, indicating significant levels of stress damage caused by HMs. In comparison to inoculating a single strain, a significant reduction was observed for the bacterial consortium with plant. The levels of the enzymes APX, GPX, and CAT were likewise noticeably reduced in (B+P). According to Kiyani et al.'s (2009) study, plants can suffer damage or have their production of antioxidant enzymes downregulated at high levels of heavy metal exposure. There isn't a

single article that shows the antioxidant activity of particular plants' root exudates. Therefore, this work is regarded as novel.

Chapter 5

Conclusions

Three key components of the global environment—soil, water, and air—are necessary for life to survive. However, contamination is constantly harming these environmental aspects; for example, a rise in heavy metal pollution in soil has become problematic to the environment and food security. To address these issues, a comparative study on the impact of bioaugmentation and phytoextraction alone and in combination has been conducted. To conclude, the cultivar *Brassica juncea* outperformed the other variety *NARC sarsoon* for the remediation of cadmium, copper, and lead in soil when a consortium of the specified strains was used. Additionally, this study reveals that employing such consortia is a quick, economical strategy that significantly contributes to the synthesis of root exudates and metal uptake. Hence, the application of this consortia can enhance the phytoremediation by the extraction of heavy metals and other pollutants along with the plant growth promotion in field.

Future Recommendations

Based on the results of this experimental study, we may draw the conclusion that the development of remediation techniques has increased our understanding of the remediation of toxic heavy metals, which have negative impacts on both human health and our ecology. This study used two brassica cultivars and bacteria for phytoremediation of Cu, Cd, and Pb from contaminated soil. For the treatment of soil contaminated with heavy metals, the bioaugmentation method with Brassica offers tremendous potential. The processes of signalling between plants and rhizospheric bacteria through root exudates and their unique role have been established. In connection to bacterial inoculation, we have not yet identified the precise genes that cause heavy metal accumulation. To optimise heavy metal accumulation processes for the restoration of polluted sites, a deeper understanding of these aspects in particular plant-microbe interactions is needed.

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